Journal of Engineering and Natural Sciences
Mühendislik ve Fen Bilimleri Dergisi

Sigma
2004/4

Invited Review Paper

FUNCTIONALLY BIOPOLYMER SYSTEMS

Mehmet MUSTAFAEV*

Department of Bioengineering, Faculty of Chemistry and Metallurgy, Yildiz Technical University, Davutpasa-İSTANBUL

Geliş/Received: 23.12.2004

ABSTRACT

Advances in functionally biopolymer systems have provided new opportunities on basic science studies and bioengineering applications. In this review, the recent data on interaction mechanisms of synthetic polyelectrolytes with proteins and physico-chemical criteria for the construction of functionally biopolymer systems are reviewed. Also, the structure and structural-chemical features of protein-polyelectrolyte complexes are discussed. A special chapter is dedicated to metal-induced polycomplexes and described the peculiarity of phase transition in these systems. Complexes of proteins with polyelectrolytes may be considered as models for specific nucleoprotein complexes and nucleotide-protein complexation research. Such reactions may simulate the antigen-antibody interactions, the processes of assembling of viruses, chromatin, ribosome strains and other cell components in complicated biological systems. Applications of functionally biopolymer systems in immunology (polymeric immunogens for vaccine innovation) and radiobiology (active radioprotectors development) are discussed.
Keywords: Protein, polyelectrolyte, biopolymer systems, immunology, radiobiology.

FONKSİYONEL BİYOPOLİMER SİSTEMLER

ÖZET

Fonksiyonel biyopolimer sistemlerdeki gelişmeler temel bilim çalışmaları ve biyomühendislik uygulamalarında yeni fırsatlara imkan sağlamışlardır. Bu yayında, sentetik polielektrolitlerin proteinlerle etkileşim mekanizması ve fonksiyonel biyopolimer sistemlerin oluşumundaki fiziko-kimyasal kriterler hakkında yakın zamanda elde edilmiş veriler bir arada incelenmiştir. Ayrıca, protein-polielektrolit komplekslerinin yapı ve kimyasal-yapı özellikleri tartışılmıştır. Metal-induced polikompleksler için özel bir bölüm ayrılmış ve bu sistemlerdeki faz geçişin özellikleri anlatılmıştır. Proteinlerin polielektrolitlerle olan kompleksleri spesifik nükleoprotein kompleksleri ve nükleotid-protein kompleksleşmesi araştırmaları için model olarak düşünülebilir. Bu tip reaksiyonlar antijen-antikor etkileşimi, virüslerin, kromatin ve ribozom zincirlerinin ayrıca karmaşık biyolojik sitemlerdeki diğer hücre bileşenleri için taklitçi model olabilirler. Fonksiyonel biyopolimer sistemlerin immünolojide (yapay aşı uygulamalı amaclı polimerik immünojenler geliştirmek) ve radyobiyolojide (etkin radyoprotektörlerin hazırlanması) uygulamaları tartışılmıştır.
Anahtar Sözcükler: Protein, polielektrolit, biyopolimer sistemler, immünoloji, radyobiyoloji.

* e-mail:mustafa@yildiz.edu.tr, Tel: (0212) 449 15 60

1. INTRODUCTION

Introduction of synthetic polymers to biomolecules has been studied for the application in the fields of medicine, pharmacy and engineering [1-18]. Polymeric microspheres and gels have various functional applications such artificial muscle, drug-release systems and recovery of cultured cells. Modification or attachment of proteins or other biomolecules with synthetic polymers can provide many benefits for both *in vivo* and *in vitro* applications. Covalent coupling of proteins to large macromolecules can alter their surface and solubility properties, creating increased water solubility or even organic solvent solubility for molecules normally sparingly miscible in such environments. Polymer modification of foreign molecules can provide increased biocompatibility, increasing in vivo stability and delaying clearance by the reticuloendothelial systems. Modification of enzymes with polymers can dramatically enhance their stability in solution; can provide cryoprotection for proteins sensitive to freezing. Polymers with multivalent reactive sites can be used to couple numerous small molecules for creating pharmacologically active agents that possess long half-life in biological systems. Polymer modification of surfaces can effectively mask the intrinsic character of the surface and thus prevent nonspecific protein adsorbtion. Finally, multifunctional polymers can serve as extended cross-linking agents for the conjugation of more than one molecule of one protein to multiple numbers of a second molecule, creating large complexes with increased sensitivity or activity in detecting or acting open target analyses. For example, synthetic and natural polymers such as polysaccharides, poly(L-lysine) and other poly (amino acids), poly (vinyl alcohols), poly (vinyl pyrrolidinones), poly (acrylic acid) derivatives, various poly (urethanes) and poly (phosphazenes) have been coupled to with a diversity of biosubstances to explore their properties. Copolymer preparations of two monomers also have been tried.

Similar complexes can be formed to create highly potent immunogens consisting of hapten-polymer conjugates [9,16-18a], for reducing the immune response and induction of an antibody response toward the hapten [19]. Biodegradable poly (lactide-co-glycolide) microspheres or microcapsules developed over the past decade for reliable, preprogrammed release of contraceptive steroids has significant potential for adaptation to antigen release for immunization [20-24]. In addition, polymeric encapsulation of antigens could prevent the acid and enzymatic degradation that has been a barrier to the development of oral vaccines. The possible mechanisms of immunopotentiation by sustained release of antigen include a depot effect analogous to that of aluminium salt adjuvants, the delivery of antigen directly to antigen-presenting cells such as macrophages or continuous exposure to antigen as occurs in chronic infections. At the same time, iminocarbonate polymers with added tyrosine, a known adjuvant, was evaluated for an adjuvant effect independent of the sustained–release effect. Despite the potential of microencapsulated vaccines, a number of unsolved questions persist. Some of them: residual solvents and monomers in the microspheres, adverse reactions with slowly released antigen, control of allergic reactions, the size of microspheres, etc. It is known that covalent attachment (and complex formation) of linear neutral (non charging) synthetic polymers to proteins reduces the immunoreactivity. Polyethylene glycol (PEG) has been widely studied for protein modification, reducing the immune reactivity and/or immunogenecity of originally antigenic protein, and improving their in vivo stability with prolonged clearance times. A few of them have already been authorized by Food and Drug Administration for clinical use.

There exist data in the literature over applications show that the charged water-soluble polymers (polyelectrolytes-PE) being injected to animals simultaneously with various antigens increase immune response and thus produce adjuvant effect [9,16,25-29]. The complexes/conjugates of such nonimmunogenic synthetic PE with microbial and viral protein and polysaccharide antigens given prior to inoculation confer protection against diseases. It provides effective immune protection without traditional classical adjuvants. The development of these approaches to modify the immunogenecity of antigens will open perspectives for the creation of

new vaccines, diagnostic, pharmaceutical and biotechnological preparations. The new vaccines will eliminate the risk associated with the isolation of large quantities of infections virus and avoid the production of improperly inactivated vaccines. Moreover, polymeric vaccines are expected to be stable and inexpensive to produce. Therefore, complexes and conjugates of proteins and various haptens with synthetic PE were shown to exhibit enhanced immunogenecity and were considered as a new artificial immunogens for vaccine innovation. Whereas similar structures with electrically neutral polymers were noted to possess much lower immunogenecity. Thus the immune response can be widely varied due to the structure and composition of the polymeric conjugates.

In the light of these findings it was very important to study the methods and mechanism of the including of antigenic epitopes (peptides, proteins, polysaccharides and other organic haptens) of different diseases into polyelectrolyte matrix (Biopolymer Systems), establish the mechanism of the binding and the structure of the form conjugates (or complexes).

2. PROTEIN-POLYELECTROLYTE COMPLEXES

Early studies of protein-polyelectrolyte complexes (PPCs) were reported by Morawetz et al. [30-32] in the 1950s. They investigated the precipitation of liver catalyses by interactions with some synthetic polyelectrolytes. Subsequently, the interactions of various proteins with various natural and synthetic polyelectrolytes have been extensively investigated by a number of groups.

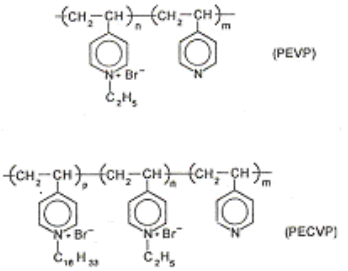
The interactions of proteins with synthetic polyelectrolytes (PE), which are controlled mainly by electrostatic forces, may result in soluble complexes [33-39], complex coacervation [40-44], or the formation of amorphous precipitates [45,46]. These polycomplexes appears to be of great importance for medicine and biotechnology in particular with respect to design of immunogenic compounds for vaccine innovation [9,17]. Studies of mechanisms of protein cooperative binding by synthetic polyelectrolytes is of interest for immobilization of enzymes, stabilization and destabilization of their tertiary structure, specific sorption of proteins on surfaces, elucidation of the mechanism of polyelectrolyte physiological activity and the modification of protein-substrate affinity [47-59]. It is also important for the establishment of general principles and physico-chemical mechanisms that are in the background of structure-property relationships. In addition, such reactions may simulate the processes of assembling of viruses, chromatin, ribosome strands and other cell components in complicated biological systems. Moreover, physicochemical studies of protein-polyelectrolyte interactions may provide some insights into interactions between proteins and nucleic acids in the transcription process [58].

All of the states of protein containing polyelectrolyte complexes may be achieved by the selection of the macromolecular components, choice of ionic strength and pH, and control of the concentration of proteins and polyelectrolytes. This chapter focuses on the effects of chemical composition, concentration of components and solution behaviour on structural and chemical peculiarities of water-soluble complexes of serum proteins with synthetic polyelectrolytes discussed in recent and current research.

2.1. Complexes of Proteins with opposite charged Polyelectrolytes

Water-soluble and insoluble polyelectrolyte complexes (PEC) of proteins can be obtained from any globular proteins and oppositely charged linear PE, when certain conditions are met. Some examples of systematic studies in this area are the complexation of negatively charged proteins with polycations: quaternized poly-4-vinylpyridine (QPVP) [33-39,60,61,65-68,77-80,83,87], polydimethyldiallylammoniumchloride (PDMDAAC) [15,44,64,81], homopolymer of (N,N,N-trimethylamino) ethyl chloride acrylate (CMA) and positive charged proteins with polyanions: dextran sulfate [44], sodium poly(styrenesulfonate)-NaPSS, polyacrylic acid–PAA,

polymethacrylic acid–PMAA, copolymers of 2-(acrylamido)-2-methylpropanesulfonateand acrylamide-poly(A/CD). Within the framework of these investigations it seemed reasonable to investigate the influence of the molecular mass distribution (polydispersity) of polyelectrolytes on their complex-forming activity, since early studies in this area were conducted with the use of unfractionated polymers. The critical point, which determined, to a large extent, the successful outcome of these studies was the use of PE fractions with a predetermined molecular mass. This very circumstance made it possible to establish differences in the water-soluble complex-forming activity of PE with proteins and use of different physicochemical methods for the study of such polycomplexes. A systematic study of water-soluble protein-polycation complexes has been reported firstly by Kabanov and Mustafaev [33-35]. When pH of the solution exceeds the isoelectric point (pI) of the protein a globule acquires a negative charge due to dissociation of the carboxyl groups being at the periphery of the globular species and able to form salt bonds with the polycation chains. In these work the fractions of quaternized poly-4-vinylpyridine with the linear dimensions considerably larger than those of the BSA globule were used.



Polycomplexes can be obtained by a direct mixing of the solutions of the oppositely charged protein and PE. There may be two versions of the component mixing: addition of BSA solution to PE solution and vice versa. These procedures were carried out as turbidimetric titration of one solution with the other. The behaviour of the mixtures of PE and BSA for two versions of mixing is shown in Figure 1 (a, b) as an example.

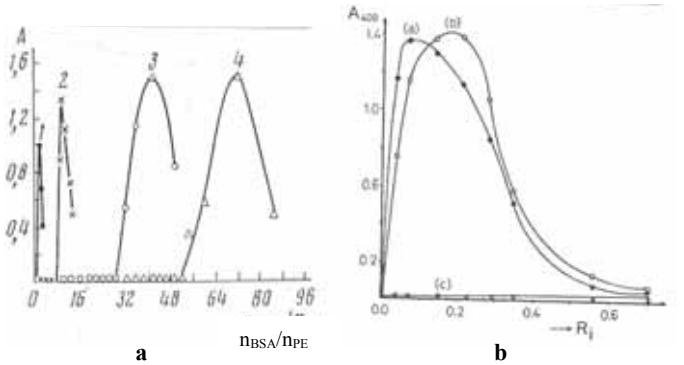


Figure 1. Dependence optical density (A) of the solutions of BSA-PVEP mixtures on the n_{BSA}/n_{PE} at the different degree of polymerization (Pw): $0{,}19.10^3$(1); $0{,}95.10^3$(2); $3{,}3.10^3$(3); $5{,}9.10^3$(4); C_{PE}=0,15 g/100ml. (a) Turbidimetric titration curves at various polymer/protein molar ratios (R_i) of (a) PVEP-HAS and (b) PECVP-HAS mixtures at pH 7; (c) both PVEP-HAS and PECVP-HAS mixtures at pH 3; n_{HSA}=5.7 μM. $R_i=n_{PE}/n_{BSA}$.

When BSA solutions are added to polycation solutions at pH 7 the formation of the complexes occurs. The system remains homogeneous over a wide range of the BSA/PE ratios, i.e. the formed complexes are soluble. The phase separation in the system occurs only at some critical protein concentrations depending on the degree of polymerization of PE. The further increase of protein content in mixture again leads to the formation of soluble polyelectrolyte complexes followed by a decrease of turbidity.

Quite a different behaviour is observed when BSA solution is titrated with PE solution (Figure 1b). In this case increasing polymer content in the mixture, leads to an increase of the system turbidity indicating the formation of polycomplex insoluble in aqueous media. The further increase of PE content in polymer-protein mixture leads to the formation of soluble polyelectrolyte complex followed by a decrease of turbidity. When the ratios of the components in solution achieve some critical values the system again became homogenous and at the further increase of PE content the phase state of the mixture remains unchanged. Thus the different ways of protein and PE mixing differ by the intermediate states of mixtures depending on the component ratio in the mixture, but at a proper ratio the both lead to the formation of soluble polyelectrolyte complex.

Homogeneous mixtures of polycations with proteins were studied by the different techniques: Sedimentation [33-35], Viscometry [34,35], Static, Quasi-Elastic and Electrophoretic Light Scattering [57,64], Electron Microscopy [34], Optical Rotary Dispersion [34], Fluorescence and Circular Dichroism Spectroscopy [66-70].

Ultracentrifugation of the homogeneous BSA-PVEP system at various ratios of the components was reported by Kabanov and Mustafaev in 1977 [34]. The typical sedimentograms of PVEP and its mixtures with BSA at different ratios of their macromolecule concentrations (n_{BSA}/n_{PE}) in the solution are given in Figure 2. The values of n_{BSA} and n_{PE} number of macromolecules of BSA and PVEP fractions in one volume of solutions respectively, calculated by equation $n = C \times 10^{-2} N_A / M$, M-molecular mass, N_A- Avogadro number, C-concentration, g/dl. As it follows from this figure in the general case the system is characterized by a bimodal distribution of sedimenting components. The comparison of the values of the sedimentation coefficients (Sc) corresponding to the peaks I and II of the mixture and of the individual components (Sc of the BSA and PVEP under the similar conditions are equal to 4.2 sved. and 1.9 sved., respectively) shows that the protein binding by takes place: the peak with the sedimentation coefficient of pure BSA is absent in the PVEP sedimentograms. One may assume that the rapidly sedimenting substance (peak II) is a complex PVEP-BSA (Sc = 3.6 sved.). The value of Sc for a slowly sedimenting substance (peak I) is 2.0 sved. i.e. It corresponds to a free –PVEP. Studies of sedimentation in the homogeneous systems at different ratios of the components permits to elucidate some important features characterizing PVEP-BSA complex formation. An increase of the number of protein molecules in the mixture (the weight concentration of PVEP is kept constant and equals 0.15g/dl) leads to a decrease of the area of the peak corresponding to the free PVEP macromolecules (peak I) while the area of the assumed peak of the complex (peak II) increases (curves 2-4).

When the ratio of the number of macromolecules is 3:1, the peak of free PVEP disappears, and only one peak characterized by Sc = 5.2 sved remains on the sedimentogram (curve 5). The described situation is typical for all studied fractions of PVEP interacting with BSA. At the same time the BSA/PVEP ratio at which one observes a disappearance of the free PVEP peak, at the fixed weight concentration of PVEP, depends on the degree of its polymerization, i.e. on the contour length of PVEP macromolecules. Figure 2 shows the dependence of the area of the free PVEP peak on the ratio of the numbers of protein macromolecules and that of PVEP (n_{BSA}/n_{PE}) for PVEP of different degree of polymerization as example. A linear decrease of the free PVEP concentration in the solution during titration with protein unambiguously shows its binding to a complex. A decrease of the area of the corresponding to the free PVEP is followed by an enhancement of the area of the second peak for soluble polyelectrolyte complex species.

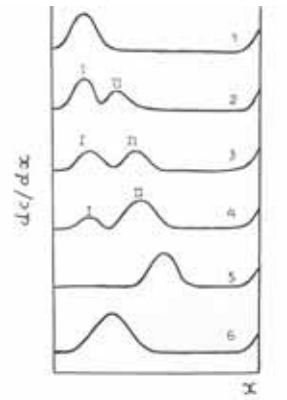


Figure 2. Sedimentograms of Q-PVP (1) and its mixtures with BSA at different ratios of the BSA macromolecule number to that of Q-PVP ($n_{BSA}/n_{Q\text{-}PVP}$): 2 - 0.75; 3 - 1.25; 4 - 2; 5 - 3; the centrifugation time (t) is 60 min.; 6 - 6; t = 20 min.; w = 56 000 rpm. The concentration gradient (dc/dx) is plotted along the ordinate; the distance from the rotation center of the rotor (x) in arbitrary units is plotted along the abscissa.

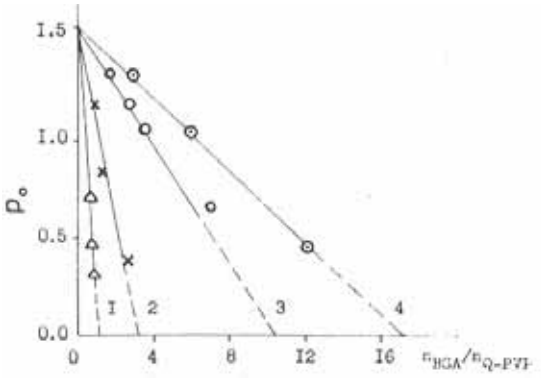


Figure 3. Dependence of the sedimentation peak area (P_o) of free Q-PVP in the Q-PVP-BSA system on $n_{BSA}/n_{Q\text{-}PVP}$ for Q-PVP with different DP. 1: 0.19 x 10^3; 2: 0.95 x 10^3; 3: 3.3 x 10^3; 4: 5.9 x 10^3; Q-PVP = 0.15 g/dl; the content of BSA is varied; 25°C; pH = 7.0.

It is important to emphasize, however, that the free PVEP still remains in the system over a sufficiently wide range of the molar ratios BSA/PE even when $n_{BSA}/n_{PE} \leq \mathbf{1}$. A similar sedimentation pattern was also found for the complexation of PEVP with a Ovalbumin [29], Gammaglobulin [38], Formiatdehidrogenase and Alcoholdehidrogenase enzymes [75-78], surface protein fractions of mycobacteria (B.tubercloses antigen) [9], fraction F1 of the plaque microbe [9], mixtures of different proteins, whole serum [38] and blood [38].

An independent experiment has shown that the free protein is absent in the system over the whole studied range of the ratios BSA/PE., i.e. all added BSA is strongly bound by the polycation. At the same time the existence of the free PE in the system under these conditions unambiguously indicates a non-random distribution of the protein molecules between the polycations. It was suggest that the reason causing just provide disturbance of the randomness of

the distribution is probably due to a positive interaction of the globules sorbed by one chain. In other words the formation of contacts between BSA globules "condensed" on the same polycation results in an additional decrease of the free energy exceeding a free energy increase caused by the disturbance of the randomness of the distribution. A theoretical description of such cooperative binding is given in [71]. The ultimate case of the non-random distribution corresponds to the situation when some polycations sorb the maximum possible under given conditions number of protein globules, while the others remain practically unpopulated. It is such type of distribution that was found previously at complexation of polymetacrylic acid with polyethylenglycol oligomer [72] and DNA with cationic polypeptides and basic proteins (histons and lysozyme) [73] in aqueous solutions. The changes of dynamic characteristics of positively charged lisocime protein globules and negatively charged polymethacrylic acid in the process of complex formation in water and water-salt solutions have been analyzed using polarized luminescence method by Anufrieva [68]. The irregular character of protein molecules distribution between polyelectrolyte chains is shown: some parts of polymethacrylic acid are filled with compactly disposed protein and some parts are free.

Let us consider now the character of the dependence of PVEP-complexes sedimentation coefficients, Sc, on the ratio n_{BSA}/n_{PE}. The corresponding curves are given in Figure 4.

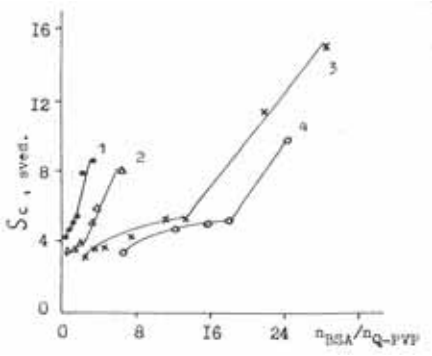


Figure 4. Dependence of sedimentation coefficients of Q-PVP-BSA complexes on $n_{BSA}/n_{Q\text{-}PVP}$ for Q-PVP with different DP. 1: 0.19 x 10^3; 2: 0.95 x 10^3; 3: 3.3 x 10^3; 4: 5.9 x 10^3; [Q-PVP] = 0.15 g/dl; the content of BSA is varied; 25°C; pH = 7.0.

It is seen that in the range of n_{BSA}/n_{PE} N_i, i.e. over the range of ratios when free polycations are still present in the system a change of Sc is relatively small. It follows from the above-mentioned data that when the amount of the added protein at [PVEP] =const is increased, the accumulation of the complexes occurs in this region. The composition of these complexes for each given PVEP is constant and is determined by the polycation contour length. Recently, Guney et al. [69] shows that in this range a change of fluorescence intensity of tryptophan residues of BSA globules at complexation with polycations also is relatively small.

Some increase of Sc for complexes (see curves 3 and 4 in Figure 4) over this range is most probably caused by a decrease of the medium viscosity because of involvement of the free PVEP in the complex with protein and not because of an increase of the mass of the complex particles. The latter is especially applicable to the system characterized by high values of N_i giving evidence that non-random distribution of the globules between the polycations when $n_{BSA}/n_{PE} = N_i$ practically satisfies the principle "all or none".

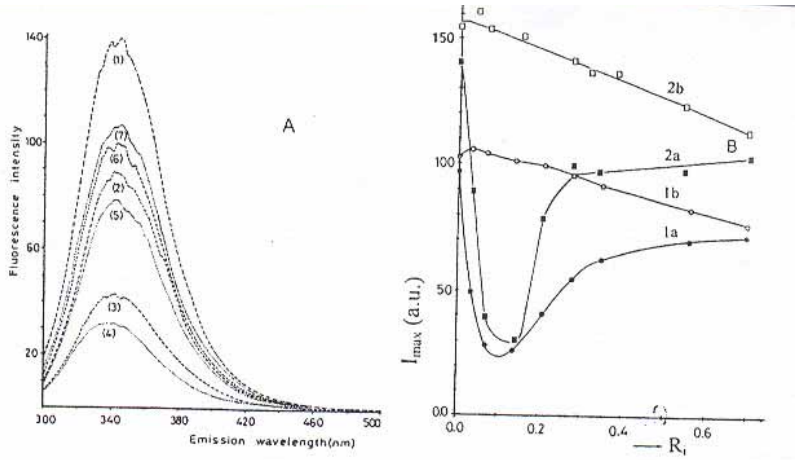


Figure 5. (A) Fluorescence spectra of BSA at 5.7 μM (1) in the presence of increasing amounts of PEVP at pH: 7.0. R_i = 0.035 (2), 0.07 (3), 0.14 (4), 0.21 (5), 0.35 (6) and 0.7 (7); pH 7.0; 20°C. Emission intensities (I_{max}) at 340 nm versus R_i for PEVP-BSA (1a, 1b) and PEVP-HSA (2a, 2b) mixtures at pH 7.0 (a) and pH 4.3 (b).

However, when the values of n_{BSA}/n_{PE} coinciding with N (disappearing of the peak I in sedimentograms) are reached a sharp increase of the sedimentation coefficients of the product of BSA interaction with PVEP occurs. The peak of the free BSA is not observed yet in the sedimentograms (see Figure 2 curve 6). It shows that in spite of the free polycations consumption protein binding continues. In other words, when an additional amount of BSA is supplied; the above described complexes find additional resources for its adsorption. Interaction between BSA and PVEP is not followed by any changes of the protein secondary structure. It follows from the data on optical rotary dispersion (see below).

Factors Influencing Protein-Polyelectrolyte Complexation. To establish the nature of protein binding by the polycation chains the polycomplexes were studied by the equilibrium dialysis technique [61]. It is well known that as a result of cooperative reactions between the opposite charged polyelectrolytes and formation of the intermacromolecular salt bonds in most cases a low molecular electrolyte is eliminated in the solution:

For the protein-polyelectrolyte mixtures:

$$(SA)^{-n} \cdot nNa + (PE)^{+n} \cdot nBr^{\Theta} \rightarrow [(SA)^{-(n+m)} \cdot (n-m)Na^{+}(PE)^{n+m} \cdot (n-m)Br^{\Theta}] + m(Na^{+}Br^{-})$$

Therefore, analyzing the content of a low molecular salt in the reaction mixture one can determine the number of salt bonds formed by a protein globule with a polyelectrolyte macromolecule. To determine the concentration of a low molecular electrolyte in the mixtures BSA-PEVP the equilibrium dialysis of polycomplex solutions was carried out. The dialyze was analyzed to determine the halide content by Ag-metric titration. The corresponding data are listed in Table 1

Table 1. Results of the Ag-metric analysis of Br^{Θ} concentration in the dialysates of Q-PVP-BSA polycomplexes with different composition ($M_{w\ Q\text{-}PVP}$ = 250 000)

Polycomplex composition	Amount of isolated Br per 1g of Q-PVP 10^3 mole	Number of salt bonds per 1 molecule of BSA
7 : 1	0.974	60
9 : 1	1.154	50
11: 1	1.374	60
13 : 1	1.634	50

It is seen that an increase in BSA content in the mixture results in a proportional increase in Br^{Θ} concentration in the dialyze. It shows, according to scheme I, an increase in the overall salt bond number between the protein globules and the polycations. If one knows a polycomplex molecular composition and a total amount of the low molecular electrolyte eliminated as a result of the intermacromolecular reaction, it is possible to calculate the number of the salt bonds per a single protein globule in the polycomplex. As it seen in Table 1 this value is practically independent on the on the reaction mixture composition and is 55+10. This result convincingly shows the equal reactivity of the BSA globules at their interaction with PVEP. It should be noted that only a half of the protein carboxyl groups determined from protein composition [82] take part in such interaction.

The influence of ionic strength and pH of the medium on the behaviour of BSA-PVEP complexes has been studied by Mustafaev [81]. The redistribution of the albumin molecules between polycations depending on the ionic strength (Figure 6) and pH (Figure 7) of the medium was found.

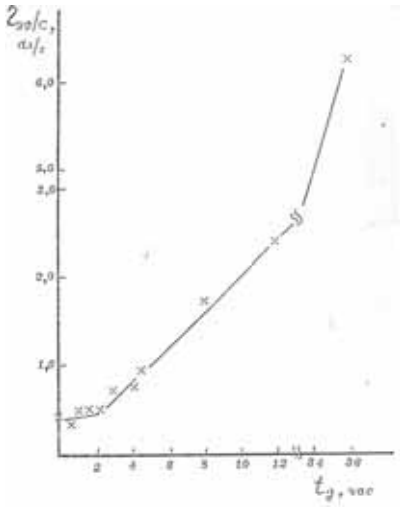


Figure 6. Sedimentograms of BSA-PVEP mixture solutions after dialysis against pure water at different times (t-hours): before dialysis (1,1); 0,5 (1,2); 2 (1,3); 3 (2); 8 (3), 36 (4); pH7 n_{BSA}/n_{PE}=3; 25˚C

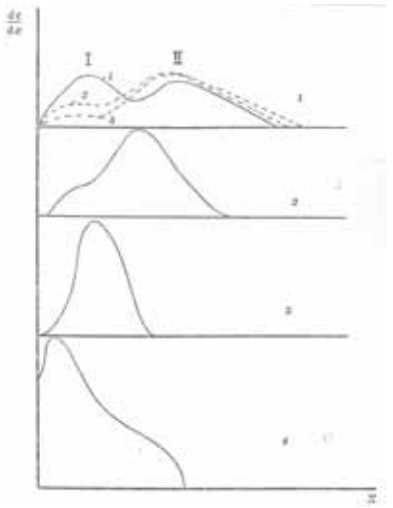


Figure 7. Dependence of inherent viscosity ($\eta_{sp/C}$) of BSA-PVEP mixtures on time of dialysis (t_d); pH 7; 25˚C

As a result of this process some polycations became free, while other did albumine globules overload. The lower and upper limits of the complex stability regard to the ionic strength were determined (1.1 x 10^{-8} x 10 mol/l). The excessive desalination (C =10 mol/l) of the system results in the electrostatic destabilization of particles of cooperative polycomplex with the formation of rather bulky non-ordered aggregates being the products of the statistical interaction of albumine with polyelectrolyte. It was found that the formation of a complex between albumine and polyelectrolyte is an equilibrium process. The analysis of the BSA-PVEP systems by the sedimentation method showed that the desalting solution of polycomplex after adding of NaCl salt was acquired the same distribution on sedimentograms as a native BSA-PVEP complex.

Let us dwell on the analysis of the formed polycomplex composition and come back to Figure 6 where the areas of free PVEP sedimentation peaks are plotted vs. the ratio of the number

of protein molecules to that of PVEP chains in the system. The intersection points obtained at the extrapolation of these plots to the zero area of the free PVEP peak correspond to n_{BSA}/n_{PE} when all polycations are bound to a complex with BSA. Taking into account the above proved fact of the quantitative binding of BSA with PVEP one may consider that $\lim(n_{BSA}/n_{PE}) = N_i$, when $P_0 \to 0$, (P_0 is the area of the free PVEP sedimentation peak). This limit equals the number of the protein molecules bound by a polycation of a given degree of polymerization under given conditions. It fallows from Figure 6 that the higher the degree of polymerization of PVEP, the higher N_i is. The dependence of N_i on DP of PVEP for the different protein-polycation systems is given in Figure 8. Within the experimental error these dependences are linear. It means that in the average the site of the polycation chain of the approximately constant definite length L is used for such globule binding.

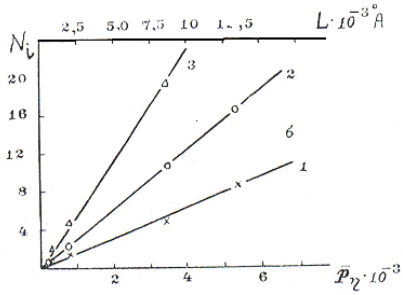


Figure 8. Dependence of N_i; on degree of polymerization (P_η) of the polycation for the FDH-PVEP (1), BSA-PVEP (2); BGG-PVEP (3).

When the composition of the mixture is $N_i = n_{BSA}/n_{PE}$ (the characteristic composition corresponding to disappears of free polycation fraction in sedimentograms) only the polycomplex particles with the characteristic composition are present in the solution. The structure of such particles may be represented as a totally of the BSA globules bound by the salt bonds with the wound on them polycation chain. Most charged groups of the polycation in the polycomplex remain free. These groups are situated in loops on the surface of the particles with the characteristic composition and provide its solubility in water. When the new protein particles are added to the reaction mixture ($n_{BSA}/n_{PE} = N_i$), the electrostatic binding of BSA globules to free sites of the polycations being in soluble polyelectrolyte complex occurs. It is followed by an increase in the particle molecular mass and sedimentation coefficient. As a result of such interaction the total positive charge of the polycomplex particles decreases resulting in a decrease of their affinity for the solvent. As it is seen in Figure 9, an increase in the protein content in the polycomplex particles results in a decrease in the inherent viscosity of their solutions being in an agreement with the considered above results. However, the polycomplex particles still keep an ability to polyelectrolyte swelling. When the content of the protein in the reaction mixture is close to the values corresponding to the point of precipitation of the polycomplexes from the solution, the quantities A_2 of polycomplex solutions have the minimal values [34,35].

From studies of the complexation of proteins with both anionic and cationic polyelectrolytes one can concluded that protein-polyelectrolyte complexes are stoichiometrically formed through salt linkages. The addition of inorganic electrolytes generally decreases the electrostatic interactions of polyions due to charge shielding effect. For complexation between protein and polyelectrolyte, the salt effect produces a decrease in the degree of initial binding, as shown in Figure 10 for NaPSS-RNA [83] and BSA-PVEP systems. Reduced protein binding with increasing ionic strength was also found for the lysozyme-PMAA system by Anufrieva [68]. The addition of salt can also affect the stability of the protein-polyelectrolyte complex. For example, Morawetz [30] found that complexes formed by catalase and polyacrylic acid more readily

precipitate upon increase in $BaCl_2$ concentration. However, an opposite effect is shown for the complexation of BSA and PDMDAAC [62], where the phase separation pH goes higher at high ionic strength. It seems that the reduction of the electrostatic interaction by adding salt can stabilize complexes or may also make complexes aggregate. The effect of ionic strength on the mechanism of protein–polyelectrolyte binding was studied for the BSA-PVEP system by Mustafaev [81] and recently for the BSA-PDMDAAC system by Dubin et al. [79]. It was found that the polycomplexes are stoichiometrically formed in low ionic strength solution. At high ionic strength the complexes are formed with a broad distribution in structure.

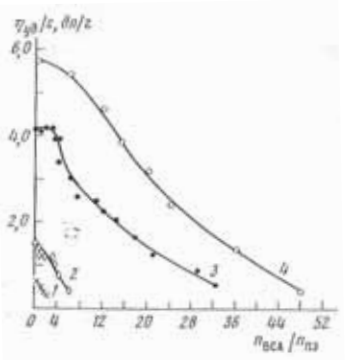


Figure 9. Dependence of inherent viscosity ($\eta_{SP/C}$) of BSA-PE mixture solutions on n_{BSA}/n_{PE} for PEVP with different degree of polymerization Pη: $0{,}19.10^3$ (1); $0{,}95.10^3$ (2); $3{,}3.10^3$(3); $5{,}6.10^3$(4); C_{PE}=0,15 g/100ml; pH 7.0; 25°C.

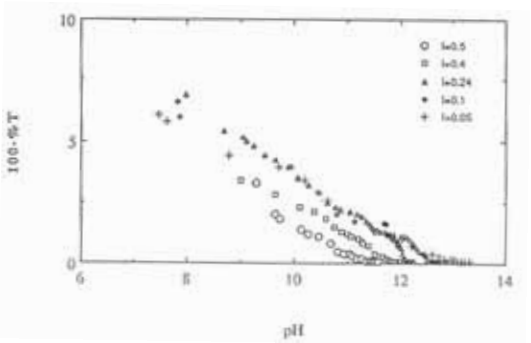


Figure 10. Turbidimetric titration of NaPSS-RNAse system in NaCl solution with various ionic strength. Note the inverse dependence of pH_c on I.

Since the complexation between proteins and polyelectrolytes is caused by Coulomb interaction. It has been evident that increasing pH promotes the formation of protein-polycation complexes, and decreasing pH enhances complexation of proteins and polyanions. Clearly, the higher the ionic strength, the lower the critical pH, since proteins (for example RNAse [79]) needs more positive charge to overcame the charge shielding at high ionic strength. Ohno and coworkers investigated the interactions of human serum albumin (HSA) and poly(N,N-dimethyltetramethyleneammonium bromide) [86]. This polycation interacts with HSA through electrostatic forces and the extent of interaction was found to be pH sensitive. The binding can be complicated due to the different polycomplex compositions that occur with wide molecular weight distributions of nonfractionated polycations. From the fluorescence study of papain–PVS

(poly(vinyl alcohol sulfate)) complex, Cha et al. [86b] and recently of serum proteins–quaternized poly-4-vinylpyridine complexes Mustafaev et al. [65] found that the intensity and emission shift for the complex strongly depends on the pH, as shown in Figure 11. Analogous results were obtained by sedimentation method [34].

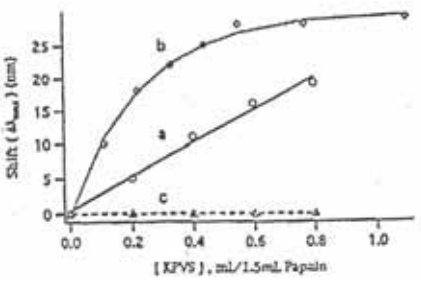


Figure 11. Effects of pH on the emission shift of papain-KPVS complexes. (*a*: pH 2, *b*: pH 7, and *c*: pH 10). (from Ref. [87]).

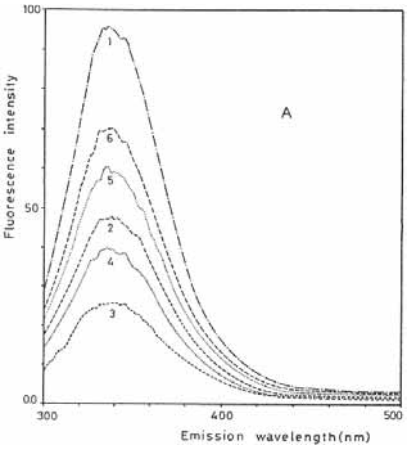


Figure 12. Fluorescence spectra of HAS at 5,7μM (1) in the presence increasing amounts of PVEP at pH 7.0. R_i (n_{PVEP}/n_{HSA})=0,035 (2); 0,14 (3); 0,21 (4); 0,35 (5); 0,7 (6); pH 7.0; 25˚C

Initial complexation between polyelectrolytes and proteins can even occur under conditions where the net protein charge is the same as that of the polyelectrolyte. From studies of the complexation of BSA, chicken in egg lysozyme, and bovine pancreas ribonuclease with anionic and cationic PE, Dubin et al. [79b] concluded that the results obtained in samely charged systems may be attributed to non-uniform protein charge patches and these may be observed by computer modeling [79b]. There is little systematic study of the effect of PE charge density and polymer concentration on protein-polyelectrolyte complexation. Samsonov [42] in 1969 by using size-exclusion chromatography method showed that the strength of the complexes formed by RNAse and copolymers of sodium vinyl pyrrolidone and vinyl sulfonate diminishes with reduction in the content of sodium vinyl sulfonate in the copolymer and with increase in the content of the Na ions in the solution. One exception is the observation of strong interaction between BSA, RNAse or lysozome and PVS, a polyanion with a large density [87]. It was found that the critical initial binding pH increases with the polyion negative charge density even when the protein binding is occurring at pH_{IEP} (isoelectric point). This was explained by recognizing that the increased electrostatic attraction between the more densely charged polyions and a local protein positive region overcomes possible repulsion between the polyanion and the global protein charge. In the study of BSA-PDMDAAC complex [79b], it was found that the initial complexation pH is insensitive to the concentration of BSA and PDMDAAC. For several protein-polyelectrolyte complexes, Kokufuta [87,87a] found that the amount of polyelectrolyte needed to precipitate a protein is linearly proportional to the amount of the protein in solution. The concentration dependence for the efficiency of protein-polyelectrolyte phase separation was also reported by Morawetz [30] for several other systems.

Protein structure in Polycomplex particles. As mentioned above the formation of polycomplexes in the mixtures PE-protein was intensively studied by different physico-chemical methods (hydrodynamic, titration, light-scattering, spectrophotometric, etc.). These methods

provide general information about the binding of components and the structure of complex particles, while the information about the structure of protein globules in these particles is practically absent.

Strelzowa et al. [70] have observed circular dichroism spectroscopy (CD) spectral changes in the range of 250-330 nm for -chymotrypsin upon mixing the protein with dextran sulfate. However, the CD spectrum of -chymotrypsin is not affected by adding dextran sulfate to the protein solution. Dubin et al. [79b] studied the complexation of PDMDAAS with papain, insulin and hemoglobin by CD spectroscopy. The CD spectra for papain and insulin show an α-helix structure change upon complexation, but the structure of hemoglobin is not affected by complexation.

On the bases of the results obtained in the BSA-PVEP mixtures by Optical Rotary Dispersion methods [34]. It was shown that interaction between BSA and PVEP is not followed by any changes of the protein secondary structure.

Fluorescence techniques have recently been used to study protein-polyelectrolyte complexation [69,88,89]. Previously reported on the mechanism of PEC formation by using fluorescence measurements [88] in anthracene and pyrene-loaded artificial polymer systems. On the other hand, the fluorescence of tryptophan (Trp) residues in proteins has been widely used as a probe of the conformational changes of the proteins [89-96]. Therefore, the spectral changes in fluorescence, especially the emission maximum (I_{max}), were used as parameters for the conformational change of proteins induced by the formation of polyelectrolyte complexes with synthetic PE. From the fluorescent emission shift of tryptophan residues in proteins, it is possible to localize the interaction between proteins and PE at certain protein domains. In recent fluorescence studies of papain complexation with potassium poly(vinyl alcohol sulfate) (KPVS), Frank et al. [74] found a blue shift in the emission spectra of papain in the presence of KPVS. These blue shifts were interpreted as the result of shielding of tryptophan residues from aqueous media by the complexed KPVS chains. At increased papain concentration, the shielding effect was found to decrease (see Figure 13).

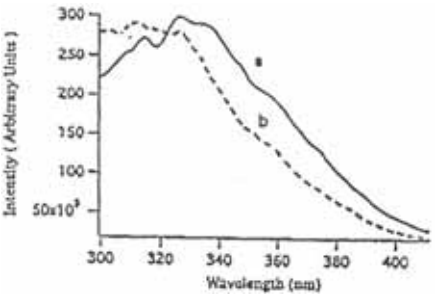


Figure 13. Fluorescence emission spectra of papain: KPVS complexes excited at 280 nm. (*a*: papain, and *b*: papain: KPVS = 1.5 : 0.8). (from Ref. [74])

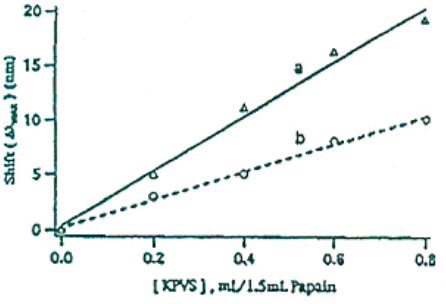


Figure 14. Effects of KPVS concentration on papain: KPVS complexation. ([papain] = *a*: 3 x 10^{-6} M, *b*: 1 x 10^{-5} M). (from Ref. [74])

Thus, a high degree of complexation at low papain content was suggested. The first structures and the conformational changes of albumin have been clarified, especially for human serum albumin (HSA) and bovine serum albumin (BSA). The BSA molecule is known to contain two tryptophan residues. One of them is located on the bottom of hydrophobic cleft between domains 1 and 3 whereas the other is on the surface of the molecule [92-94]. HSA contains a naturally occurring single Trp residue, which is surrounded by layers of an amorphous and permeable protein matrix [94]. Teramoto et al. [88] studied the interaction of BSA and HSA with

polyelectrolytes using fluorescence measurement. Figure 16 shows the pH dependence of the emission maximum (I_{max}) of the Trp of BSA in the presence of polycations (A) or polyanions (B).

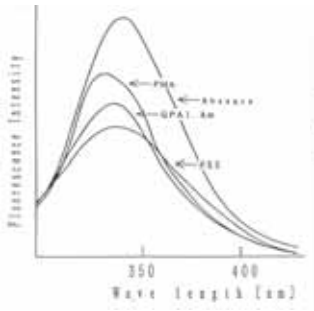


Figure 15. Fluorescence spectra of BSA in the absence or presence of polyelectrolytes at pH 7.4. [BSA] = 4 x10^{-2} (g/L). [Polyelectrolyte] = 2 x 10^{-4} (unit mol/L).

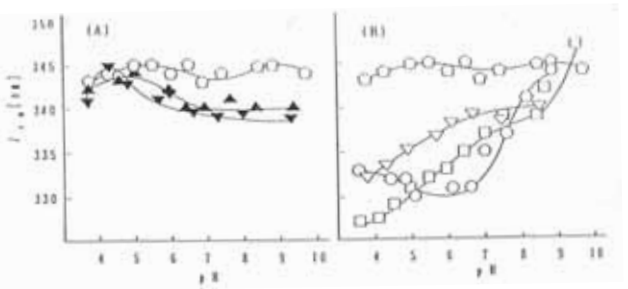


Figure 16. pH dependence of λ_{em} of tryptophan of BSA in the presence of polyelectrolytes in water. (A) polycations, (B) polyanions. (⌂) BSA, (▲) BSA+ QPA1.Am, (▼) BSA + PQE, (○) BSA + PMA, (□) BSA + PSS, (Δ) BSA + PNMA. [BSA] = 4 x 10^{-2} (g/L). [Polyelectrolyte] = 2 x 10^{-4} (unit mol/L).

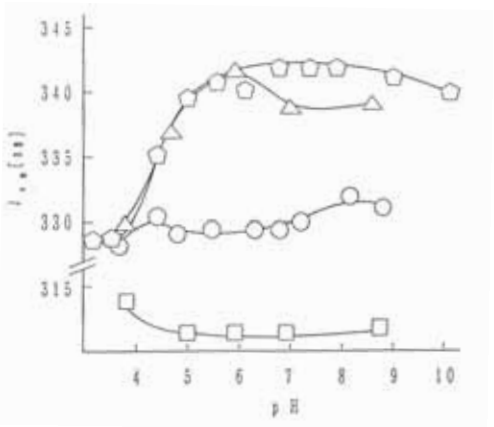


Figure 17. pH dependence of λ_{em} of tryptophan of HSA in the presence of polyelectrolytes in water. (⌂) HSA, (▲) HSA + QPA1.Am, (○) HSA + PMA, (□) BSA + PSS, [HSA] = 4 x 10^{-2} (g/L). [Polyelectrolyte] = 1 x 10^{-4} (unit mol/L).

QPA1.Am-poly (allyltrimethylammoniumchloride), PQE-poly (2-acryloylethyltrimethylammonium chloride), PMA-poly(methacrylic acid), PNMA-poly(N-Methacryloyl- - d,l-alanine), PSS-poly(sodium styrenesulfonate) After adding polycations, a slight blue shift of λ_{em} was observed only in the pH region higher than the isoelectric point (pI=4.7) of BSA. The difference of the structural formulas of polycations exerted little influence upon λ_{em}. Thus it is found that polycations can interact randomly with anionic sites of BSA through electrostatic interaction and induce a slight conformational change around the Trp of BSA. On the other hand, polyanions induce a marked blue shift of λ_{em} in pH regions lower than 9, where the net charge of BSA is negative. It is well known that albumin contains specific binding

sites for drugs, where basic amino acids are localized. Acidic compounds (e.g., anionic surfactants, fatty acids, and so on) are bound to these binding sites more easily than are cationic compounds [88]. BSA has two Trp's; one (Trp212) of the two exists in the neighborhood of drug binding Site II [88]. These results suggest that polyanions interact selectively with Site II of BSA even in the alkaline pH region to change the orientation of Trp212. However, λ_{em} was shifted further toward a shorter wavelength with a decrease of pH. This seemed to be due to a conformational change around another Trp (Trp134) that occurs when the net charge of BSA becomes positive to facilitate the formation of PEC with polyanions. The interaction mechanisms between BSA and polyelectrolytes are summarized schematically in Figure 21.

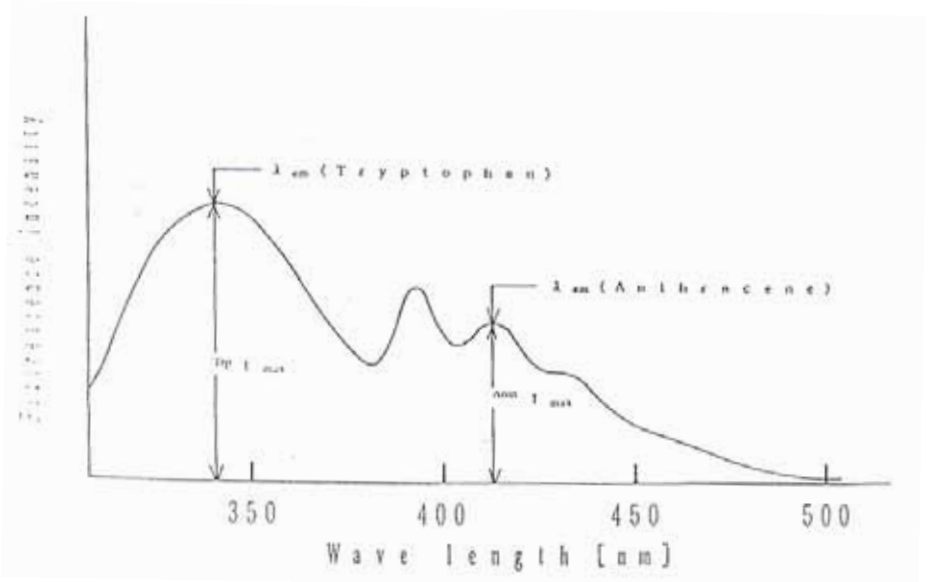


Figure 18. Typical fluorescence spectrum of BSA with Anth-loaded polyelectrolytes. [BSA] = 4 x 10^{-2} (g/L). [Polyelectrolyte] = 2 x 10^{-4} (unit mol/L).

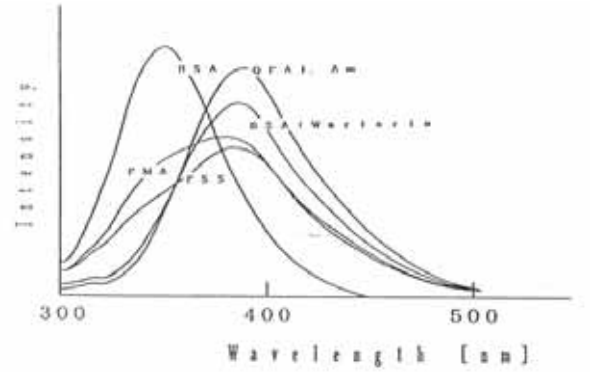


Figure 19. Fluorescence spectra of warfarin-bound BSA in the presence of polyelectrolytes at pH 7.4. [BSA] = 4x10^{-2} (g/L). [Warfarin] = 3 x 10^{-5} (mol/L). [Polyelectrolyte] = 1 x 10^{-4} (unit mol/L).

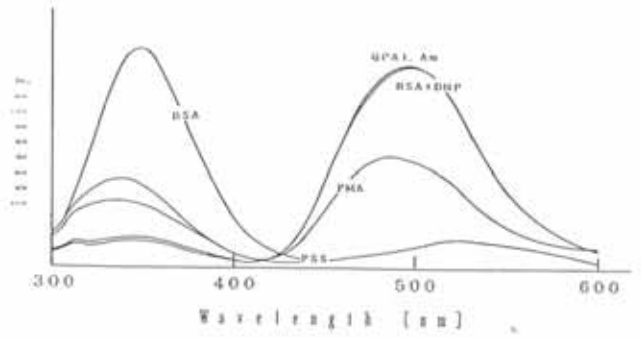


Figure 20. Fluorescence spectra of dansyl-L-proline (DNP) BSA in the presence of polyelectrolytes at pH 7.4. [BSA] = $4x10^{-2}$ (g/L). [DNP] = $3x10^{-5}$ (mol/L). [Polyelectrolyte] = 1 x 10^{-4} (unit mol/L).

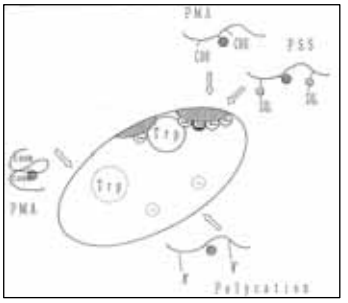


Figure 21. Schematic representation of the complexation of albumin with polyelectrolytes.

Study of a BSA and HSA tryptophan fluorescence for the mixtures of proteins with quaternized poly-4-vinylpyridine salts in homogenous systems at different component ratios permits elucidation of some important features characterizing polycomplex formation [69]. As shown in Figure 22 the fluorescence intensity (I_{max}) of BSA at pH 7 decreases (quenching) with adding of PE solution to BSA solution. Depending on the ratio of components the quenching passed through a minimum and then increased to a constant value.

However, as the fluorescence is quenched by increasing PE concentration, the wavelength values at the maximum of emission (λ_{max}) practically do not change and simply for pure proteins. These results indicate that the emission intensity quenching is due to the binding of protein molecules to the polycation chain and complexing is do not induce a essentially conformational change around the Trp of protein globules. The maximum number of BSA and HSA molecules are bound to the polymer at about n_{BSA}/n_{PE} = 10; this is based on the maximum reduction in fluorescence intensity and have been in a pretty correlation with the results obtained by sedimentation analysis [34].

Structures of the Protein-Polyelectrolyte Complexes. Hydrodynamic (Viscosity and Sedimentation) and Light Scattering Results Mustafaev reported a considerable decrease in the reduced viscosity ($\eta_{sp/C}$) of quaternized poly-4-vinylpyridines (PVEP) in pH 7 aqueous solution, upon addition of BSA [34]. The decrease in viscosity was explained by the formation of polycation-BSA soluble complexes. The dependences of the inherent viscosity of PVEP-BSA polycomplex solutions with the compositions N = n_{BSA}/n_{PE} (characteristic ratio) and N on their concentrations are given in Figure 22 It is seen that a decrease in the solution concentration is

fallowed by an increase of the inherent viscosity showing the ability of the polycomplex particles to polyelectrolyte swelling.

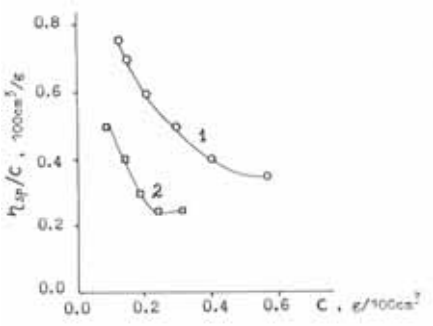


Figure 22. Dependence of the inherent viscosity of Q-PVP-BSA solution on SPEC concentration. $\xi = 3.3 \times 10^2$ (1), $\xi = 2.5 \times 10^2$ (2), $DP_{Q\text{-}PVP} = 10^3$.

In a more recent study of human hemoglobin-dextran sulfate mixtures, Nguyen: found that $\eta_{sp/C}$ decreases with the polymer concentration, as shown in Figure 22. In contrast to the behaviour of polyelectrolytes in pure water, as embodied by the relationship $\eta_{sp/C}$ on C, the ionic interactions between particles seems to be reduced through complexation, which is reflection of the process of particularly charge neutralization. Similar behaviour also described by Kuramoto [ ] for BSA in the presence of poly(acrylic acid). The dependence of sedimentation coefficients of PVEP-BSA complexes on n_{BSA}/n_{PE} for PVEP with different degree of polymerization (DP) is given in Figure 22.

It is seen that in the range of $n_{BSA}/n_{PE} = N$, i.e. over the range of ratios when free polycations are still present in the system a change of S (coefficient sedimentation) is relatively small .It follows from the above mentioned data that when the amount of the added protein at [PVEP]=const is increased, the accumulation of the complexes occurs in this region. The composition of these complexes for each given PE is constant and is determined by the polycation contour length. Remarkable that in this region of ratios a change of fluorescence intensity (I_{max}) also is relatively small. However, when the values of n_{BSA}/n_{PE} coinciding with N are reached a sharp increase of the sedimentation coefficients of the product of BSA interaction with polycations occurs. The peak of the free BSA is not observed yet in the sedimentograms (see Figure 2, curve 6). It shows that in spite of the free PVEP consumption protein binding continues. In other words, the above described complexes, when an additional amount of BSA is supplied; find additional resources for its adsorption.

The further definition of the PVEP-BSA complex structure is obtained from the comparison of the inherent viscosities and the sedimentation coefficients for these complexes formed by the polycations with different length when $n_{BSA}/n_{PE} = N_i$. It is seen in Figure 23. That the viscosity of the solutions sufficiently sharply increases with increasing degree of polymerization of the polycation. At the same time the sedimentation coefficient remains unchanged.

The relation of this kind may exist only for sufficiently extended particles at their elongation (strictly speaking for extended rigid rods). To determine the shape of such particles an investigation of the intrinsic viscosity of the solutions of the indicated polycomplexes with the composition N was carried out. To exclude the effect of polyelectrolyte swelling the viscosity measurements were performed in the regime of izoionicdilution. Figure 24 shows the dependences of the inherent viscosity for the solutions of PVEP-BSA polycomplexes with the characteristic composition on the solution concentration.

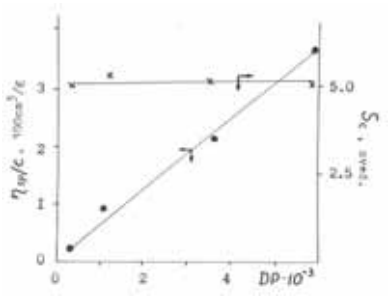


Figure 23. Dependence of the inherent viscosities (η_{sp}/C) and the sedimentation coefficients for Q-PVP-BSA polycomplexes when $n_{BSA}/n_{Q\text{-}PVP} = N_i$ on DP of the polycation.

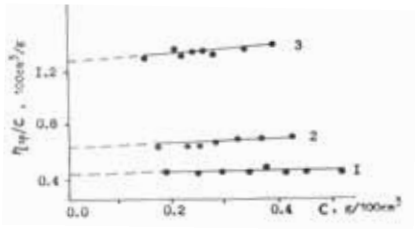


Figure 24. Dependence of the inherent viscosity of Q-PVP -BSA polycomplex solution on its concentration. Isotonic dilution. ξ = 3.3 x10^2 (1), $DP_{Q\text{-}PVP}$= 10^3 (1); 2.3 x 10^3 (2); 2.6 x 10^3 (3).

It is seen that such dependences are linear over the whole range of the solution concentration indicating the absence of polyelectrolyte swelling of polycomplex particles.

As it was above mentioned only the particles of the soluble complexes are present in the BSA-PVEP homogeneous mixtures at the protein content equal or somewhat exceeding the characteristic composition. It permits to study such solutions by light scattering. The molecular parameters of a macromolecule are usually obtained from static light scattering results by using Zimm-diagrams corresponding to Equation:

Where c is the mass concentration of polymer, K is a constant which contains the optical parameters of the system; Mw and R_g are the weight average molecular weight and the root mean square radius of gyration of the macromolecule, respectively; and A_2 is the second viral coefficient. The molecular characteristics of the particles of the soluble PVEP-BSA complexes with the composition $n_{BSA}/n_{PE} = N_i$ were determined by light scattering measurements using the double extrapolation method [34,35,66]. For all investigated polycations over the whole studied range of concentrations the dependence of K_c/R_0 on the concentration was linear, indicating the absence of dissociation of the polycomplexes at dilution. In other words, the studied soluble PVEP-BSA polycomplexes are stable over a wide range of the solution concentrations and polycomplex composition.

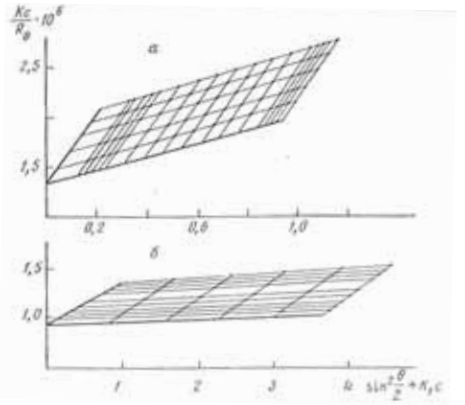


Figure 25. Zimm-diagrams for BSA-PVEP mixtures at different nBSA/nPE: 7(a); 11 (b); Pη=2,5.10^3; İ=0,01 mol/l; $K_ı$ (optical constant of solution)=10^2; pH 7.

The results of the studies of PVEP–BSA complex solutions with different composition formed by polycations with a different molecular mass, Mw, are listed in Table 2

Table 2. Molecular characteristics of the particles of soluble Q-PVP-BSA polycomplexes with different composition at pH = 7, in 0.01 N NaBr.

$M_{w_{Q\text{-}PVP}}$	Mixture composition $\xi \cdot 10^{-2}$	$n_{BSA}/n_{Q\text{-}PVP}$	$(\partial n/\partial c)_{\mu}$ dl/g	$M_w \cdot 10^{-5}$	$A_2 \cdot 10^4$	Polycomplex composition $n_{BSA}/n_{Q\text{-}PVP}$
$1.2 \cdot 10^5$	3.3	3:1	0.188	3.6	3.25	3.4:1
	2.5	4:1	0.179	4.2	-	4.3:1
	2.0	5:1	0.181	5.1	3.33	5.5:1
	1.8	6:1	0.200	5.7	2.08	6.4:1
$2.5 \cdot 10^5$	3.3	7:1	0.188	7.6	2.16	7.3:1
	2.5	9:1	0.179	8.6	0.94	8.7:1
	2.0	11:1	0.181	10.5	0.72	10.3:1
	1.7	12:1	0.200	11.2	0.50	12.4:1
$3.2 \cdot 10^5$	3.3	8.1	0.188	8.9	0.82	8.1:1
	2.5	11:1	0.179	11.3	0.23	11.6:1
	2.0	14:1	0.181	13.2	0.17	14.3:1
	1.8	16:1	0.200	14.1	0.01	15.6:1

As it is seen an increase in the complex molecular mass with increasing protein content in the reaction mixture is characteristic of all the studied PVEP-BSA systems. If to know the molecular masses of the molecular masses of the complex particles and those of individual components it is possible to calculate the molecular composition of the complex particles. The results of such calculations are listed in Table 2. It is seen that at any content of protein in the mixture being in excess of the characteristic composition the soluble polyelectrolyte complex composition within the experimental error coincides with that of the reaction mixture. This fact is in agreement with the high speed sedimentation data and indicates that only one PVEP macromolecule enters each polycomplex particle over the studied range of concentrations and component ratios. Since an increase in the polycation DP results in an enhancement of the number of BSA globules in the polycomplex and the number of protein molecules increases proportionally to the polycation length, it is also convenient to express the polycomplex composition in terms of the ratio $\xi = \frac{DP}{N_i}$, where N_i is the number of BSA molecules sorbed by one chain of PVEP. This ratio does not depend on the polycation molecular mass. Note that the polycomplexes with the characteristic composition formed by BSA and the polycation chains of different length correspond to the composition $\xi=3.3 \times 10^2$ An increase in BSA content in the polycomplex results in a decrease in the ξ value.

As it seen in Table 2 PVEP-BSA polycomplex particles of the characteristic composition have the minimum molecular mass and the greatest affinity for the solvent. Addition of the protein to the reaction mixture in amounts exceeding the characteristic ones causes together with an increase in molecular mass of polycomplex particles a decrease in the values of the second viral coefficients of the solutions. This behaviour of the systems permits to assume that the addition of the protein to the solution in amounts greater than N_i causes a reaction in which the particles of the polycomplexes with the characteristic composition and free protein globules are interacting components. Since under the conditions of the reaction when pH=7 BSA globules are negatively charged (pI = 4.9) it is responsible to assume that ionogenic groups of the polycation included to the polycomplex and having not formed salt bonds with protein molecules

are responsible for BSA binding to polycomplexes with the composition $\xi=3.3 \times 10^2$. The existence of the charged polycation sequences in the soluble polycomplexes directly fallows from the viscometric data.

The further definition of the PVEP-BSA complex structure is obtained from the comparison of the intrinsic viscosity and the molecular weight for these complexes. Figure 26 shows the dependence of the intrinsic viscosity logarithm for solutions of the polycomplexes with the characteristic composition on the logarithm of their molecular weight.

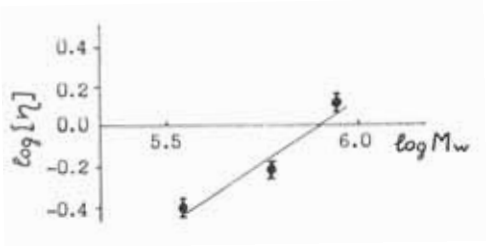


Figure 26. The dependence of log[η] on log M_w for the Q-PVP-BSA SPEC. $\xi = 3.3 \times 10^2$.

This dependence obeys Mark-Kuhn-Houwink equation:

$[\eta]=KM^a$ with the parameters $K=0{,}11.10^{-5}$ and $a=1.4 \pm a^3$

The latter value corresponds to rather rigid asymmetrical rods and suggests a rod-like model for the soluble polyelectrolyte complex particles. To determine the shape of the polycomplex particles with the composition $\xi=3.3x10^2$ the dependences of the root mean square radii of gyration on their molecular mass Mw were studied by light scattering. It is seen in Figure 27 that these dependences are linear for all studied compositions.

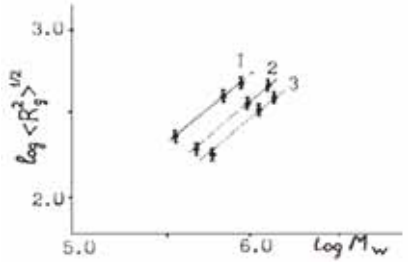


Figure 27. Dependence of log $<R^2>^{1/2}$ on $\log M_w$ for the particles of the Q-PVP-BSA complexes. $\xi = 3.3 \times 10^2$ (1); $\xi = 2.0 \times 10^2$ (2); $\xi=1.8 \times 10^2$ (3).

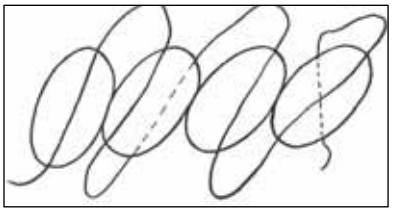


Figure 28. Proposed structure of Q-PVP-BSA soluble complex with characteristic composition.

It means that the polycomplexes with the similar composition may be considered as the representatives of one polymer homologous series. The dependence of the root mean square radius of gyration on the molecular mass of the polycomplex particles can be described by a known equation: $[R_g^2]^{1/2}=\text{const}.M^{\alpha}$ where $\alpha= 1 + 0.15$ that also corresponds to the asymmetrical rods.

Model of the Structure of Polycomplex particles with characteristic composition (N_i). Based on above-mentioned results Kabanov and Mustafaev proposed a rod-like model for the soluble polyelectrolyte complex particles with characteristic composition. This is shown in Figure 28.It should be accepted that protein globules join with each other in some way forming an asymmetric

stack. The latter may be approximated by a cylinder. The polycation chain encircles the butt-joined protein globules and the longer the polycation chain the longer the cylinder is.
It is remarkable that the described above scheme of the complex structure, obtained as a result of the analysis of the overall physical-chemical measurement data is confirmed by the data of electron microscopy. The complex micrograph obtained for the systems (BSA-PVEP and BGG-PVEP) in which $n_{BSA}/n_{PE}= N_i$ are given in Figure 30.

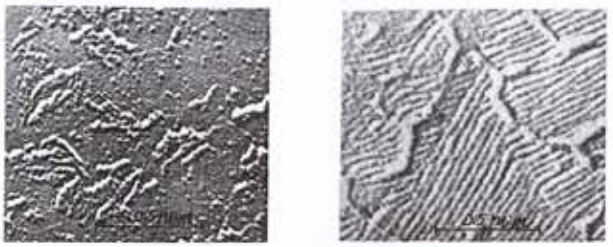


Figure 29. Micrographs of Q-PVP-BSA polycomplex when $n_{BSA}/n_{Q\text{-}PVP} = N_i$ DP=10^3. a-sample is prepared by putting a drop of the complex solution (0.01 g/dl) on the preparative grid with a substrate and removal of the solution bulk with filtering paper; b- sample is prepared by a slow evaporation of the solvent from the complex dialyzed solution (0.01 g/dl).

Extended linear formations consisting of globular particles coupled one with the other is distinctly seen in the micrograph. The thickness of each rod consisting of globules is about 100 A°. The solubility of the complex was attributed to the hydrophilic contribution from the loops of the bound polymer chain.

Figure 30. Electron micrographs of PVEP-BGG polycomplex when n_{BGG}/n_{PE}=2, DP=0,95.10^3. (Sample preparation as in the case of BSA-PVEP).

Model of the Structure of Polycomplex particles over the component ratios corresponding characteristic composition (at $N > N_i$). As shown in Figure 31an increase of the number of protein molecules in the mixture (the weight concentration of polycation is kept constant) leads to a decrease of the values of the reduced viscosity (η_{sp}/C) of PVEP-BSA mixtures When the ratio of components is $N=N_s$ the successive addition of BSA result in sharply decreases of the reduced viscosity and the (η_{sp}/C) characterized by very low values (0.03-0.05). The described situation is typical for all studied fractions of PVEP interacting with BSA. Comparison of the inherent viscosities and the sedimentation coefficients for these complexes formed by the polycations with different length when $n_{BSA}/n_{PE}=N_s$ shows that as the viscosity of the solutions remains practically unchanged with increasing degree of polymerization of the poycation the sedimentation coefficients for polycomplexes increases. As it fallows from light scattering data of the polycomplexes at any content of protein in the mixture being in excess of the characteristic

composition ($N > N_i$) an increase in the complex molecular mass (Mw) is characteristic of all the studied PVEP-BSA systems (Table 2) From obtained values of Mw of complexes and ratios of components it fallows that only one polycation chain is contained in the particles of BSA-PVEP complexes. Thus, the obtained relation of this kind may exist for sufficiently high compactness of particles of soluble polycomplexes at their elongation. These data allow to consider the structure of water-soluble PVEP-BSA complexes formed at $N > N_i$ (N_s) also as conglomerates of BSA globules assembled by one chain of PVEP. In this model the bound polycation chains are localized in a hydrophobic center wherein the positive charges from PVEP are neutralized by the negative BSA charge; the hydrophobic center is also surrounded by negative charged BSA, which provides a net complex surface charge and is thus responsible for the solubility of the complex (Figure 31).

The phase separation in the PVEP-BSA systems occurs only at some critical protein concentrations ($n_{BSA}/n_{PE} \geq N_s$) depending on the degree of polymerization of polycation (see Figure 31). As it follows from the curve of turbidimetric titration of a PVEP solution with a solution of proteins (BSA, OA, BGG) and vice versa titration of protein solutions (BSA, HSA, BGG) and mixtures of different proteins (BSA+BGG, BSA+β_1-G; β_1-G+BGG, BSA+β_1-G+BGG, whole serum and blood) with solutions of polycations one can distinguish three regions of reaction mixture composition (Figure 30).

In region I (for the systems when polymer solutions were titrated with protein) the solutions remains homogenous due to formation of water-soluble polyelectrolyte complexes. The successive addition of protein molecules at $n_{BSA}/n_{PE} > N$ leads to phase separation-region II. The maximum value of turbidity, as it was noted in numerous studied polyelectrolyte-protein systems, is reached at equivalent ratio of oppositely charged groups of proteins and polycations. The addition of the protein above this equivalent ratio is accompanied by a decrease in the turbidity of the solution, and at some critical ratios (region III) the system again becomes homogeneous. The soluble fractions of PVEP-BSA mixtures (supernatants) was studied by sedimentation analysis after removing of the precipitate. Figure 31 shows the dependence of the amount of precipitate (1), concentration (area of the peaks) (2) and coefficient sedimentation (3) of soluble complexes and concentration of free protein molecules in the supernatants (4) on the ratios of components.

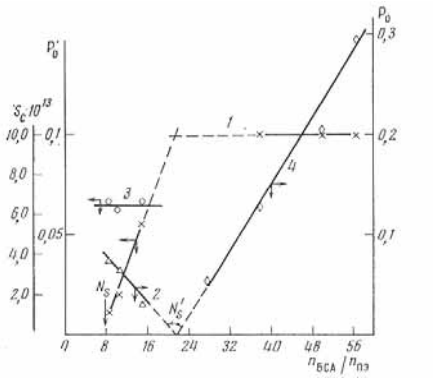


Figure 31. Dependence of the amount (P_o^1) of concentration (2) and sedimentation coefficient (3) of soluble complexes and concentration of free protein molecules in matrix solutions (4) on n_{BSA}/n_{PE}; CPE=0,15 g/dl; pH 7.0.

From the linear form of these dependences it follows that an increase protein concentration in mixture leads to a corresponding increase of the amount of precipitate and then attains a limiting value, while the concentration of soluble complexes decrease and their peak on sedimentograms disappears at N_s ratios (region II.). Sedimentation coefficients of the peaks remain the same; the composition of soluble complexes remains virtually unchanged. At the

ratios corresponding to maximum value of precipitate there are no reaction components and products (the protein, the polycation or water-soluble polycomplexes) in the solution. The addition of the protein above this ratio is accompanied by a decrease in the turbidity of the solution, and at some critical values of ratios (region III) the system again becomes homogeneous.

As it mentioned above about 55 negatively charged carboxylate groups of the native BSA molecules are able to form salt bonds with the polycation, though the total number of carboxylic acid groups of BSA is 125 (50 Asp +75 Glu) [97]. It means that only part of the carboxylic groups settled on the surface of BSA globule is accessible for the interaction. The composition (Q) of polycomplex [98,99] was expressed as the ratio of the total quantity of negative charges on the protein molecules (a) to the total number of positive charges on the polycations (b) in the mixture. The value of Q is related to the mass concentration of the components C (g/l) by the equation:

$$Q=a^-/b^+ = 55 \times (C_{BSA}/M_{BSA}) \times (M_{PEVP}/C_{PEVP})$$

Figure 32 shows the dependence of relative optical density A/A_{max} of a mixture of BSA and PVEP on the mixture composition Q. On sedimentograms of the solutions (supernatants) obtained at Q>1 two peaks are observed. The sedimentation coefficient 4.3 S of slowly sedimenting particles ("slow" peak) virtually coincided with that one of free BSA while quickly sedimenting particles were characterized by rather large value 25 S ("fast" peak). This means that at 2.5 > Q > 1 free protein coexists with newly formed water-soluble PVEP-BSA complex particles and insoluble PVEP-BSA complex particles. Addition of BSA till Q=2.5 is accompanied by a concurrent increase in the areas of both peak, and the area of "fast" peak is much greater than that one of "slow" peak. The successive addition of BSA after complete solution of the precipitate (Q > 2.5) does not lead to noticeable change of the "fast" peak area, while the rate of growth of "slow" peak area increases. Sedimentation coefficients of both peaks at Q > 1 (in both heterogeneous and homogeneous regions) remains the same, 4.3-4.5 S and 25-26 S respectively. The character of turbidimetric titrations virtually did not change under variation of PVEP degree of polymerization in the studied region P_{PEVP} = 200-1500.

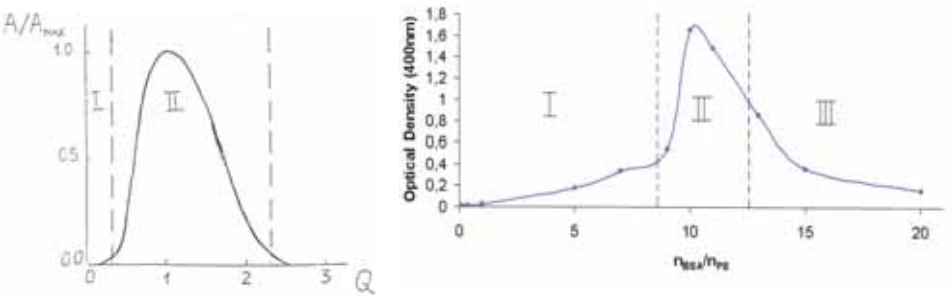


Figure 32A. Dependence of relative optical density A/A_{max} of a mixture of BSA and PEVP or the mixture composition Q. [PEVP] = 1 g/l, Pη=1,3.10^3

B. Dependence of optical density (OD_{400} nm*)* of a mixture of BSA and PEVP on the n_{BSA}/n_{PE} Pη=10^3; pH 7. (unpublished results of Dr. Zeynep Mustafaeva and Eray Dalgakıran).

From the areas of sedimentation peaks of soluble complexes BSA-PVEP and free BSA calculated the amount of BSA (C_{BSA}) including in the complexes and then a composition φ of the complexes, that is expressed as the ratio of total quantity of negative charges on the protein molecules (a^-) to the total number of positive charges on the polycations (b^+) in the complexes:

$$\varphi = a^-/ b^+ = 55 \times (C_{BSA}/M_{BSA}) \times (M_{PVEP}/C_{PVEP})$$

The composition of complexes provide to be not influenced by both P^{-}_{PVEP} and mixture composition Q in the whole studied region Q> 1. The value of this characteristic composition φ_c was equal to 2.0 + 0.3.

Figure 33 shows the dependence of the average molecular mass M_{sd} of the water-soluble BSA-PEVP complex on the degree of polymerization of polycation. The values of average molecular mass M_{sd} of the complexes were calculated from both the sedimentation data and QELS data using Svedberg's formula: $M_{sd} = \frac{RT}{(1-vp)} x(s/D)$, where R is the universal gas constant, T is the temperature (293K), p is solvent density, s and D are respectively the constants of sedimentation and diffusion of the complex particles. The specific partial volumes v of the BSA-PVEP complexes and free BSA virtually coincide, v = 0.75 + 0.03 [100]. From the linear form of this dependence it follows that an increase in the chain length leads to a corresponding increase in the number of protein molecules in the complex, while its composition φ_{sd} remains virtually unchanged. The average value of φ_{sd} calculated from the tangent of the slope of the line in Figure 33 is 1.9 + 0.3 that is in a good agreement with the value φ calculated from sedimentation peaks areas. The number of BSA molecules N_{BSA} and polycation molecules N_{PVEP} in the complex which were calculated from known values of M_{sd}, and the molecular mass of the components are given in Table 2.

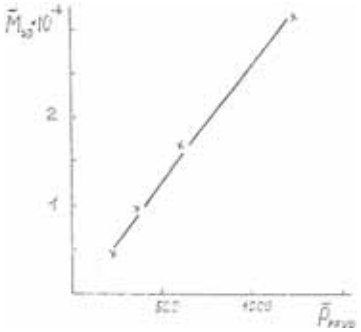


Figure 33. Dependence of the molecular mass $\overline{M}_{sd}$ of the BSA-PVEP complex on the degree of polymerization of the PVEP.

Table 3. Molecular characteristics BSA-PVEP complexes

P_{PEVP}	$\bar{M}_{sd} \times 10^{-3}$, Da	N_{PEVP}	N_{BSA}
250	700	1	10
400	1100	1	15
650	1600	1	20
1300	3000	1	40

From these data is follows that only one polycation chain is contained in the particles of dissolved BSA-PVEP complex.

One can distinguish such three regions of reaction mixture composition also in the solutions of polycations containing the different proteins. Figure 34 shows the curve of turbidimetric titration of a BSA + BGG mixture solutions with a solution of PVEP. Curves of the titration are presented as the dependence of the amount of the precipitate (m). On the concentration of PE which were added to solution of BSA+BGG mixtures with constant concentrations. The dependence passes through a maximum. Starting with very low concentrations of polycation in protein mixtures (Cproteins = 0.5g/dl BSA+0.5g/dl BGG; C_{PE}=0.025 g/dl PVEP Cprot./C_{PE} = 40) phase separation took place, which indicates the formation of an insoluble triple polycomplexes.

On further increase in polycation concentration, the amount of the precipitate increases and then attains a limiting value. In this region as it fallows from sedimentation and electrophoretic datas of the mixtures, after removing of the precipitate, free protein fractions (BSA, sedimentation coefficient 4.5 S and electrophoretic mobility u = 6.0 sm.v.sec.; BGG, 7.2 S and u=1) coexists with soluble and insoluble polycomplexes containing both BSA and BGG. At the ratio of components, which correspond to situation of maximum amount precipitation in mixture, all components were included in composition of insoluble polycomplex particles. In matrix solution there are no polymeric compounds. On sedimentogram of the supernatant solution at C_{prot}/C_{PE}=13 only one peak (sedimentation coefficient 8S) which correspond to soluble polycomplex particles is observed (Figure.curve 4). One can assume that its correspond to mixed ternary PVEP-BSA-BGG complex This means that at C_{prot}/C_{PE}=13 ternary water-soluble complexes coexist with insoluble ternary complexes. After complete solution of the precipitate the soluble complexes protein-PE are formed by separate distribution of individual proteins at the matrix (it is remarkable that decrease of polycation molecules in the system results in the formation of a soluble complex of mixed composition). These polycomplexes (BSA-PVEP and BGG-PVEP) coexists with free polycation macromolecules. Such situation corresponds to those ratios where uneven distribution of protein molecules between polycation chains is observed [34].

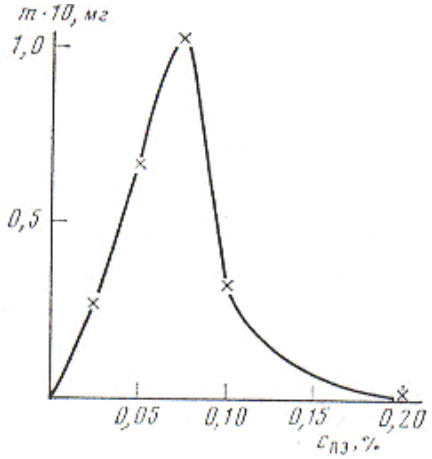


Figure 34. Dependence of the amount (m) of the insoluble complexes on the initial concentrations of adding PVEP (C_{PE}); C_{BSA}=0,5g/dl.

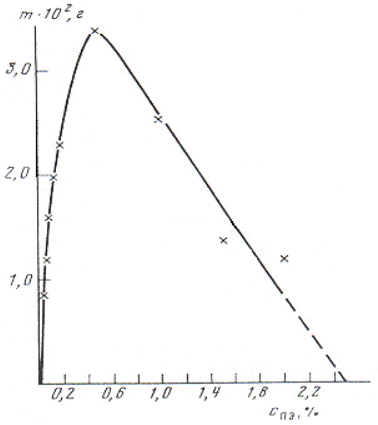


Figure 35. Dependence of the amount (m) of the insoluble complexes on the concentrations of adding PVEP (C_{PE}) in serum-PE mixtures. Initial serum is diluated 1:3 ratio. pH 7.5.

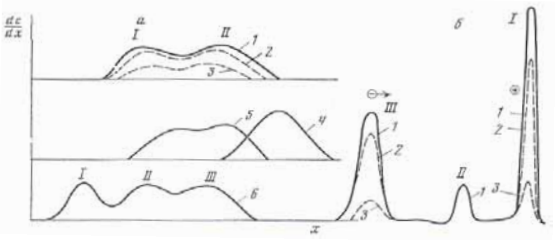


Figure 36. Sedimentograms (a) and electroforegrams (b) of the artificial mixtures BSA+BGG in the absence (1) and in the presence of PE at the different C_{PE}: 0.025 (2); 0.05 (3); 0.075 (4); 0,1 (5); 0.3 g/dl (6); $C_{Proteins}$= 0.5 g/dl BSA+ 0.5 g/dl BGG.

It has been shown that above mentioned dissolve character of insoluble polycomplexes and selectivity at binding protein fractions is observed in both artificially prepared systems (BSA+BGG, β_1-G + BGG, BSA + β_1-G + BGG) (Figure 36) and in serum and whole blood [38]. As it seen in Figure 37 one can observed the same peaks on sedimentograms of the supernatants of PVEP-serum and PVEP-whole blood, which are corresponds to soluble polycation-proteins complexes. Decrease of polycation concentration in the systems results in the formation of a soluble complex of mixed composition. When an insoluble complex is formed serum-PVEP selective sorption of β_1-globulin fractions is observed. These phenomenons were systematically studied by Mustafaev [33, 38].

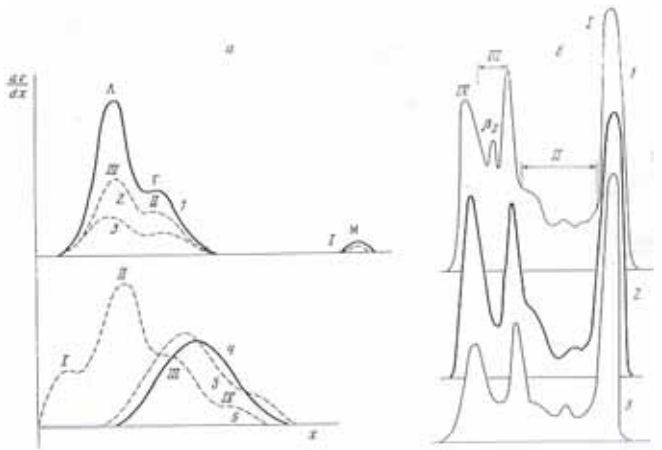


Figure 37. Sedimentograms (a) and electroforegrams (b) initial whole serum and matrix solutions of serum+PE, at different concentrations of PE (g/dl): 0.1 (2); 0.3 (3); 1.0 (4); 1,5 (5); 2.5 (6); pH 7.5; A-albumin, G-globulin, M-macro globulin, serum is diluated 1:3 ratio, with 0,025N NaCl solution.

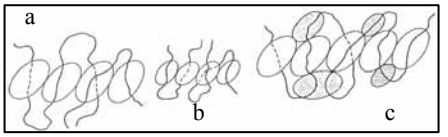


Figure 38. Proposed structures of complexes protein-PE: a-(BGG-PE) at $n_{BGG}/n_{PE} \leq N_i^1$, b-(BSA-PE), (α-globulin-PE), (β-globulin-PE) at $< N_i$; c-structure of polycomplex particles with different protein fraction which form in multicomponent mixtures at $n_{protein}/n_{PE} > N_i$

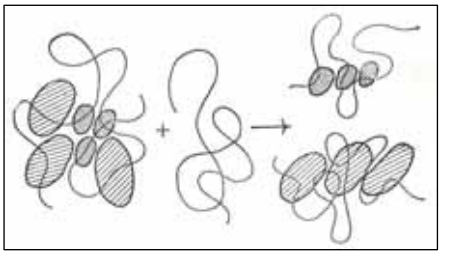


Figure 39. Redistribution of different protein globules between linear polycations

In [34] an important property of protein-polycation complexes was noted, that is the solubility in water when there is more than three-fold excess of positively charged PVEP units relative to negatively charged protein carboxylate groups. Segments of PVEP chains carrying excess charge act as the lyophilizing fragments of the complex particles. Region I in Figure 36

corresponds to formation of such positively charged water-soluble protein-polyelectrolyte complexes (see structure in Figure 38).

The maximum amount of precipitate is reached at equivalent ratio of oppositely charged groups of proteins and polycations (Q=1, n_{BSA}/n_{PE} = N's). The values of N_s and N_s are actually correspond to maximum number of protein globules which can join in composition of water soluble and insoluble polycomplexes at the given degree of polymerization of polycations, correspondingly. As it seen in Table 3 N_s and N_s as well as N_i is linear increased by increasing degree of polymerization, i.e. the length of counter of polycation. It means that in the average the site of the polycation chain of the approximately constant definite length is used for such globule binding, i.e. the number of additionally binding protein molecules per one protein in composition of water-soluble and insoluble polycomplex particles with characteristic composition is constant and equal 2 ($(N_s-N_i)/N_i$=2) and 3, correspondingly. Data obtained in [34] allow to consider the structure of such complexes in this region. Positively charged rod-like polycomplex particles at certain critical n_{BSA}/n_{PE} ratios aggregate with additional number of protein molecules and form soluble and insoluble cooperative complex particles with a more compact structures (Figure 39). In this strongly hydrophobic structure, which is on the limit of solubility, largely charged groups of polycation chain participate in formation of ionic bonds with protein globules.

Region III corresponds to formation of negatively charged water-soluble protein-polyelectrolyte complexes. The precipitate is dissolved by means of formation of the complex, which is almost doubly enriched in the protein. The characteristic composition φ =2 of the dissolved complex is determined by the minimum amount of protein necessary for attachment to the complex to provide the hydrophilicity of the particle as a whole. It is clear that with an increase in Q at Q > 1 ($n_{BSA}/n_{PE} = N_i$) the total number of salt bonds calculated per polycation remains unchanged: only their number taken over one protein globule decreases. Therefore the conclusion of an additional number of globules in the soluble complex of composition, does not occur, since this would lead to a decrease in the total number of particles that is unprofitable entropy wise, and is not compensated for by decrease in the enthalpy of the system. Data obtained in different systems [34,35,98,99] allow to consider the structure of negatively charged water-soluble protein-polyelectrolyte complexes formed at Q > 1 as a conglomerates of BSA globules assembled and reinforced by one chain of polycation. In this model the bound PVEP polycation chains are localized in a hydrophobic center wherein the almost all positive charges from polycation are neutralized by the negative protein charge; the hydrophobic center is also surrounded by negative charged proteins, which provides a net complex surface charge (negative) and is thus responsible for the solubility of the complex (Figure 40).

Figure 40. Proposed structure of the soluble BSA-PVEP complexes by Zaitsev. (from Ref. [98])

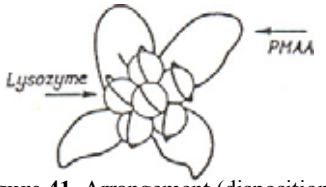


Figure 41. Arrangement (disposition) of structured parts of the lysozyme-PMMA polycomplex in the aqueous solution when the relative amount of protein in the solution is 1 and α_{PMAA} = 0.4) (from Ref. [68]).

For the lysozyme-PMAA complex, Anufrieva [68] recently suggested that some PMAA domains are filled with protein, while other domains remain unoccupied (Figure 41) for the excess protein case.

Soluble protein-polyelectrolyte complexes are usually formed at a pH close to the (protein) isoelectric point of the protein (IEP), and the soluble complexes aggregate to form coacervates toward phase separation by adjusting pH. It was suggested the existence of the primary intrapolymer complexes (in which a single polymer chain is bound to several protein molecules) for the excess protein case. These primary complexes could aggregate to form interpolymer complexes, in which several polymer chains are involved, as shown in Figure 42. The interpolymer complex could be soluble up to the point of the large-scale aggregation.

Kokufuta [87a] proposed a model for stoichiometric insoluble protein-polyelectrolyte complexes in salt-free system (see below).

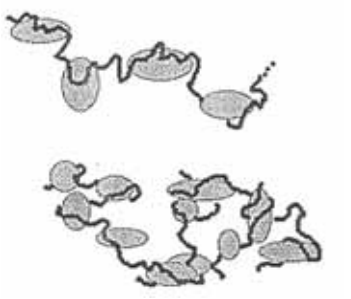


Figure 42. Intrapolymer and interpolymer structures of protein-polyelectrolyte complexes (from Ref. [79b]).

2.2. Water-soluble Complexes of Polyelectrolytes with Samely Charged Proteins

It is generally believed that the electrostatic interactions constitute the primary driving force for the formation of protein-polyelectrolyte complexes. Association of proteins with polyelectrolytes at pH > pI for polycations, and at pH < pI for polyanions has been attributed to salt linkage.

Complexes of Polyanions. It was found that some polyanions bind proteins at pH > pI [37, 57, 78]. It has been shown by high-velocity sedimentation that BSA and FDH form soluble complexes with polyacrylic acid (PAA), polymethacrylic acid (PMAA) and sodium polystyrene sulfonate (PSSNa) in neutral water solutions [37]. The typical sedimentograms of PSSNa and its mixtures with BSA at different ratios of their macromolecule concentrations in the solution as example are given in Figure 43. As it fallows from this figure in the general case the system is characterized by a unimodal distribution (by one peak) of sedimenting components. An increase of the number of protein molecules in the mixture leads to an increase both of the area of the peak and sedimentation coefficient of singular peak. These results unambiguously show protein and polyanion binding to a complex. Protein heterogeneous charge distribution (and strong binding capacity of $-SO_3$ anions [37]) and possible hydrophobic interaction of protein globules with hydrophobic polystyrene fragments can help to interpret this observation.

The analysis of the BSA-PAA, BSA-PSSNa and FDG-PSSNa systems by the sedimentation method showed that after adding NaCl salt, the soluble protein-polyanion complex particles lose some protein molecules in consequence with the compactization of complex particles.

Recently, the effects of protein charge heterogeneity in protein-polyelectrolyte complexation was described by Dubin [57]. The interaction between three monomeric globular proteins of substantially different isoelectric points (BSA, chicken egg lysozyme, and bovine pancreas ribonuclease) and synthetic polyelectrolytes with different chemical composition was investigated by turbidimetry and quasielastic light scattering (QELS) techniques in water-salt solutions. It was examine the association behaviour of two basic (RNAse and lysozyme) and one acidic (BSA) protein with polycations and polyanions of varying linear charge densities. As shown in the results of computer modeling for proteins RNAse can simultaneously have both

positive and negative potential regions in aqueous solution. This local non-uniform potential region or protein charge patch is believed to provide an attraction force that overcomes the repulsion between the global protein charge and the polyelectrolyte. Therefore, the complexes formed by polyanions with global negatively charged proteins are suggested to be a manifestation of local interaction between the protein charge patch and polyelectrolyte.

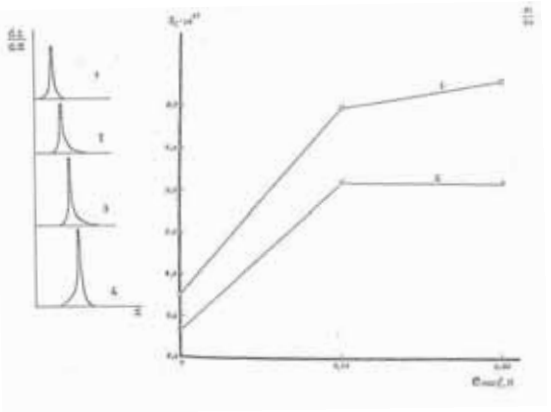


Figure 43. Sedimentograms (a) for the PSSNa-BSA mixtures at different n_{BSA}/n_{PE}: 0 (1); 1 (2); 10 (3); 20 (4); t=40 min; pH 7; b-dependence of sedimentation coefficients for the PSSNa-BSA mixtures (1) and pure PSSNa (2) on adding concentrations of NaCl. C_{PE}=0.15 g/dl; n_{BSA}/n_{PE}=10; pH 7.

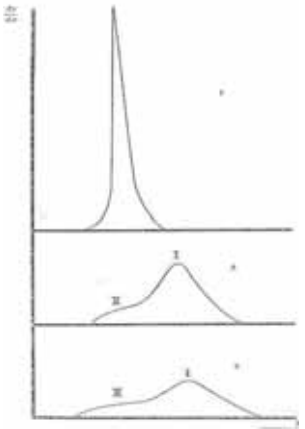


Figure 44. Sedimentograms for BSA-PSSNa mixtures at different concentrations of adding NaCl (C_{NaCl}): 0 (1); 0.15N (2); 0.3N (3); n_{BSA}/n_{PE}=10; pH 7.

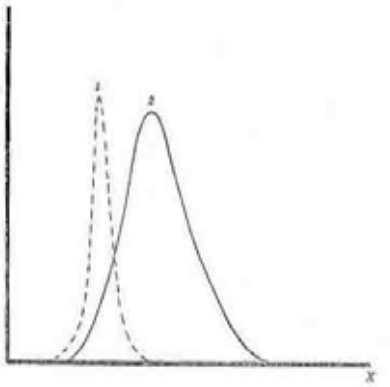


Figure 45. Sedimentograms for PSSNa (1) and its mixture with FDH (2): n_{FDH}/n_{PE} =10; pH 7.5; C_{PE}=0.15 g/dl.

On the bases of the results obtained by sedimentation analysis it was suggested that the complex formation between proteins and polyanions occurs by the random distribution of protein globules between polyion chains. Interaction between proteins and polyanions is not followed by any changes of protein α-helix structure (Figure 46). The data on Optical Rotary Dispersion and Circular Dichroism Spectroscopy of BSA and FDG were not affected by adding polyanion solutions to the protein solution. Strelzowa et al. [70] have observed CD spectral changes in the

range of 250-330 nm for α-chymotrypsin upon mixing the protein with dextran sulfate. However, the CD spectrum of α-chymotrypsin is not affected by adding dextran sulfate to the protein solution. The linear dimensions of the fractions of polyanions are considerably larger than those of the binding protein globules and every polyanion chain can bind some protein globules in succession. It may be deduced that this type complexes are formed as a result of the uniform all loading of protein globules between the polyelectrolyte chains, i.e., the protein molecules are randomly distributed between the adsorbing polyions. One polyelectrolyte molecule forms a complex with many of the protein molecules until the polyion fragments are populated with the protein globules. An outline of such a situation is given by the schematic illustration shown in Figure 46. In this case the structure of water-soluble complexes of proteins with samely (negative) charged polyelectrolytes being formed the protein molecules are randomly distributed along polyanion chains which retains the conformation of a statistical coil of the polyelectrolyte carrier. This scheme is conformed with the results of viscosity data of protein-polyanion mixtures. (The inherent viscosity of BSA-PSSNa mixtures is not essentially changing by the titration with protein solutions).

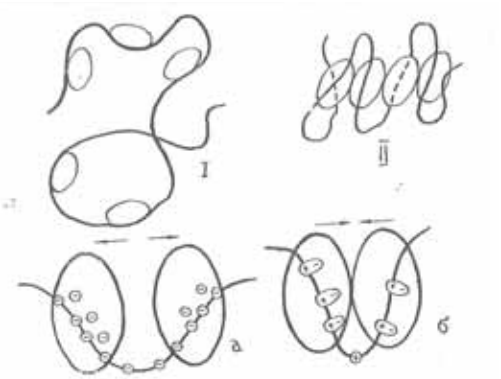


Figure 46. Proposed structures of complexes protein-PE: II-(PVEP-Protein); $n_{Protein}/n_{PE} \leq N_i$; I-(PAA)PSSNa)-Protein); "a" and "b" schematic illustration of the complex formation.

Thus, depending on the chemical nature of the polymeric carrier, protein composition and environmental conditions, two types of soluble polyelectrolyte-protein complexes may be constructed. Complexes of the first type (structure I) is on principle differ from the complexes of the second type (structure II). This difference between two types of the structures of polycomplexes by origin dictates by acting of the factors, which controls the interaction (by attraction or repulsion) between adsorbing protein globules on the polyelectrolyte chains. Initial protein globules are negatively charged in both cases, i.e. electrostatic factor prevent theirs draw together. At the same time, it is known that the non-polar interaction is the promoting factor for the association of protein molecules in water solutions. The formation of electrostatic (salt) bonds between negatively charged groups on the surface of protein globules and positively charged monomer units of polycations constitute the primary driving force at the sorption of the proteins by polycations. As a result the negative charges of protein molecules are neutralized (screening) and do not prevent to join of the bound globules, i.e. manifestation of non-polar interaction. At the same time it is achieved the partial neutralization and screening of the charge of polycations which promote draw together separate part of polycations at the self organization of the structure I. At the sorption of proteins by polyanions of polyacid (for example, PSSNa) the situation is quite different. Binding of negative charged protein globule with the polyelectrolytes takes place despite of electrostatic repulsion. Under this the efficient of negative charge of every sorbing protein globules may only increases at the expence of "stuck" units of polyanion. Then the

"docking "of globules in the polycomplex particles is not profitable (or less profitable) and they settle down separate each other by forming the structure II.

As it was mentioned above the excessive desalination of the system BSA-PVEP results in the electrostatic destabilization of particles of cooperative polycomplex (structure I) with the formation of rather bulky non-ordered aggregates being the products of the statistical interaction of albumin with the polycation (like structure II). Complexation of HSA, human or bovine hemoglobin (Hb), and bovine trypsin (BT) with poly (diallyldimethylammonium chloride) (PDDA) and potassium poly(vinyl alcohol sulfate) (KPVS), in a salt-free system were recently reported by Kokufuta [87a]. A model for stoichiometric complexes of proteins with polyelectrolytes is proposed on the bases of the results obtained (Figure 47).

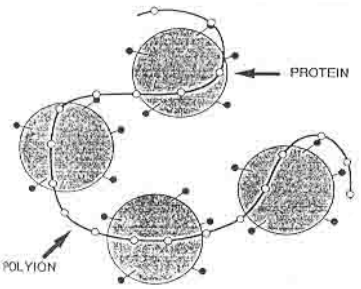
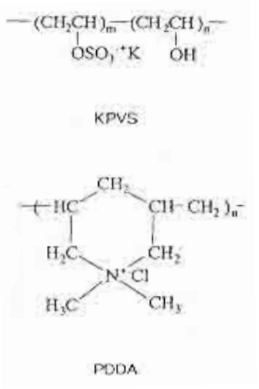


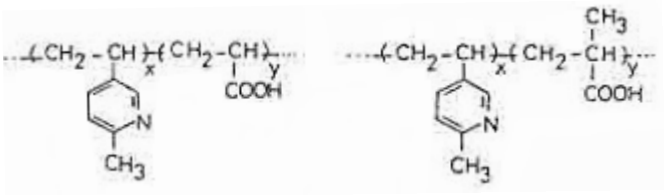
Figure 47. Schematic illustration of stoichiometric complex consisting of protein and polyion. Open and solid circles represent ionizable groups in the polyion and protein, respectively [87a].



Structures of polyelectrolytes studied. The physical data for KPVS and PDDA are as follows: DP_n for KPVS, 1500; [η] (in 1 N NaCl at 25°C) for PDDA, 1,67 $dl.g^{-1}$; equivalent weight referring to the molecular weight of polymer assigned to one mole of ionizable groups, 166 for KPVS and 158 for PDDA; degree of esterification of KPVS which is expressed as *m/(m+n),* 0.925. Both KPVS and PDDA maintain a completely dissociated state in the pH range 2 to 13.

In this model one polyelectrolyte molecule forms a complex with many of the protein molecules until all of the polyion charges are stoichiometrically neutralized with the opposite charges of the proteins. An outline of such a situation is given by the schematic illustration shown in Figure 47 in which the complex consists of a number of inflexible and global protein molecules bridged with loosely extended polyelectrolyte ions. The salt linkages maintaining the structure of the complex as an amorphous precipitate seem to be very "loose", because changes in pH or additions of other polyions sever some of the salt linkages, particularly the linkages between the protein imidazolyl and the KPVS sulfate groups. This looseness may make it possible for the protein and polyion molecules to undergo stoichiometric neutralization or 1:1 binding with their oppositely charged groups through thermal motion.

Complexes of Polyampholytes. Interaction of BSA, BGG and FDH with copolymers (CP) 2-methyl-5-vinylpyridine (MVP),acrylic (AA) and methacrylic acids (MAA) in different proportions in aqueous solutions was studied by Kabanov and Mustafaev [36].



The physico-chemical characteristics of CP are shown in Table 4

Table 4. The physico-chemical characteristics of CP

Copolimer	$[\eta_{sp}/C]$, dl/g	So, 10^{-13}	$M_{sd(\eta)}$	Isoelectric point (pI)
KP-I-66(x)	0,4	2	30000	6
KP-I-50	0,4	1,6	20000	5,5
KP-I-40	0,3	3,8	70000	5,2
KP-I-40	0,7	2,5	60000	4,5 + 6
KP-I-30	0,5	5,4	160000	4,5 + 6

These MVP and AA copolymers (CP-I) contained 66 (CP-I-66),50 (CP-I-50) or 34 (CP-I-34) mol% links of MVP. The MVP and MAA copolymers (CP-II) contained 30 (CP-II-30) and 40 (CP-II-40) mol% links of MVP. These copolymers are characterized with higher polydispersity [36]. According to this, the ratio of the components (n_p/Z) can be calculated as follows:

$$\overline{Z} = C_{cp}.10^{-2}.N_A/[\alpha.M_{AA} + (1-\alpha).M_{MVP}]$$

$$\eta_p/\overline{Z} = C_p.[\alpha.M_{AA} + (1-\alpha).M_{MVP}] \Big/ C_{cp}.M_P$$

Where C-concentration, g/100ml; N_A-Avogadro number; M_o-molecular weight of protein; $M_{AA(MAA)}$ and M_{vp} – molecular mass of AA(MAA) and MVP monomer units of CP correspondingly; α-part of AA(MAA) monomer units in composition of CP.

As it follows from the Table 4 both CP at pH 7 is the above of their isoelectric point and charged negatively. Under identical conditions the molecules of BSA, BGG and FDH also carry a summary of negative charges and preexisting electrostatic repulsive forces between CP and proteins will prevent the formation of stable polycomplexes. However, analysis of interactions revealed that the tightness of their binding to each other depended critically on the MVP monomer units in composition of copolymers.

The typical sedimentograms of CP-I and its mixtures with protein at different ratios of components (n_P/Z-the number of protein macromolecules per number of monomer units of CP) are given in Figure 48. In the general case the system is characterized by a bimodal distribution of sedimenting components. The value of Sc for a slowly sedimenting substance (peak I) is 2.0 saved, i.e. it corresponds to a free CP-I -66. One may assume that rapidly sedimenting substance (peak II) is a complex CP-BSA. Figure shows the dependence of the area of the free CP peak on the ratio of components for CP of a different composition.

A linear decrease of the free CP concentration in the solution during titration with protein in the case of CP-I-66 unambiguously shows its binding to a complex (Figure 48 curve 1). The CP-I-50 also forms the complex with protein molecules. As it follows from this figure, the area of the peak corresponding to free CP-I-50 is decreased by increasing protein concentration in the mixture (Figure 48 curve 2). However, the rate of decreasing of the area of free copolymer

fraction in mixture protein-CP-I-50 is lower than those of the copolymer containing 66 mol % MVP monomer unites. CP-I-40 show only relatively weak tendency to bind with BSA. Decreasing of the area of free CP-I-40 peak quickly end. The intersection points obtained at the extrapolation of the plots in Figure 50 to the zero area of the free CP peak correspond to $(n_{BSA}/Z)x10^3$ when all copolymer macromolecules are bound to a complex with BSA. One may consider that lim $(n_{BSA}/Z)x10^3=N_i$, when $P_0 \rightarrow 0$. This limit equals the number of protein molecules bound by a 1000 monomer units of copolymer in polycomplex particles. The corresponding characteristics for CP-I-66 and CP-I-50 are N_i=6 and 14, respectively. The value of N_i in the case of CP-I-50 is practically twice as much than those of CP-I-66.One can assume that this result may indicate on the more dence surrounding protein globules by chains of CP-I-66 (higher number of intermolecular contacts per protein globule). Another possible reason– increasing the number of intramolecular contacts between hydrophobic methylvinylpyridine monomer unites of CP with the formation of compact structure ("cluster" or "tack"), which are not participating in binding with protein.

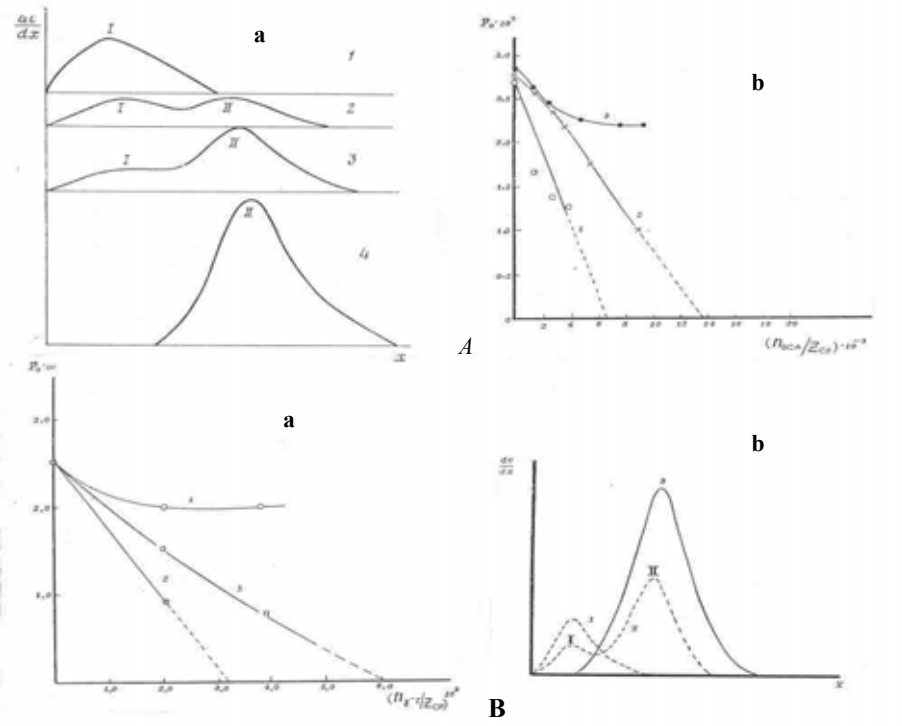


Figure 48 A. Sedimentograms (a) and dependence (b) of the sedimentation peak area (P_o) of free CP in the CP-BSA system on $(n_{BSA/z}).10^3$; a-correspond to CP-1-66; ratios: 1(2); 3 (3); 9 (4); b-3 (CP-1-40); 2(CP-1-50); 3 (CP-1-66); pH 7.0.
B. Dependence of the sedimentation peak area (P_o) of free CP on ratios $(n_{BSA/Z}).10^3$ for CP: (CP-1-66) (1), (CP-1-50) (2); (CP-1-40) (3) (a); Sedimentograms of free CP-1-66 (1) and its mixtures with FDH at different $(n_{FDH/Z}).10^3$: 1 (2); 10 (3); t=70 min. (b).

It is important to emphasize, that the free CP-I-66 and CP-I-50 still remains in the system over a sufficiently wide range of the molar ratios n_{BSA}/Z .It was suggest that these results indicate a non-random distribution of the protein molecules between the amphoteric polyions.

Therefore, it was shown, that in a wide range of ratio of the components soluble cooperative complexes were formed. Ability of amphoteric copolymers to form complexes with proteins depend both on the composition of the copolymers and the pH of the medium. Copolymers of methacrylic acid with MVP (CP-II-40) in contrary to CP-I-40 form a stable complex with proteins (Figure 49) and ability of CP-I-40 to form stable complexes increased with decreasing pH of the solution.

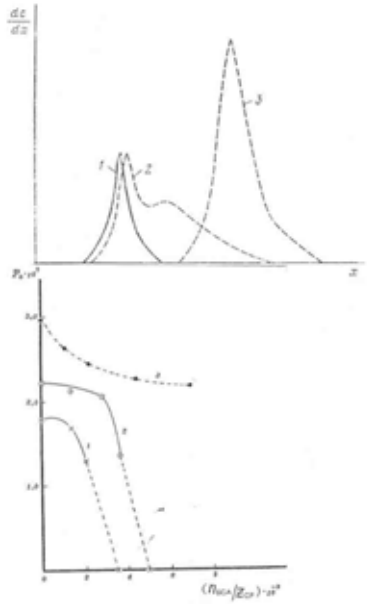


Figure 49. Sedimentograms for free CP-11-40 (1) and its mixtures with BSA at different $(n_{BSA/Z}).10^3$: 3 (2); 6 (3); Dependence of the sedimentation peak (P_0) of free CP in the CP-BSA system on ($n_{BSA/Z}$) ratios. (BSA- (CP-11-40) (1); (BSA-(CP-11-30) (2); (BSA-(CP-1-40)) (3); pH 7.0.

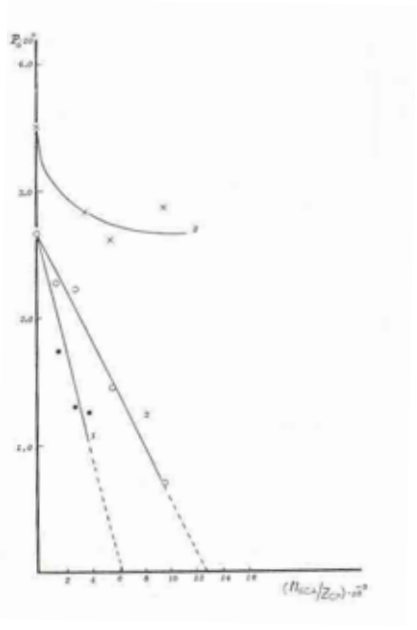


Figure 50. Dependence of the sedimentation peak (P_0) of free CP on $n_{BSA/Z}$ in the BSA-(CP-1-66) system at different pH 7 (1); 5 (2); 4 (3).

It was suggest that, under conditions where both CP and proteins have negative charges formation of hydrogen bonds and nonpolar interactions of MVP monomer units with protein globules promote the formation of stable water-soluble complex. The non-polar interactions of the protein globules with hydrocarbonic 2-methyl-5-vinylpyridine chain fragments of the polyampholyte play an important role in the association of similarly charged particles. It was shown that besides this effect of chain fragments of 2-methyl-5-vinylpyridine a significant contribution in the association is made by non-polar interactions, created by methyl groups in copolymer 2-methyl-5-vinylpyridine and methacrylic acid. It is confirmed with the fact that these complexes are not destroyed in the presence of sufficient amount of NaCl. At the some time the values of inherent viscosity of free copolymers and its mixtures with proteins decreased with increasing of the concentrations of NaCl (Figure 52).It means that in the structure of polycomplex particles exist the charged free polyion sequences, which was not involved in protein interaction.

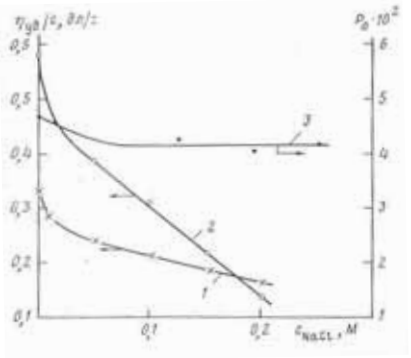


Figure 51. Dependence of the inherent viscosity ($\eta_{Sp/C}$) and sedimentation coefficient (Sc) for free BSA, CP (1-5) and complexes (1'-5') on $n_{BSA/Z}$: 1,1'-BSA+ (CP-1-66); 2, 2'-BSA+(CP-1-50), pH 7.0; 3, 3'-BSA+ (CP-1-66), pH 5.0; 4, 4'-BSA+(CP-11-40), 5, 5'-BSA+ (CP-11-30); pH 7.0.

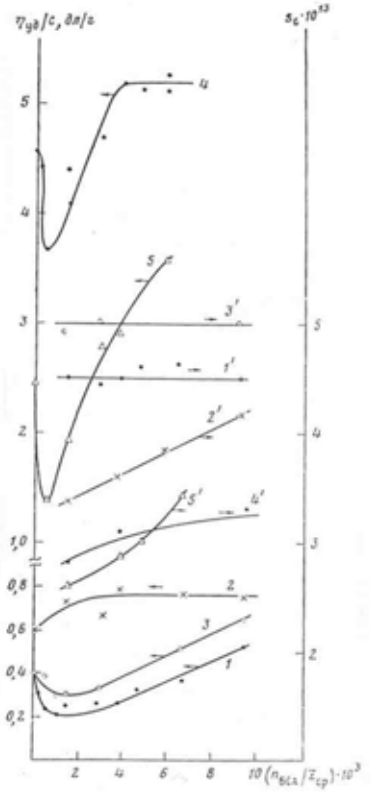


Figure 52. Dependence of the inherent viscosity ($\eta_{Sp/C}$) for CP-1-66 (1), complex (BSA+(CP-1-66) (2) and area of peak (P_0) of complexes (3) on the adding concentrations of NaCl. $(n_{BSA}/Z).10^3=6$; pH 7.0.

The association mechanism and structure of the formed complexes was studied. The dependence of the inherent viscosity of CP-BSA complex solution on the ratios is given in Figure 51.

It is seen that an increase of protein concentration in the mixture is followed by an increase of the inherent viscosity showing the formation of the polycomplex particles with more asymmetric structure than the coils of free copolymer polyions. At the same time the sedimentation coefficient changes insignificantly that conforms to the data of viscosity. Asymmetry of the structure of BSA-CP-I-66 complex is confirmed by the data of electron microscopy. The complex micrograph obtained for the system in which $n_{BSA}/Z=N_i$ is given in Figure 52. Extended linear formations are distinctly seen in the micrograph. The thickness of each rod consisting of globules is about 100 A^o. The rods, in all probability, form from contacting protein globules, which are "stick together" by the chains of linear polyampholytes.

Figure 53 shows the proposed structure of soluble protein-copolymer complex particles. In this model complex formation is accompanied by non-equal distribution of the chain copolymer between the protein globules and appearance of asymmetric particles of the complex. A correlation between the ability of polyampholytes to bind protein and their physiological action was found (see below).

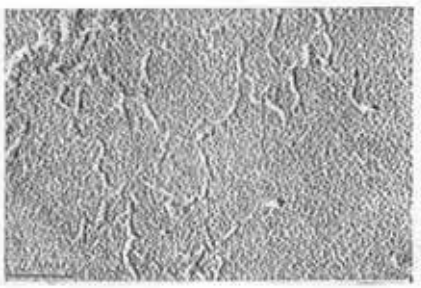


Figure 53 Electron micrographs of BSA+CP-1-66 complexes at $(n_{BSA/Z}).10^3$=6,4. (2x22000).

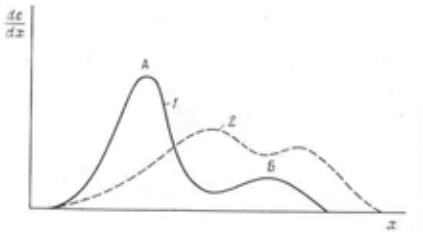


Figure 54. Sedimentograms of the initial serum (1) and its mixture with CP-11-40 (2). pH 7.5; C_{CP}=0,15 g/dl.

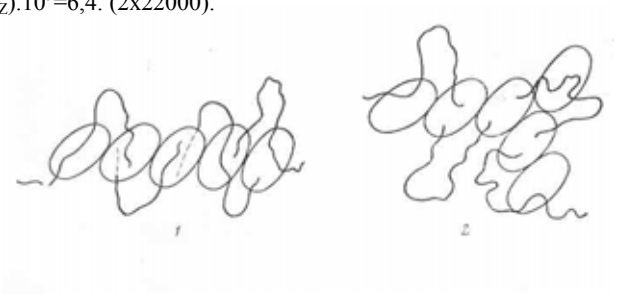
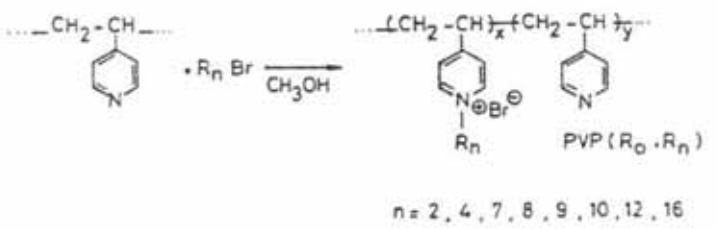


Figure 55. Proposed structures of BSA with copolymers at $n_{BSA/Z}=N_i$; 1-BSA+(CP-1-66), BSA+(CP-1-40); and BSA+ (CP-11-30), pH 7.0; 2-BSA+(CP-1-50); pH 7.0; and BSA+ (CP-1-66); pH 5.0.

Complexes of Polycations. A detailed analysis of physico-chemical properties of the mixtures of serum proteins (bovine serum albumin –BSA, human serum albumin-HSA) with poly-4-vinylpyridine (PVP) derivatives revealed that the chemical structure (charge density, hydrophobic-hydrophilic balance) of PE strongly affects their interaction with the proteins and, correspondingly, the stability of the polymer-protein complexes were formed thereby. It was found that in acidic media (pH 4.3) PVP acquires a weak positive charge and thus becomes unable to form complexes with positively charge BSA (isoelectric points of BSA pI=4.9) [97]. However after the loading of PVP with lateral hydrophobic radicals (PVP-Rn) the former acquire the ability to form complexes with BSA. The complex-forming capacity of PE molecules is different and depends both on the lengths and the amount of N-alkyl radicals.

These products whose general formula appears as:



where x/(x+y).100=7-8%, were obtained by quaternization of the PVP fraction (Pn=10^3) by corresponding alkyl bromides (from C_2 to C_{16}) as described previously [101-105]. For

convenience's sake these products will further be termed as PVP-Rn, where "n" is the number of carbon atoms in the N-alkyl fragment. It was obtained from the hydrodynamic analysis data that the viscosity of the solution of PVP-Rn whose hydrophobic radical length does not exceed eight carbon atoms is equal to the viscosity of the original PVP, i.e., in this sequence the size of the macromolecule does not change. The situation is quite different at n>8.In the range of R_{10},R_{16} the drastic decrease of viscosity is paralleled with an increase in the value of sedimentation coefficients, which facilitates the transition from the coil to the compact structure. (Figure 56)

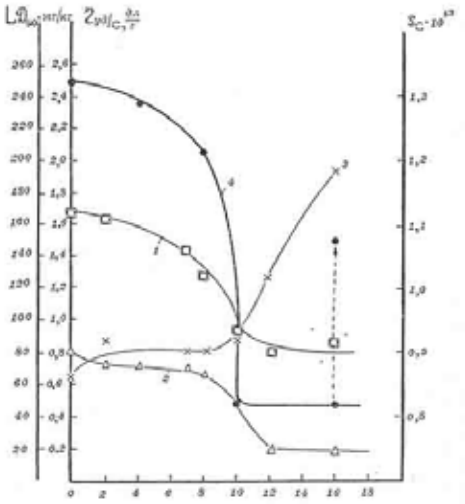


Figure 56. Dependence of the inherent viscosity ($\eta_{Sp/C}$) and sedimentation coefficient (Sc) on number of carbon atoms (n) in the N-alkyl fragment of PVP solution 0,5 % CH_3COOH (1,3) and 0,5 % CH_3COOH+0,2N NaCl (2); pH 4.3. Curve 4- correspond to toxicity (LD_{50}) of PVP(R_0, R_n).

In the next series of our experiments covalent conjugates of PVP(Ro, R_{16}) were obtained by quaternization of the PVP molecule with cetyl bromide ($C_{16}H_{33}$),a hydrophobic radical having a permanent length [101-105].In the case when the N-alkyl group represented rather a lengthy carbon chain and certain "critical" concentrations of N-alkylated bonds such PE underwent conformational transitions in dilute solutions, eventually resulting in the compactization of the macromolecular coils. It was suggested that the hydrophobic cooperative interactions between N-alkyl fragments play an important role in the transition of macromolecules from the coil to the compact structure.

PVP(Ro, Rn) conjugates thus obtained were further used for the study of complex formation with BSA. It was obtained that the complex-forming capacity of hydrophobically modified PVP in acidic media (pH 4.3) is different and depends on the length as well as on the number of alkyl radicals. Figure 57 shows plots of the area of the peaks corresponding to the free polyelectrolyte in the BSA-PVP(Ro, Rn) mixture is obtained from sedimentograms (ultracentrifugation experiments) of homogeneous systems.

An increase in the BSA concentration at a constant PVP-(Ro, Rn) concentration leads to a decrease in the area of the peak corresponding to the free PE, while the area of the presumed peak of the complex increases. At the peak of free PE disappears, and only one peak remains on the sedimentograms. It was also shown that the BSA/PE ratio at which the free PE disappears, at a fixed weight concentration of PE, depends on the length of alkyl radicals, i.e. the longer the radical length the less BSA are needed to make the free PE peak disappears. These results

indicate that, even at pHs < pI where net charges of PE and BSA are positive, electrostatic interaction is conjointly in effect with hydrophobic interaction of N-alkyl radicals for the binding (Figure 57). Hydrophobic interaction between N-alkyl fragments of PE, which are not including interaction with protein molecules, can help the stabilization of the complex structure as a whole.

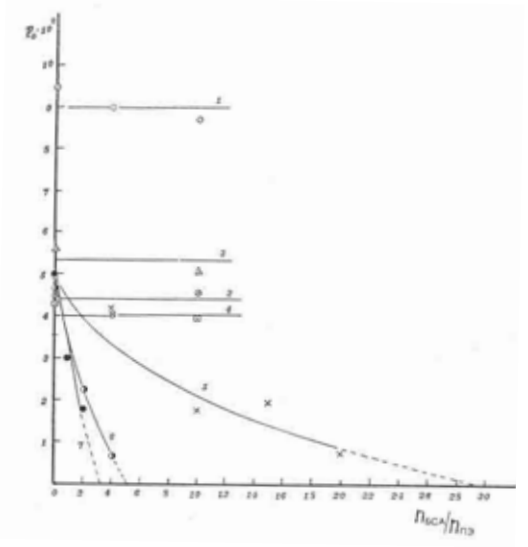


Figure 57. Dependence of the peak area (P_0) of free PVP(R_0, R_n) on n_{BSA}/n_{PE} in PVP(R_0, R_n)-BSA systems: 1-PVP(R_0); 2- PVP(R_0,R_2); 3- PVP(R_0, R_7); 4- PVP(R_0, R_9); 5- PVP(R_0, R_{10}); 6-PVP(R_0, R_{12}); PVP(R_0, R_{16}); pH 4.3 β=7-8%.

Noticeably, the binding of BSA to PVP(Ro, Rn) take place at a Rn, which corresponds to N-alkyl radicals realizing conformational transition of polymeric chains, i.e. exist the relationship between the complex-forming capacity of PE and the conformational transition in polyelectrolyte chains. Polycations of PVP(Ro, Rn) whose N-alkyl radicals are abundant enough to induce the formation of PE-BSA complexes but which are insoluble in neutral aqueous media can form stable electrostatic and hydrophobic complexes with BSA in acidic aqueous solutions. However, at physiological values of the ionic strength and pH such complexes lose, to a certain extent, their stability: some part of the protein molecules dissociate from the main complex to form an insoluble pellet, in which one polyionic chain corresponds to one protein molecule (so-called stoichiometric complexes). This phenomenon is very important also in view of the fact that electrostatic and hydrophobic cooperative interactions play an important role in many biological systems; hydrophobic effects on PEC formation of proteins should be an important problem to be investigated.

The interaction of BSA with various fractions of the copolymer of 4-vinyl-N-cetylpyridinium bromides has been studied depending on the length of macromolecules and the content of side hydrophobic cetyl fragments:

Introduction of hydrophobic cetyl radicals into PVP chains lead to compactization of macromolecular chains (Figure 57). The inherent viscosity decreased by increasing the amount of the cetyl radicals. As seen from Figure 58 curve this changing is acquire the character of conformation transition in salt containing water solution.

When BSA solutions are added to PVP(R_0, R_{16}) solutions at pH 4.3 the inherent viscosity of the mixtures was considerably increased (Figure 59).The degree of increasing of inherent viscosity so much the stronger the more compact of the conformation of initial PVP(R_0,

R_{16}) ,i.e. more the amount of cetyl radicals in the composition of polycation. So far as the viscosity of free BSA at corresponding concentrations is neglecting few this results direct testify on the binding of protein molecules with polycations. At pH 4.3 the globules of BSA acquires a positive charge (pI of BSA equal 4.9) and therefore one can assume that the interaction of hydrophobic N-cetyl radicals with hydrophobic section on the surface of protein globules is the driving force at the formation of protein-polycation complexes.

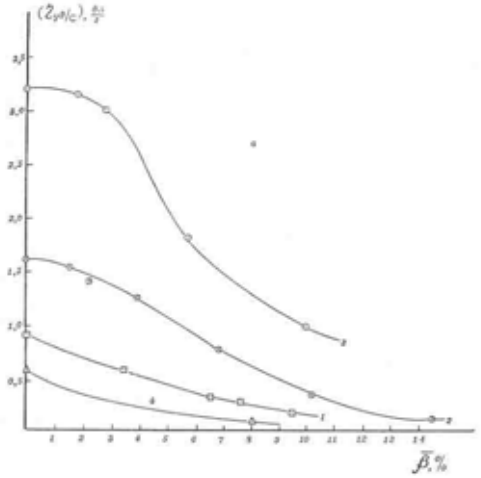


Figure 58. Dependence of inherent viscosity ($\eta_{Sp/}C$) of polycations on degree of quaternization (β) with ceytyl bromide at different P_w; 10^3: 1-0.6; 2-1.0; 3-2.15; 4-0.4; pH 4,3.

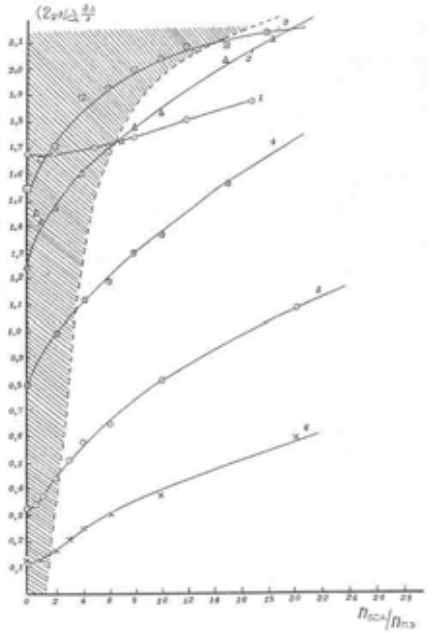


Figure 59. Dependence of ($\eta_{Sp/C}$) BSA-PVP(R_0, R_{16}) mixtures on n_{BSA}/n_{PE} at different β (Pη=const): 0 (1); 4 (2); 1,5 (3); 7 (4); 10 (5); 14,5 (6).

As it follows from sedimentograms of BSA-PVP(R_0, R_{16}) mixtures, in the general case the system is characterized by a bimodal distribution of sedimenting components (Figure 60). The free polycations coexist with the water-soluble protein-polymer complexes. An independent experiment has shown that the free protein is absent in the system when polycation chains contain some critical concentration of cetyl radicals ($\overline{\beta}$ ~7%) over the whole studied range of the ratios n_{BSA}/n_{PVP}. This results indicates that the non-uniform distribution of protein globules between polycations-sorbent is revealed.

P(R_0, R_{16}) sedimentation peaks plotted vs. the ratio of the number of protein molecules to that of polycation chains in the system.

As seen from this figure the function P_0= f(n_{BSA}/n_{PVP}) are not linear in contrary to above described protein-polycation mixtures. It means that there is polycation present in the system with different "capacity" as regards protein globules at given degree of polymerization and quaternization ($\overline{\beta}$). This phenomenon may be have to do with compositional heterogenecity of polycations as the degree of quaternization have strong influence on the binding capacity of

PVP(R_0, R_{16}). Therefore, the values N_i, which were obtained from Figure 61 are less exact than in the case of protein-PVP(R_2) and its characterizes the average number of protein molecules bound one polycation chain belonging to a given fraction of PVP(R_0, R_{16}). The dependence of N_i on degree of polymerization of polycations with different degree of quaternization ($\overline{P}_\eta$) is given in Figure 62.

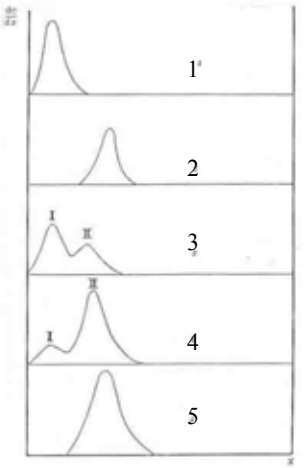


Figure 60. Sedimentograms of PVP(R_0,R_{16}) (1) and its mixtures with BSA (2) at different n_{BSA}/n_{PE}: 1 (3), 2 (4), 3 (5).

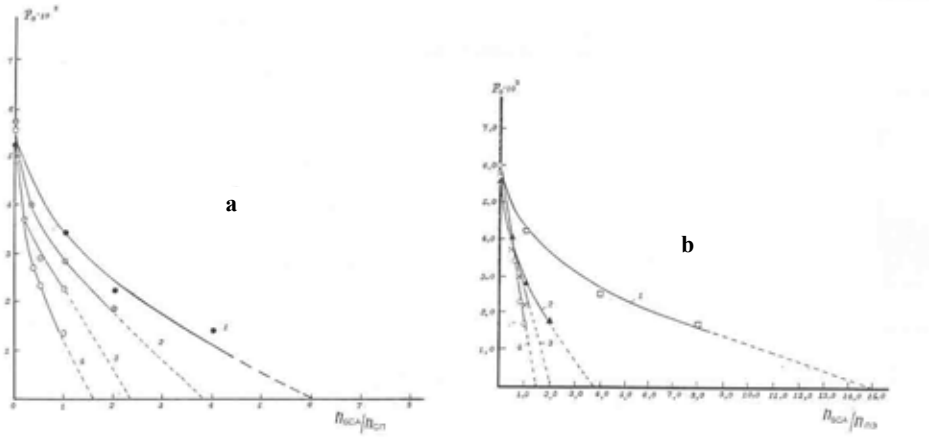


Figure 61. Dependence of the sedimentation peak area (P_0) of free PVP(R_0, R_{16}) in the BSA- PVP(R_0, R_{16}) system on n_{BSA}/n_{PE} for PVP(R_0, R_{16}) with different Pη, 10^3: 2,15 (1); 1,0 (2); 0,6 (3); a- β=4%; b- β=7%. (0,4 (4).

Within the experimental error these dependences are linear. The rate of this rise so much the higher than the lowest value of " $\overline{\beta}$ " in the system over the whole studied range of the " $\overline{\beta}$ ". From the data in Figure 62 one can approximately estimate the average number of

monomer units ($\bar{l} = \overline{P_\eta}$ /N_i) or the average length of the section of macromolecules of polycation per one-protein globules in composition of polycomplex particle. It follows from this figure that the higher the % content of N-cetyl fragments in composition of polycation, the higher the "$\bar{l}$ " is (Figure 62).

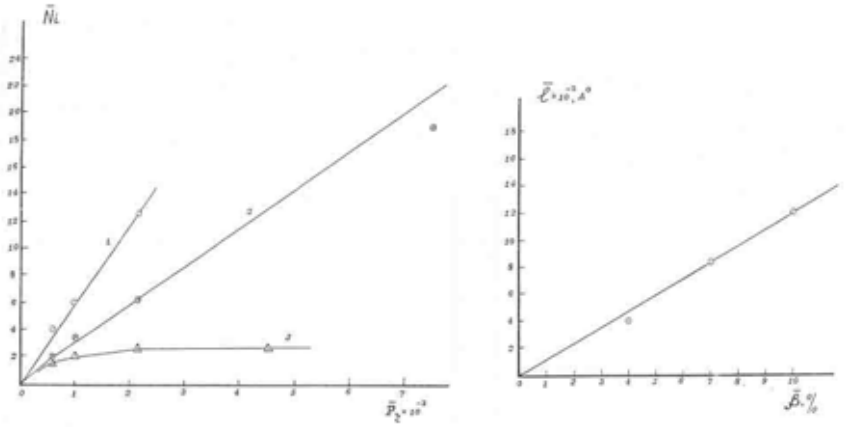


Figure 62A. Dependence of characteristic composition (N_i) of polycomplexes on degree of polymerization of PVP(R_0,R_{16}) at different β, %: 4 (1), 7 (2), 10 (3).

B. Dependence of the average value ($\bar{l}$) of the length of PVP(R_0, R_{16}) per one protein globule in polycomplex particles with composition of N_i on β (the results was obtained from Figure 62A)

The molecular characteristics of the particles of the soluble BSA-PVP(R_0, R_{16}) complexes with the composition $n_{BSA}/n_{PVP} = N_i$ were determined by light scattering measurements. For all investigated polycations over the whole studied range of concentrations the dependence of KxC/R_θ on the concentration was linear, indicating the absence of dissociation of the polycomplexes at dilution (Figure 63). In other words, the studied soluble BSA-PVP(R_0, R_{16}) polycomplexes are stable over a wide range of the solution concentrations and polycomplex composition.

Table 5.

Компоненты	dn/dc	$\bar{M}_w \cdot 10^{-3}$	$\bar{M}_z \cdot 10^{-3}$	$\bar{N}_c$	
				светорассеяние	седиментация
БСА	I,8	70	–	–	–
ПВП(R_0, R_{16})-I-6,5	2,2I	70	70	–	–
ПВП(R_0, R_{16})-II-7,0	2,I9	I20	II6	–	–
ПВП(R_0, R_{16})-II-8,0	2,I7	I25	II8	–	–
ПВП(R_0, R_{16})-II-I4,0	2,68	I30	I26	–	–
БСА+ПВП(R_0, R_{16})-I-6,5	2,I5	I20	I40	0,7	I,0
БСА+ПВП(R_0, R_{16})-II-7,0	I,85	320	336	3,5	3,0
БСА+ПВП(R_0, R_{16})-II-8,0	I,93	240	290	2,5	2,0
БСА+ПВП(R_0, R_{16})-II-I4,0	2,32	250	260	2,0	2,0

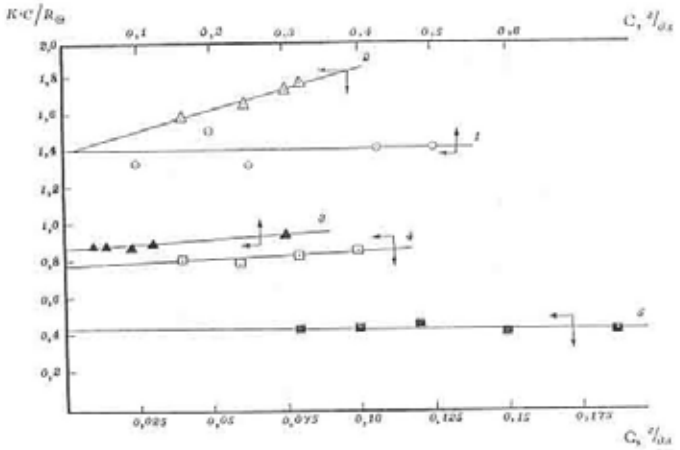


Figure 63. Dependence of K.c/R0 on C. 1-BSA; 2- PVP(R_0, R_{16}); (β=6,5), 3-BSA+PVP(R_0, R_{16}) (β=6,5); $n_{BSA}/_{PE}$=1 4-PVP(R_0, R_{16}); (β=8); 5-(BSA+PVP(R_0, R_{16}) (β=8) $n_{BSA}/_{PE}$=3; 0,5% CH_3COOH; pH 4.3 λ=4360A°

The results of the studies of BSA-PVP(Ro,R_{16}) complex solutions with different composition formed by polycations with a different molecular mass, Mw, and degree of quaternization " $\overline{\beta}$ " are listed in Table 5

As it is seen from the Table 5 the average values of molecular weights calculated from the sedimentation data, $M_s = (M)_{PVP} + N_i \times M_{BSA}$ are in a good agreement with the values Mw obtained by light scattering measurements. From these data it follows that only one polycation chain is contained in the particles of soluble BSA-PVP(R_0, R_{16}) complex.

Some information about the structure of the BSA-PVP(R_0, R_{16}) complexes are obtained from the comparison of the inherent viscosities and the sedimentation coefficients for these complexes formed by the polycations with different length at given degree of quaternization when $n_{BSA}/n_{PVP}=N_i$. It is seen in Figure 64 that the viscosity of the solutions sufficiently and strongly increases with increasing degree of polymerization of the polycation. At the same time the sedimentation coefficient changes insignificantly. The relation of this kind may exist for sufficiently extended particles at their elongation. The result obtained from the electron microscopy is quite conform with this conclusion (Figure 64): The separate particles of polycomplex with length 400-500 Å and thickness 100 Å are distinctly seen in the micrograph (the contour length of polycation is about $2.5x10^3A^o$, " $\overline{\beta}$ "=8 mol%).

Interaction between proteins and PVP(R_0, R_n) is not followed by any changes of the protein secondary structure (Figure 65)

Thus, the bindings of protein globules with polycations were realized by the results of introducing of some N-cetyl radicals (R_{16}) into hydrophobic parts of protein molecules. One can assume that, the molecules of protein in composition of polycomplex particles are in contact with each other and such positive interaction results in an additional decrease of the free energy exceeding; a free energy increase caused by the disturbance of the randomness of the distribution. In present situation, however, the last approval is not so evident, since non-uniform distribution may be also cause of necessity of partial destruction of initial compact structure of polycation-sorbent. Moreover, for the explanation of the correlation of protein binding capacity of PVP(R_0, R_n) with its conformation transition "coil-compact structure" one can suggest that the fragments

of initial compact structure of polyelectrolyte remain also in structure of polycomplex particles and is of importance of its stabilization. These fragments, probably, are the intramolecular clusters forming by the lateral hydrophobic R_n radicals, which are not directly involved in the complex formation (Figure 66).

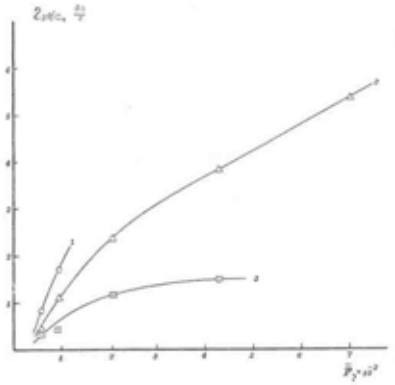


Figure 64. Dependence of inherent viscosity ($\eta_{Sp/C}$) of PVP(R_0,R_{16})-BSA complexes $n_{BSA}/_{PE}=N_i$ on Pη of PVP(R_0,R_{16}) at different β, %: 4 (1), 7 (2), 10 (3).

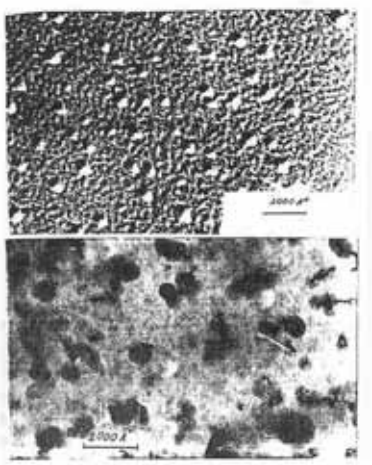


Figure 65 Electron micrographs of PVP(R_0, R_{16})-BSA complexes a-method freezing-etching; b-method thermal attachment. (0,01 g/dl). $n_{BSA/PE}=3$; $P\eta=10^3$; β~8%

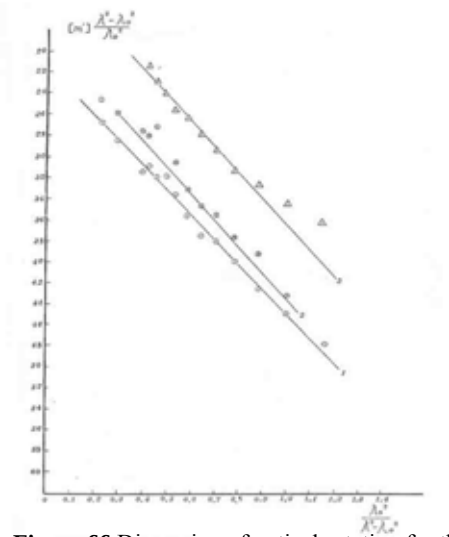


Figure 66 Dispersion of optical rotation for the solution of free BSA (1) and PVP(R_0, R_{16})-BSA complexes at different n_{BSA}/PE: 1 (2); 4 (3); processing with Moffite equation.

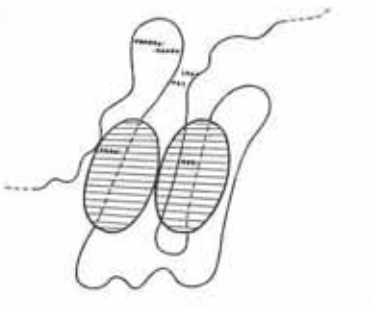


Figure 67 Proposed structure of BSA-PVP(R_0, R_n) complexes

It is clear that the self-organization of a whole structure requests the overcome of the electrostatic repulsion at the approach of the positive charged protein molecules and positive charged section of PVP(R_0, R_n) chains. It is only possible in the case when the R_n radicals are

characterized with sufficient length and amount, i.e. contribution of hydrophobic interaction energy must be predominating. The susceptibility of polycations to compactization is increased at the transition of the length of lateral radicals from R_{10} to R_{16} and at $\overline{\beta} \geq 3\%$: the number of protein globules sorbing by one polymer chain with given counter length is increased correspondingly (i.e. N_i).

An increase in protein content (at the ratios $n_{BSA}/n_{PVP} > N_i$) in mixtures results by the "polymerization" of polycomplex particles, which leads to the increasing of its molecular mass and asymmetry. Moreover, only a few of excess protein molecules were included to this process; the most part of them remains free and characterized as a separate peak on sedimentograms.

When NaCl solutions are added to polycomplex solutions at pH 4.3 above its critical concentrations (C_{NaCl} 0.154 M) and (or) pH of solutions were increased (pH $\geq$ 5) the water-soluble complex lost part of protein molecules and transform to insoluble state at what only one protein molecules bound by one polymer chains. These results are very important for the creation of stable polycomplexes in the conditions (ionic strength and pH) of living organism.

Modeling of nucleoprotein complexes

Complexes of proteins and linear natural polyions, which include chromatin, ribosomes and other cell components as well as viruses, are widely distributed in nature and fulfill an important function. It can be assumed that the basic contribution to the stabilization of the complexes is from cooperative electrostatic interactions involving ionogenic groups of proteins exposed on the surface of the globule. These interactions may result in soluble complex formation between nucleic acids and histones, complex coacervation, or the formation of amorphous precipitates. Such phenomena are also undoubtedly significant in the cell, where the Coulombic association of DNA with basic histones leads to the collapse of the nucleic acid and where basic polypeptides such as polylysine are thought to profoundly influence DNA behaviour. Similar electrostatic interactions between proteins and nucleic acid are likely to play a role in the transcription process.

It was found that protein interaction with poly-N-ethyl-4-vinylpyridinium bromide in aqueous solution results in self assembling of the asymmetrical aggregates [33-35,36-39]. Such aggregates may be considered as models of specific nucleoproteid complexes i.e. viruses, chromatin, ribosome strands and other cell components in complicated biological systems. It is quite probable that the principle laid in the basis of such nucleoproteid structure construction, is actually very general. To realize this principle it suffices to fulfill two thermodynamical conditions: 1) a possible cooperative coupling, i.e. "sticking" of a globule to an open linear chain; 2) sufficiently "strong" positive interaction of the protein adsorbed globules with each other. A necessary kinetic condition for self assembling is a possibility of slipping of the adsorbed globules along the chain-sorbent in the process of complex formation, in order to find optimal contacts among them, and using the trial and error method to permit the whole system to acquire the structure corresponding to the minimum of the free energy.

3. POLYELECTROLYTE-METAL-PROTEIN COMPLEXES

As it was mentioned above, polymer-protein complexes (PPC) is formed as a result of the interaction of polyion chains with the oppositely charged groups of the protein molecule during these reactions. The extent of the interaction is found to be pH and ionic strength sensitive and dependent on the isoelectric points of the proteins. Such systems include complexes stabilized by cooperative electrostatic and hydrophobic interactions between the fragments of PE and protein molecules and conjugates in which the functional groups of the components are linked by covalent bonds. In those cases where PE macromolecules do not contain the corresponding electrostatic or hydrophobic groups for protein binding, it is necessary to modify the carrier polymer (or protein), which can give rise to changes in its effect(s) upon biological systems. Moreover, covalent conjugation of polyelectrolytes with proteins may lead to partial changes in

the chemical structure of the antigenic (or catalytic, etc.) determinants as a result of their involvement in the formation of chemical covalent bonds. This approach is not technological, however, since the free components formed in the reaction system during the crosslinking procedure have to be separated from the main product, which requires additional labour expenditure and effort.

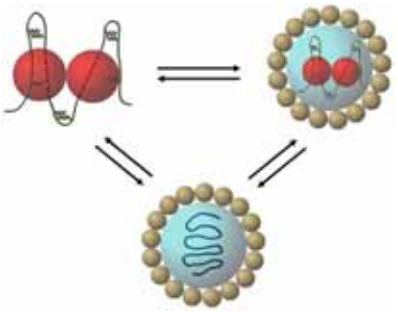
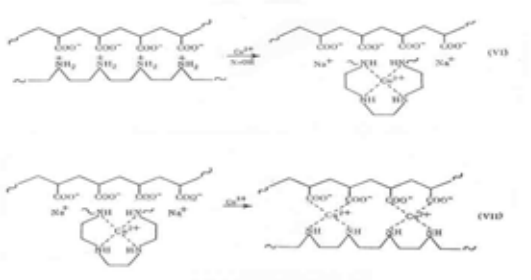


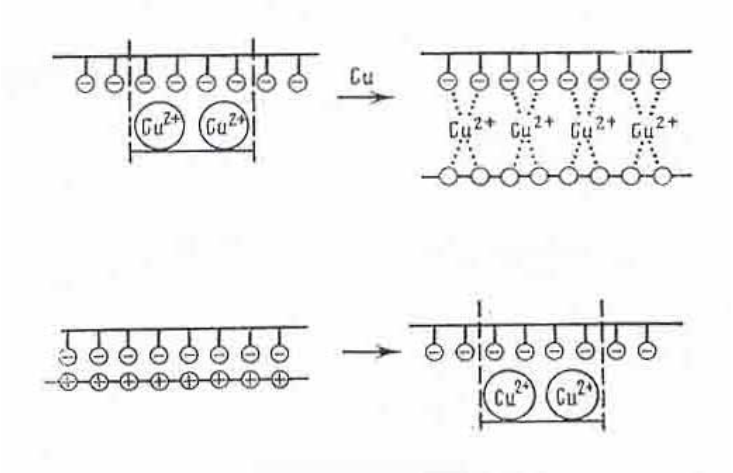
Figure 68. A schematic presentation of the structure formation in protein-polyelectrolyte mixtures at different protein/polyelectrolyte ratios (redistribution of protein and polyelectreolyte molecules in the structure formation in protein-polyelectrolyte complexes depending on the concentrations of protein globules).

A relatively new technique involves the use of transition metal (Me) compounds as means of activating the support surface and allowing direct coupling of proteins without prior to derivatization of the activated support, through formation of chelates [106,107]. Evidence has recently been presented for the existence of a ternary complex between proteins, Cu^{2+} ions, and amino acids [107]. Some publications in the current literature are devoted to the construction of drugs based on such Me-mediated complexes of natural PE and antigens [108-112]. These authors succeeded in demonstrating that polysaccharide-protein mixtures supplemented with metal ions are effective means of prophylaxis and treatment of some microbial infections in animal and human.

Metal chelate formation may proceed via binding with functional groups of two different macromolecules (usually one of them provides “acidic” functional groups, and the second “basic” groups). These studies are not numerous in number, although metal binding with mixed biopolymers, the process that is of great importance for biological reactions (replacement of histons from DNA, association of the ribosome components, etc.), is intensively investigated.
Both polymer-polymer compositions and interpolymer complexes are used as chelating agents for synthesis of such chelates. It has been shown by studying of the systems polyethylenimine (PEI)-PAA-Me (Me=Cu, Co, Ni) that Cu(II) ions give polycomplexes of two types [113-117]. The first ones contain four coordinated amino groups of PEI and cooperatively bind polyanions of PAA (at pH values above 10); other polycomplexes have two amino and two carboxylic groups in Cu(II) coordinative sphere (pH=7-9). Stabilization of these complexes is provided by formation of both salt-like and coordinative bonds:



Interpolymer complexes are usually formed within a short range of pH values. Thus, in the system PAA-P4VP-Cu(II) at equimolar ratio of pyridine and carboxylic groups only complexes of Cu(II) with P4VP are formed in acidic medium, whereas at pH 4.0-5.0 "mixed" complexes with chelate node $Cu(Py)_3(COO)$ are formed:



The effect of stability of restrictions of "triple" polycomplexes with regard to pH values can be used to carry out selective extraction of several metal ions (e.g., of Cu(II) ions by the systems PAA-PVP or by PEI-polyepichlorohydrin [115]).

Mixed ligand macromolecular metal complexes derived from a combination of synthetic and biopolymers are of special interest. Several investigators have studied the solubility of polyelectrolyte-metal-protein mixtures in water medium. In particular, insoluble complexes of BSA with nonfractionated polyacrylic acid (PAA) in the presence of Ba^{2+} have been described by Morawetz *et al*. [30]. The soluble and insoluble ternary complexes of the positively (or negatively) charged narrow fractions of polyelectrolytes with same charged proteins in the presence of different transient metal ions have been firstly reported and systematically studied by Mustafaev [118-129]. Such systems include the same charged polyelectrolyte and protein mixtures and do not possess the ability for complexation without metal ions. Poly-4-vinylpyridine (PVP), poly N-vinylimidazole (PVI), PAA, poly(N-isopropylacrylamide) (PNIPAAm), polyacrylamides (PAAm) which contain amino acid end groups and copolymers of acrylic acid with 4-vinylpyridine, 2-methyl-5-vinylpyridine, vinylimidazole, N-isopropylacrylamide, N-vinylpyrolidone, (maleic anhydride-N-vinylpyrrolidone copolymers) and some others with different composition were chosen as polyelectrolytes. Proteins with different pI such as BSA, HSA, BGG, hemoglobin (Hb), globin (GL), transferrin (Tr), (Table 6), ovalbumin (OA), superoxide dismutase (SOD), surface antigens of the influenza virus, protein fraction of the BCG Mycobacteria cell, and some others were chosen as biopolymers.

As it was mentioned above the routes of ternary PE-Me-Protein complex preparation are very simple. Thus, for example, polycomplex can be obtained by a direct mixing of the solutions of the polymer-metal complexes with the solutions of proteins.

Polyelectrolyte-Metal Complexes. The formation of PE-metal complexes in mixtures of P4VP-Cu^{2+}, PAA-Cu^{2+}, PNIPAAm-Cu^{2+} and in mixtures of different copolymers with different transient metal ions were analyzed by the titration shift method and measurements by HPLC, cyclic

votammetry, spectroscopy, equilibrium dialysis, and fast sedimentation methods [124,130-135]. It was shown that water-soluble and insoluble stable polyelectrolyte-metal complexes are formed in the wide range of pH, and the solubility of polycomplexes depends on the metal/polymer ratios and pH of solutions.

The solutions of partially ionized polyelectrolytes, containing Cu^{2+} ions, were analyzed by the titration shift method, cyclic votammetry and HPLC measurements recently [124]. These data have implicated the presence of chelate with four pyridine groups in P4VP-Cu^{2+} and two carboxylate groups in PAA-Cu^{2+} mixtures bound to a copper ion, correspondingly. Fast sedimentation and quenching of luminescence have been employed to study the binding Cu^{2+} by PAA recent1y [136]. It was shown that the metal ions are unevenly distributed between the macromolecules. However, these findings were obtained only by the rather indirect method of quenching of luminescence. Therefore, in this investigation, the interactions between Cu^{2+} and PAA were at first analyzed by HPLC, which allows study of the fraction composition at relatively low concentrations of mixture. Addition of copper ions did not affect the solubility of PAA within a certain range of n_{Cu}/n_{AA} values at pH 7. The phase separation in the system PAA-Cu^{2+} occurred only at some critical metal concentrations ($n_{Cu}/n_{AA} = 0.25$). A typical HPLC analysis of PAA and of its soluble mixture with Cu^{2+} ions at different ratios of their molecular concentrations is given in Figure 69. The mixture of PAA-Cu^{2+} was characterized in chromatograms by two peaks. Moreover, the presence of Cu^{2+} gave rise to an increase the optical density of the mixture. The increase in the optical density (A_{280}) of the solution may reflect the involvement of the part of PAA in polymer-metal complexes (peak II). The bimodal distribution of components may lie in the uneven distribution of Cu^{2+} between the polymer coils, which appears to move more slowly than free PAA (peak I). The elution volume *(Ve)* corresponding to peak I does not change and remains equal to that of free PAA, but the values for the *Ve* of peak II differed from those values of the individual PAA peak. Free Cu^{2+} ions were hereby absent in the PAA-Cu^{2+} mixture as indicated by cycle votammetry (see below under Figure 69). Moreover, analysis by atomic absorption spectroscopy indicated an uneven distribution of Cu^{2+} among the peak fraction obtained by Sephadex G-IOO filtration of PAA-Cu^{2+} mixture [Figure 69(b)]. These findings indicated a nonrandom distribution of the copper ions between the polyanions under the experimental conditions ($n_{Cu}/n_{AA} < 1$). PAA-Cu^{2+} mixtures are, thus, implicated to consist of two fractions: PMC-I (PAA-Cu^{2+} complexes with relatively low concentration of Cu^{2+} ions and/or free PAA) and PMC-II (PAA-Cu^{2+} complexes with relatively high concentration of Cu^{2+} ions). When the ratio n_{Cu}/n_{AA} is 0.25, a phase separation took place in the system and the area of the peaks in the matrix solution was decreased. Both peaks disappeared when the ratio of n_{Cu}/n_{AA} is 0.4. It follows from these data that, at relatively high concentrations ($n_{Cu}/n_{AA} \geq 0.25$), Cu^{2+} promotes the crosslinking of the macromolecule, as a result of which the system loses its solubility.

3.1. Ternary Polyanion-Metal-Protein Systems

Insoluble Complexes. To produce a polymer-metal complex, various concentrations of the metal salts (for example $CuSO_4x5H_2O$, pH: 4) solution were added to PE, dissolved in phosphate buffer. The pH values were adjusted with 1M NaOH to the desired pH. The ternary complexes were, in turn, prepared by adding protein solutions to the polymer-metal complex (PMC) solution. Water-insoluble products of the complexes were investigated spectrophotometrically at 400 nm and by weighing dry amounts of pellet. Protein/polymer (n_p/n_{AA}) and Cu^{2+}/AA (n_{Cu}/n_{AA}) ratios were calculated using the equation $n=CN_A/M$, where n is the number of the molecules in 1 ml; M is the

molecular weight of components; N_A is the Avogadro number; C is the concentration in g/100ml. Curves of the turbidimetric titration of a copolymers solution in the presence of metal ions with a solution of proteins are presented as the dependence of optical density (or amount of precipitate) of the solution on the n_P/n_{AA} and C_P/C_{CP} ratios (C_P and C_{CP} -weight concentration of protein and copolymer correspondingly).

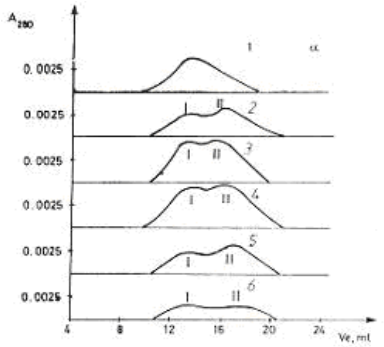


Figure 69. (a) Gel filtration HPL chromatograms of PAA (1) and of its mixtures with Cu^{2+} at different ratios of Cu^{+2} ions to acrilic acid monomers (AA) ((n_{Cu}/n_{AA}):2-0.08;3-0.10;4-0.15;5-0.20;6-0.25;(5-6)-the phase separation in the system takes plase. The concentration of PAA was 0.1 g/dL; PH7. Bio-Sil Sac 25 column was used in the experiment. (b) Distribution of the amount of Cu^{2+} in the mixtures PAA-Cu^{2+}

Table 6. Some physico-chemical properties of proteins

Protein	Origins	Molecular Weight (kDa)	Isoelectric Point (pI)
BSA	Bovine	67	4.7
HSA	Human	88	5.0
Tr	Human	88	5.9
Hb	Human	64	7.0
Gl	Human	16	6.8

Figure 71 presents the results of the turbidimetric titration for different ternary PE-M-Protein mixtures. As it follows from these figures in the general case the system is characterized by a three regions of reaction mixture composition and solubility; dependence of the optical density (A_{400} nm or precipitate amount) of the ternary mixtures on protein concentrations (at constant polymer-metal concentrations) passes through a maximum.

The system remains homogenous over a wide range of the protein/PMC ratios, i.e. the formed ternary complexes are soluble (region I) for the excess PMC case. The phase separation in the system occurs only at some critical protein concentrations depending on the degree of polymerization of PE, and metal concentrations. In this region II phase separation takes place, which indicates the formation of an insoluble triple complex. On further increase in C_p/C_{pe} the optical density increases and then attained a limiting value. The pattern changes significantly on further increase in protein concentrations. Depending on the protein concentration in the mixture, partial or complete prevention of phase separation then takes place and at some critical ratios of protein/PMC (region III) the system again becomes homogeneous.

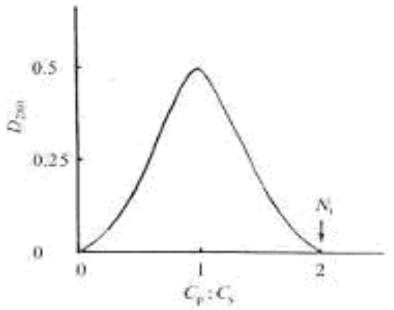


Figure 70. Dependence of the relative optical density (D) of the ternary mixture of protein (BSA), CP-1, and Cu^{2+} on the amount of protein (C_p) added at constant concentrations of CP-1 and Cu^{2+} (C_s).Point N_4 indicates the minimum weight of protein which must be introduced into the system to prevent precipitation.

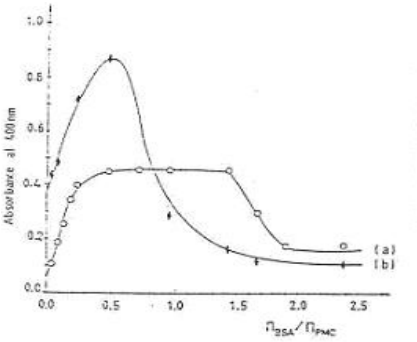


Figure 71. The Dependence of A_{400} on the ratio of components for the mixture PAAm-Cu^{2+}-BSA. The BSA solution at different concentration added to the solution of PMC at constant concentrations of PMC (n_{BSA}/n_{PMC}). Mw(PAAm)=20 000; 20 C°; pH 7; C_{PAAm}=0.15 g/dL;C_{CuSO4}-= 0.05 g/dL.(b) The PMC solution at different polymer concentrations of BSA (n_{PMC}/n_{BSA}) CBSA=0.5 g/dL; $n_{Cu^{2+}}/n_{PAAm}$ =0.1; ($C_{PAAm}\leq$3 g/dL;$C_{CuSO4}\leq$1.05 g/dL)

The solubility of the mixture depends on the nature (or pI) of the proteins and pH value of the solutions. Figure 70 presents the dependence of the optical density of the ternary mixture of different proteins, PAA and Cu^{2+}, on the amount of added proteins at constant concentrations of the PAA and Cu^{2+} (PMC). Starting with the ratio n_{Cu}/n_{AA} = 0.25, a phase separation took place in the systems Hb-Cu^{2+}-PAA and GL-Cu^{2+}-PAA at pH 7 even at very low concentrations of copper ions (n_{Cu}/n_{AA} =0.08). However, Hb and GL solutions mixed with PMC at higher values of pH gave rise to soluble ternary mixtures (Figure 71b). As for the systems BSA-Cu^{2+}-PAA, HSA-Cu^{2+}-PAA and Tr-Cu^{2+}-PAA, observations provided a different picture. The values of A_{400} did not change considerably over a wide range of metal concentration ($0 < n_{Cu}/n_{AA} < 0.25$) in the system with increase in the amount of protein added. However, there existed a critical concentration of Cu^{2+} ions in ternary mixtures ($n_{Cu}/n_{AA} \geq 0.25$), at which the system lost the homogeneity with formation of insoluble particles (see Figure 71). In conclusion of the results presented in this section it is suggested that the solubility of the ternary mixtures is closely correlated with the pI values of protein molecules (Table 6) and depends on the concentration of components in the mixture.

Studies of the role of different factors of reaction, environment permits to elucidate some important features characterizing ternary complex formation. The homogenous mixture of ternary systems shows some different behavior depending on the order of addition of protein to PMC or PMC to BSA (Figure 72). For mixture prepared by the addition of various protein concentrations to PMC solutions in the early stage, all systems were water soluble and homogeneous. However, depending on time and on the ratio of these mixtures, the homogeneity was lost and insoluble particles appeared in the system. For this case the absorbance of mixtures was measured after 24 hours. At the addition of PMC solutions to protein solution formation of the precipitate occurs on the same manner. However, as seen from Figure 74, the width of the curve of turbidimetric titration is narrower in last case. At the same time in both cases, independent of the method of preparation, the same character of the formation of soluble and insoluble ternary complexes versus ratios of components are observed. Thus the different ways of protein and PMC mixing differ by the intermediate states of ternary mixtures depending on the

component ratio in the mixture, but at a proper ratio the both lead to the formation of soluble polycomplexes.

It is critical that insoluble complexes, unlike soluble complexes which form instantaneously, take place depending on time. These results permit investigation of the velocity of the formation of the ternary insoluble complex by the spectrophotometric method at different reaction conditions (the effects of the preparing mixture, ratio of the components, low molecular salts, temperature and stirring, molecular weight of polyelectrolyte and Cu^{2+} concentration). Figure 75 shows the variations of the absorbance at 400 nm versus time for the mixtures, prepared by different methods and ratio of components. Obtained results showed that, the velocity of the formation of insoluble complexes in the case when BSA solution was added to PMC solution is faster. In this case the concentration of BSA was less than the concentration of PMC at the initial moment, in the system water soluble complex occurred. When PMC solution was added to BSA solution, because the concentration of BSA is small at the beginning of titration, insoluble ternary complex formed, therefore the reaction rate increased slowly, i.e. the diffusion process decreased. As can be seen with increasing ratios, the rate of reaction increases to reach a maximum value and then decreases. For a ratios which corresponds to soluble complexes, the value of dA/dt =0 (dA/dt-the rate of the reaction was obtained from the tangent to the curves in the early stages of the reaction).

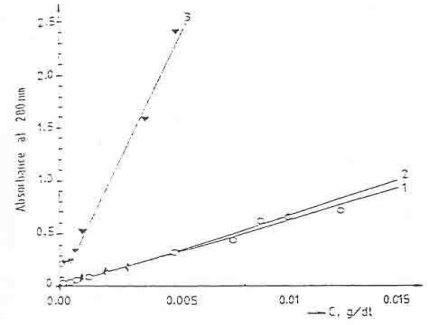


Figure 72. The dependence of λ_{280} of mixtures on concentration of BSA. The correlation coefficient (r)of straight lines are 0.9901 (1.BSA-PAAm), 0.9972 (2.BSA) and 0.9948 (3. BSA-Cu2+- PAAm), Mw=20 000; C_{PAAm} = 0.15 g/dL C_{CuSO4} = 0.05 g/dl)

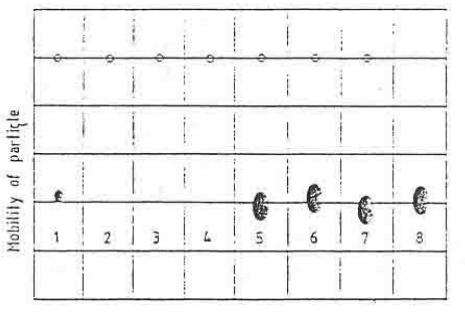


Figure 73. Electrophoresis of BSA and the mixtures of BSA-Cu^{2+}-PAAm (t=60 mins, V=300 volt). C_{BSA}=0.5g/dl (1); The concentrations of BSA in the mixtures of BSA in the mixtures of BSA-Cu^{2+}- PAAm are 0.075 g/dl (2), 0.125 g/dl (4), 0.375 g/dl (5), 0.5 g/dl (6), 0.875 g/dl (7) and 1.0 g/dl (8); C_{PAAm}=0.15g/dl; C_{CuSO4}=0.05 g/dl.

The mixture PMC-BSA was prepared at the different concentration of the sodium chloride to investigate the effect of the low molecular weight salts (Figure 76). With increasing NaCl concentration, the slopes of curves decrease and at higher concentration of the NaCl the ternary system becomes homogeneous and the absorbance against time practically does not change. Thus, the concentrations of NaCl affect not only a solubility of the system but also the velocity formation of the colloid particles.

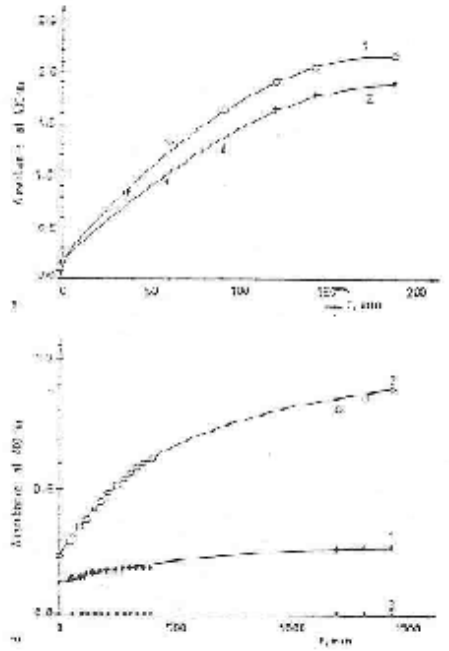


Figure 74. (a) The dependence of A_{400} of two mixtures BSA- Cu^{2+}-PAAm on times 1)BSA was added to the solution of PMC; 2)PMC was added to the solution of BSA; b)The dependence of the A_{400} of PAAm-Cu^{2+}-BSA mixtures on the time at three different (n_{BSA}/n_{PMC})=0.15 (1.dA/dt=0.15x10^{-3}); 1.0 (2. dA/dt=1x10^{-3}); 2.0 (3. dA/dt=0), (BSA was added to the solution of PMC) Mw (PAAm)=20 000.

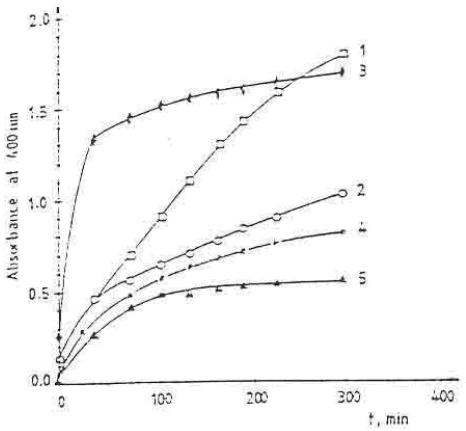


Figure 75. The dilution effect on the formation of the insoluble ternary mixture. The initial concentration of the mixtures of BSA-Cu^{2+}-PAAm; C_{BSA}=0.5 g/dl; C_{CuSO4}=0.05 g/dl; C_{PAAm}=0.15 g/dl; pH=7; 20 C°. C (1.dA/dt=0.67 x 10^{-3}); C/3 (2.dA/dt=0.89 x10^{-3}); C/5 (3.dA/dt=2.98 x10^{-3}); C/9 (4.dA/dt=1.02x10^{-3});C/17 (5.dA/dt=0.68x10^{-3})

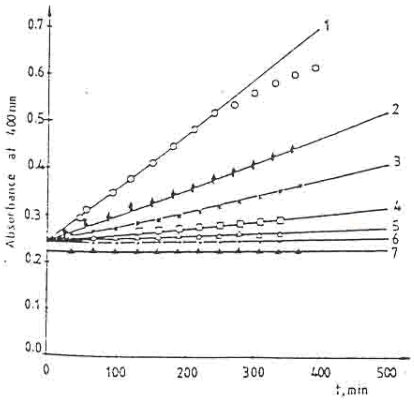


Figure 76. The dependence of A_{400} for the NaCl concentration in the mixture of BSA-Cu^{2+}-PAAm. pH = 7.0; 20°C; C_{PAAm} = 0.15 g/dl; C_{CuSo4} = 0.05 g/dl; C_{BSA}= 0.5 g/dl. 0 M NaCl (1.dA/dt = 117.7 x 10^{-5}); 0.01 M NaCl (2. dA/dt = 56 x 10^{-5}); 0.025 M NaCl (3. dA/dt = 42 x 10^{-5}); 0.05 M NaCl (4. dA/dt = 15.4 x 10^{-5}); 0.1 M NaCl (5. dA/dt = 6.25 x 10^{-5}); 0.125 M NaCl (6. dA/dt = 3.37x 10^{-5}); 0.5 M NaCl (7. dA/dt = 1.78 x 10^{-5}).

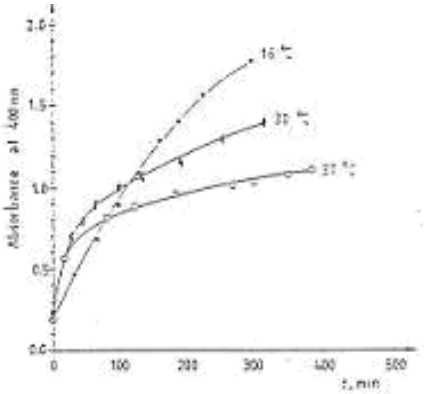


Figure 77. The effect of temperature on the formation of the insoluble ternary mixture pH 7; C_{PAAm}=0.15 g/dl; C_{BSA}=0.5 g/dl;C_{CuSO4}=0.05 g/dl

The formation rate of insoluble ternary complexes increases by increasing both the temperature and stirring of the mixture (Figure 77).

The effect of the molecular weight of polymer on the formation of soluble and insoluble ternary complexes has been studied at different molecular weight of P4VP (see below) and PAAm: 20000 (PMC1), 45000 (PMC2), and 230000 (PMC3). In all cases, the ratio of n_{Cu}/n_{AA} was kept constant at 0.1. The dependence of A_{400} of the ternary system on the ratio of components at different molecular weights of PAAm is shown in Figure 78.

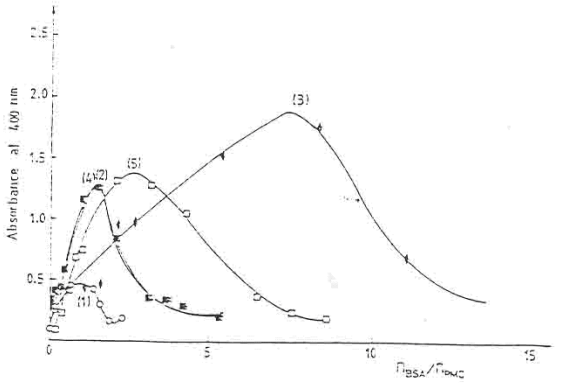


Figure 78. The dependence of A_{400} on molecular weight of PAAm and Cu^{2+} concentration for the mixture of BSA-Cu^{2+}-PAAm. pH = 7.0, 20°C. M_v = 20.000 (1), 45.000 (2) and 230.000 (3): C_{PAAm} = 0.15 g/dl; C_{CuSo4} = 0.05 g/dl (4); C_{CuSO4} = 0.1 g/dl (5).

The absorbance value of the ternary systems depends on the molecular weight of PAAm. At each of the three states, this dependence passes through a maximum. However with increasing of molecular weight of PAAm (Figure 78 curves 1-3), the maximum value of absorbance increases and soluble system takes place at higher values of n_{BSA}/n_{PMC}, i.e. it is necessary to use more BSA molecules to obtain soluble ternary system for high molecular weight of PAAm in ternary mixture. As it seen from this Figure 78 the increase of the Cu^{2+} concentration in ternary system at constant M=45000 is proportional to the amount of BSA on the ratio of n_{BSA}/n_{PMC} to obtain soluble ternary system. With increasing copper concentration in the system, the amount of Cu^{2+} which is bound to polymer increased, i.e. the capacity of binding of PMC molecule to protein increased. According to this idea, to obtain a soluble system in the case of greater Cu^{2+} concentration, it was necessary to add more BSA to system.

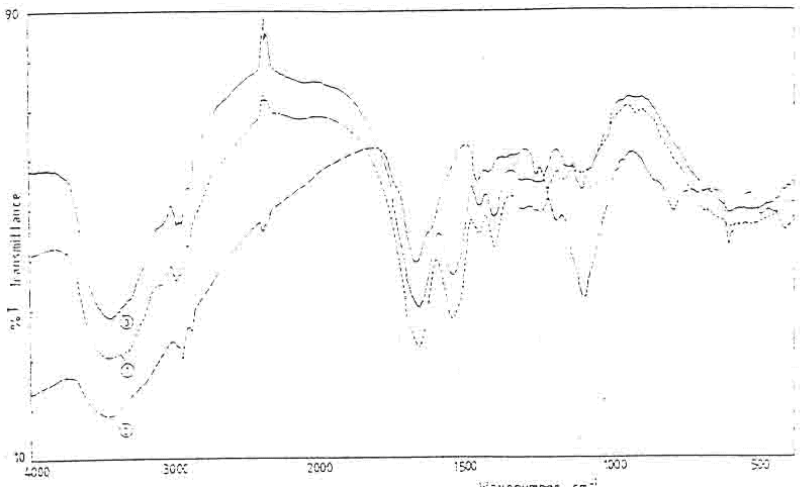


Figure 79. FT-IR spectra of PAAm (Mv =20.000) (1) and PMC1

The formation of insoluble ternary protein-metal-polymer complexes were investigated from FT-IR spectrophotometric measurements (Figure 79). As seen from the results in Figure 79 in ternary BSA-Cu^{2+}-PAAm mixture spectra the peaks at 592, 610 and 800 cm^{-1} corresponding to polymer-metal complexes are invisible. Also, the peak at 1100 cm^{-1} which is characteristic for the PAAm-Cu^{2+} strongly decreases. These results showed that the metal ions bound with PAAm after adding protein molecules partially bound with proteins. The FT-IR of the insoluble products of the ternary mixture of BSA with poly (N-isoporopylacrylamide-co-acrylic acid) copolymers in the presence of copper ions is shown in Figure 79. The copolymer spectrum has absorbances at 1240, 1500, 1580, 1650 and 1740 cm^{-1}, respectively. The BSA spectrum has absorbances at 1580 and 1650 cm^{-1}. In the CP-Cu^{2+}-BSA spectrum the band 1700 cm^{-1} disappeared and the 1580 and 1680 cm^{-1} bands shifted to 1650 and 1550 cm^{-1}, respectively. The intensity of the bands at the (1100-1200 cm^{-1}) strongly increased and changed their character. The bands 1200-1100 cm^{-1} correspond to the SO_4 counter ions of Cu^{2+} in solution. These results suggest that the copper ion is bound to the ---COOH, ---C=O, (--C—N) and (C—O) bands and mediated ternary complex formation between C_P and BSA.

The formation of the insoluble products in the mixture has influence on the surface tension of polymer solutions. (Table 7). Adding BSA molecules to the PAAm-Cu^{2+} solution initially (n_{BSA}/n_{PAAm} I) the surface tension of polymer solution decreases and at the n_{BSA}/n_{PAAm} I the surface tension takes a limiting value. These results indicate the formation of a new type colloid particle in systems; i.e., PAAm-Cu^{2+}-BSA complex.

Table 7. The dependence of surface tension to n_{BSA}/n_{PAAm}

n_{BSA}/n_{PAAm}	0.055	0.143	0.476	0.952	1.667	1.904
Surface tension (dyn/cm)	72.17	67.19	63.65	61.88	63.15	62.89

M_w(PAAm) = 20 000; 20 °C; pH = 7; C_{PAAm} = 0.15 g/dl; C_{BSA} = 0.05 g/dL

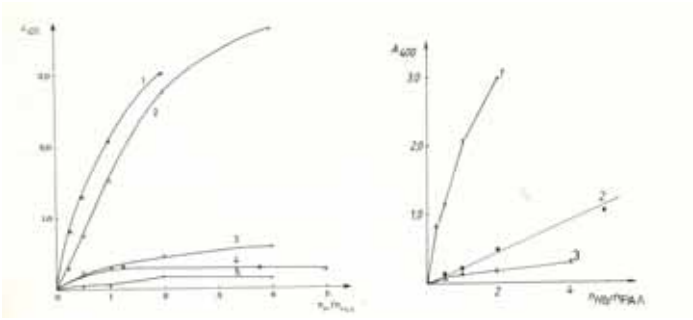


Figure 80. (a) Dependence of the optical density (A_{400}) of the ternary mixtures of different proteins with Cu^{2+} and PAA on n_p/n_{pAA} at pH 7.0. (1) Hb-Cu^{2+}-PAA; (2) Gl-Cu^{2+}-PAA; (3) Tr-Cu^{2+}-PAA; (4) HSA-Cu^{2+}-PAA; (5) BSA-Cu^{2+}-PAA. C_{PAA} = 0.1 g/dL, n_{Cu}/n_{pAA} = 0.08. **(b)** Dependence of the optical density (A_{400}) of the ternary mixture Hb-Cu^{2+} -PAA on n_{Hb}/n_{pAA} at different pH values. (1) pH 7.0; (2) pH 8.0; (3) pH 10. C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; T = 25°C.

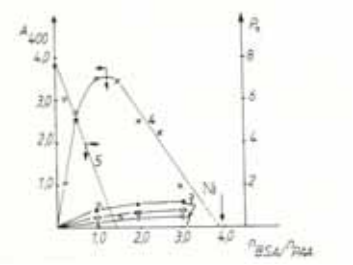


Figure 81. Turbidimetric titration of PAA-Cu^{2+} mixture with the solution of BSA. (1-4) Dependence of optical density (A_{400}) of ternary mixture PAA-Cu^{2+}-BSA on n_{BSA}/n_{pAA} at difference n_{Cu}/n_{AA}: 0.08(1); 0.1 (2); 0.2 (3); 0.3 (4). (5) Dependence of the area of the free PMC peaks (Po) on the ratio $n_{ESA}In_{FAA'}$ obtained in matrix solution of ternary mixture at n_{Cu}/n_{AA} = 0.3 C_{PAA} = 0.1 g/dL; pH 7; 25°C.

Water Soluble Complexes. The water-soluble mixtures of polyanion-metal complexes with negatively charged proteins were analyzed by different physico-chemical methods under different experimental conditions. For the analysis of the matrix solutions of the mixtures PAAm-PMC was used electrophoretic method (Figure 73). An electrophoretic study of this system showed that the proteins in the solutions are absent at the $n_{BSA}/n_{PMC\ I}$. At the $n_{BSA}/n_{PMC\ I}$ of mixture, the water soluble ternary complexes have been detected in the electrophoretic diagrams (at the same concentration, free BSA and ternary mixtures were shown to have different electrophoretic

mobility and optical density). The results plotted in this figure could be interpreted in terms of water soluble and insoluble ternary complexes formed simultaneously, at the $n_{BSA}/n_{PMC\ I}$. Further increasing the ratio n_{BSA}/n_{PMC} leads to the amount of the insoluble ternary complexes decreasing and soluble ternary complexes increasing.

The water soluble complex formation between proteins and polyanions in the presence of divalent copper ions were at first analyzed by HPLC method by Mustafaev [124]. Figure 82 presents an example of HPLC for the soluble mixtures BSA-Cu^{2+}-PAA at different n_{BSA}/n_{pAA} and n_{Cu}/n_{AA}. As suggested by the slight increase in peak I, the interaction between BSA and PAA at the pH 7.0 was weak, if not negligible, in the absence of copper ions (B). The results are consistent with the results obtained by sedimentation analysis of PAA-BSA systems.

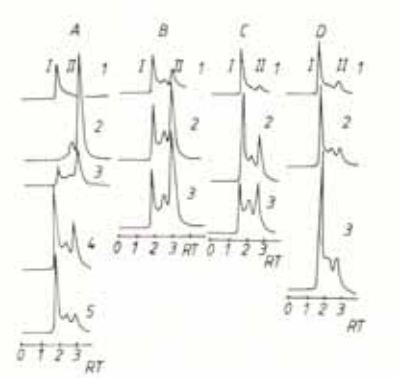


Figure 82. HPLC analysis of the formation of polymer-protein complexes in the presence of Cu^{2+}. Polymer-metal, (Cu^{2+})-protein complexes were prepared and HPLC analysis on gel filtration column performed as described in [124]. (A) Dependence of the complex formation upon n_{Cu}/n_{AA}: (1) 0.1 g/$_{100}$ mL PAA; (2) 0.1 g/$_{100}$ mL BSA; (3) (0.1 g/$_{100}$ mL PAA and 0.1 g/$_{100}$ mL BSA) plus Cu^{2+} (n_{Cu}/n_{AA} = 0.1); (4), as (3), with n_{Cu}/n_{AA} = 0.15; (5), as (3), with n_{Cu}/n_{AA} = 0.2. n_{BSA}/n_{pAA} = 1.0. (B) Chromatograms of PAA-BSA mixture in the absence of Cu^{2+}; (C) Chromatograms of PAA-Cu^{2+}-BSA mixtures at n_{Cu}/n_{AA} = 0.1. (D) Chromatograms of PAA-Cu^{2+}-BSA mixtures at n_{Cu}/n_{AA} = 0.2. For all (B, C, D) systems n_{BSA}/n_{pAA} = 0.5 (1); 1.0 (2); 3.0 (3). Diagrams represent normalized A_{280} values. (RT = retention time)

Stable complexation took place, however, upon addition of copper ions (A, C, D). The extent of complex formation was dependent on the amount of Cu^{2+} added and nearly quantitative under the experimental conditions at n_{Cu}/n_{AA} = 0.2. Thus, under conditions where both PAA and BSA have negative charges and are incapable of binding to one another, the divalent Cu^{2+} ions act as "fasteners", promoting the formation of fairly stable water-soluble ternary complex.

The participation of Cu^{2+} in the complex formation with PAA, BSA, and the ternary mixture was investigated by cyclic voltammetry analysis of soluble mixtures at pH 7. There were no peaks for PAA and BSA solution in the range of 700-800 mV.

Cyclic voltammograms of Cu^{2+} ions, PAA-Cu^{2+}, BSA-Cu^{2+}, and PAA-Cu^{2+}-BSA mixture were given in Figure 83. As it can be seen from the Figure 83, for the Cu^{2+} solution a single cathodic peak was formed at about 100 mV and the reverse scan exhibit an anodic peak at 250 mV. In the presence of PAA and BSA (PAA-Cu^{2+} and BSA-Cu^{2+}) the peak potentials correspond to reduction shift to more cathodic direction and peak currents decrease. When BSA in equal molar concentration with PAA (n_{BSA}/n_{AA} = 1)] was added to the PAA-Cu^{2+} mixture at pH= 7, the anodic and cathodic peaks disappeared practically completely. The cathodic peak was attributed to reduction of Cu^{2+} to Cu^{o} and reverse peak corresponds to its oxidation.

When the PMC solution is titrated with protein solution (n_{BSA}/n_{PAA} < 1), BSA is complexed with the polyion via copper ions. Some of the copper ion form intramolecular crosslinks in the free sections of the polyion and, thus, stabilize the structure as a whole.

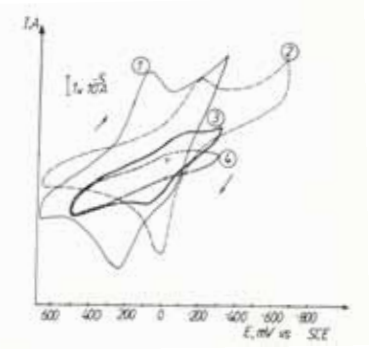


Figure 83. Cyclic voltammograms of 1.16 X ıo-3M Cu (I); mixtures of PAA-Cu^{2+} (2), BSA-CU^{2+} (3), and BSA-Cu^{2+} - PAA (4). The concentration of Cu^{2+} in all mixtures are constant and equal to concentration of Cu^{2+} in (1); n_{Cu}/n_{AA} = 0.15; working electrode = GCE; ionic strength = O.l *N* $NaClO_4$; pH 7.

The pattern changes significantly on further increase in ratio, n_{BSA}/n_{PAA} > 1 [Figure 84 (A) and (C)]. Under this condition, depending on the concentration of Cu^{2+}, the reaction between PMC and BSA may follow either of two different ways.

At low Cu^{2+} concentration (n_{Cu}/n_{AA} < 0.1), intensity of peak I first increased (n_{BSA}/n_{pAA} = 0.5 (1); 1 (2)) upon addition of BSA to the PMC solution. A further increase in BSA content (n_{BSA}/n_{pAA} = 2.0 (3)) led then to the decrease of the peak intensity nearly equal to that of free PAA at initial concentration [Figure 84 (C)]. The intensity of the peak II (monomer form) appeared to be at first essentially lower than the peak of the free BSA with equal concentration (diagram A,2 vs. C,2).

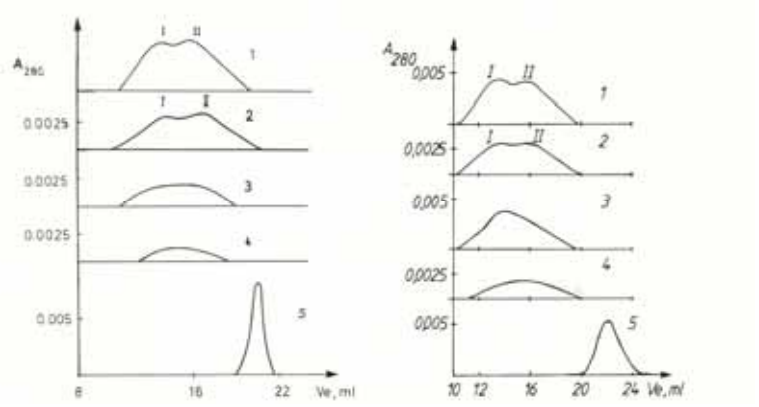


Figure 84. Gel filtration HPL chromatograms of free PMC and at the matrix solution of its ternary mixture (Hb-Cu^{2+}-PAA) (a) and (Gl-Cu^{2+}-PAA) (b) at different $n_p/n_{pAA'}$ (a) (1) Free PMC; 2.8 (2); 6 (3); 10 (4) (5) free Hb. (b) (1) free PMC; 2.8 (2); 6 (3); 10 (4); (5) free Gl. pH = 7.0; C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; *T* = 25°C. Bio Sil Sac 250 column was used in the experiment.

At n_{BSA}/n_{PAA} = 2.0, the intensity of peak II corresponding to free (monomer form) of BSA increased only slightly. Notice that the intensity of peak II with *Ve* corresponding to the dimer form of BSA increased considerably. Thus, it can be proposed that a further increase in BSA content to the breakdown of the complex as in mechanism (1) by the formation of BSA-Cu^{2+}-BSA and BSA-Cu^{2+} complexes and free PAA-Cu(II) (or PAA):

$$[\text{BSA-Cu}^{2+}\text{-PAA}] \xrightarrow{\text{BSA}} [(\text{BSA})_2\text{-Cu}^{2+}] + \text{PAA}(\text{Cu}^{2+}) \quad \text{(mechanism 1)}$$

As is known from the literature [124], BSA form in the presence of Cu^{2+}-soluble protein-metal-complexes with *Ve* corresponding to those of the monomer and dimer form of BSA. The higher capacity of BSA in complex formation with Cu^{2+} than PAA is consistent with this proposal.

At higher Cu^{2+} concentrations (n_{Cu}/n_{AA} > 0.15) [Figure 84 (A) and (D)/ a further increase of BSA continued to increase in the area of peak I, and the area of peak II did not change particularly. Therefore, a further increase of BSA leads to the formation of nonstoichiometric polycomplexes, for instance, the number of BSA molecules bound per polyion chain exceeds 1 [mechanism (2)].

$$[\text{BSA-Cu}^{2+}\text{-PAA}] \xrightarrow{\text{BSA}} [(\text{BSA})_n\text{- Cu}^{2+}\text{-PAA}] \quad \text{(mechanism 2)}$$

A migration of Cu^{2+} from the free to protein bound sections of PAA may contribute to this letter process. This is explained by the fact that the filled and free PAA macromolecules may exchange Cu^{2+} ions:

$\text{PAA*- Cu}^{2+} + \text{PAA} \rightarrow \text{PAA*} + \text{PAA- Cu}^{2+}$ and $\text{PAA- Cu}^{2+} + \text{PAA*} \rightarrow \text{PAA} + \text{PAA*- Cu}^{2+}$ where PAA * is an anthryl label containing poly(acrylic acid).

Analysis of the supernatant of the insoluble ternary protein-metal-polyanion mixtures was carried out with HPLC-gel filtration methods (Figure 84). Two peaks were hereby seen in the chromatograms of the matrix solution in both cases of Protein-Cu^{2+}-PAA corresponding to PMC-I and PMC-II. The area of these peaks depended on the concentration of protein and decreased simultaneously with the increase in the ratio n_{pr}/n_{PAA}. The turbidity (A_{400} nm) increased also in proportion with the increase in n_{pr}/n_{pAA} (Figure 84 (a)). The peaks with *Ve* corresponding to elution volume of free Hb (Figure 84 (a), diagram 5) and Gl (Figure 84 (b), diagram 5) were absent in the chromatograms of the supernatants. The absence of free protein molecules in the matrix solution indicated that all the added protein molecules are strongly bound by the PMC, resulting in the formation of insoluble ternary complexes PAA- Cu^{2+}-protein. (Indeed, the absence of complexed protein in the matrix solution was also reflected in lack of reaction with the Folin phenol reagent method.)

It can be seen that, when the ratio n_{pr}/n_{pAA} > 1, protein-free fractions of PMC remain in the matrix solution. The existence of the PMC under these conditions unambiguously indicates a nonrandom distribution of the protein molecules between the coils of polyions.

An analysis of the formed insoluble polycomplex composition deserves some consideration. Dependence of the chromatography peak area *(Po)* of free PMC (the sum of the peaks corresponding both PMC-I and PMC-II) in the Protein-Cu^{2+}-PAA system on n_{pr}/n_{pAA} is shown in Figure 85. The intersection points obtained by the extrapolation of these plots to the zero area of the free PMC peak correspond to n_{pr}/n_{pAA} when all PMC macromolecules are bound to a complex with Hb and GL Taking into account the above-indicated fact of the quantitative binding of proteins to PMC, one may consider that lim (n_{pr}/n_{pAA}) = Ni, when P_0 = 0. This limit equals the number *(Ni)* of the protein molecules bound by a PAA of a given degree of polymerization under given conditions.

At the $n_{pr}/n_{pAA} > Ni$, only one peak was seen in chromatograms in both cases (Hb-Cu^{2+}-

PAA, GI-Cu^{2+} -PAA), corresponding to the free protein; therefore, after $n_{pr}/n_{pAA} > Ni$, all PMC molecules were trapped in the fraction of insoluble complexes and the surplus of protein molecules remained in the matrix solution.

Analysis of matrix solutions of BSA-Cu^{2+}-PAA mixture revealed the fact that only an individual free PMC component was observed at $0 < n_{BSA}/n_{pE} < 1$ in matrix solutions. The increase of BSA content in polyelectrolyte mixture leads to a decrease of amount of free PMC and at about ratio of $n_{BSA}/n_{pAA} = 1$ the concentration of free PMC in mixture is equal to zero. This results show that in this case:

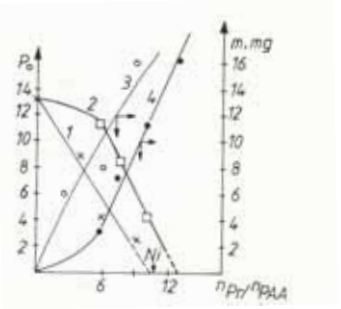
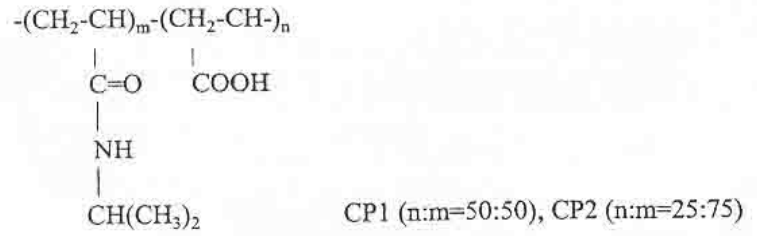


Figure 85. Dependence of the chromatogram peak area (Po) of free PMC and the amount (m) of the precipitation in Hb-Cu^{2+}-PAA (1,3) and GI-Cu^{2+}-PAA (2,4) on n_{pr}/n_{pAA}; C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; pH 7.

the formation of a stoichiometric polycomplex (BSA : PMC = 1 : 1) insoluble in aqueous media has taken place. At maximum precipitation all the protein and PMC are completely incorporated to an insoluble ternary complex. When the ratio of the components in the solution is $n_{BSA}/n_{PE} > 1$, in matrix solution a transformation of insoluble complexes into soluble BSA-Cu^{2+}-PE complexes is observed.

The HPLC-ion exchange analysis of the matrix solutions of these ternary mixtures showed that the water-soluble products in the matrix solution were obtained as one peak in the free eluent volume (Figure 86). Therefore, in the ternary mixture at these concentrations of added metal ions, insoluble, and soluble ternary complexes appear to be formed simultaneously.

"Intelligent" polycomplexes. Cu^{2+}–mediated interpolyelectrolyte complex formation of BSA and water-born poly(N-isopropylacrylamide-co-acrylic acides) (CP) copolymers:



were studied by HPLC, UV-Visible spectrophotometer and fluorescence methods by Mustafaev [127,128]. HPLC analysis confirmed that at pH 7 BSA does not bind CP, since both protein and copolymer macromolecules are negatively charged at this pH and electrostatic repulsion forces prevent complex formation. However, complexes were formed following the addition of copper ions (Figure 86). The incorporation of BSA in the soluble complex with CP1-Cu^{2+} (PMC) was indicated by a peak shift corresponding to BSA on the chromatograms. The extend of complex formation was dependent on the composition of CP, the C_{BSA}/C_{CP} ratio and the amount of Cu^{2+}

added. Only one peak, corresponding to the stable water soluble ternary complexes, was observed for the C_{P1} at n_{Cu}/n_{AA} =0.2 and C_{bsa}/C_{cp}=1.0 in water and in 0.154 M NaCl. However, there is a critical concentration of Cu (n_{Cu}/n_{AA} =0.2) in ternary mixtures, (C_{BSA}/C_{CP}=1) at which the system lost homogeneity with the formation of insoluble ternary polycomplexes. CP2, which contains less acrylic acid comonomers, with n_{Cu}/n_{AA} 0.08, formed only insoluble ternary complexes. These results suggest that the hydrophobic N-isopropylacrylamide comonomers in composition of macromolecules decreased the solubility of their ternary complexes with proteins.

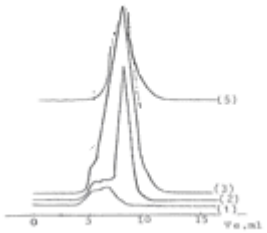


Figure 86. HPLC analysis of the soluble CP1-Cu^{2+} (1), CP1-BSA (2) and CP1-Cu^{2+}-BSA (3. 4) mixtures at different n_{cu}/n_{AA}: 0.1 (3); 0.18 (4); (5) corresponds to free BSA. CP at 280 nm does not absorb at pH 7.0. Solution is water without added NaCl. C_{BSA}/C_{CP} = 1.0; C_{CP} = 0.1 g/100 mL.

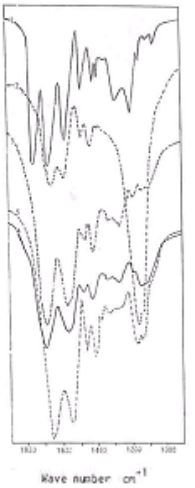


Figure 87. FT-IR spectra of (1)-CP1; (2)-CP1+Cu^{2+}; (3)-BSA; (4)-BSA+Cu^{2+} (5)-CP1 Cu^{2+}-BSA (n_{Cu}/n_{AA}=0.2)

Studies of thermal collapse property of CP-Cu^{2+}-BSA ternary mixtures in water and in the presence of low molecular weight salt permits to elucidate some important features characterizing ternary complex formation. The temperature dependence of OD_{500} values for the solutions of CP1-Cu^{2+}-BSA mixtures in water and in the presence 0.154 M NaCl, prepared at n_{Cu}/n_{AA} =0.2 and C_{BSA}/C_{CP}=1 are shown in Figure 86.

At pH = 7 the phase state of the ternary mixture prepared in water as CP and CP-Cu^{2+} complex was independent of temperature increases and remained soluble in water (they showed thermal collapse property at pH 3.0). In contrast, in solutions of 0.154 M NaCl the ternary mixture revealed increasingly higher OD_{500} values with increased temperatures. The ternary CP1-Cu^{2+}-BSA mixture in salt solution exhibited a negative temperature solubility coefficient and a phase transition on heating (near body temperature). The next series of experiments were devoted to analysis of the composition of the insoluble fraction of the CP1-Cu^{2+}-BSA mixture after transformation by temperature. HPLC results for the soluble mixture CP1-Cu^{2+}-BSA which was prepared by dissolution at room temperature of the precipitate obtained after heating the ternary mixture at 37° C are shown in Figure 87, 88, 89.

The single chromatogram peak showed that all of the reaction components after thermal collapse are composed of CP1-Cu^{2+}-BSA complexes. The absence of a soluble fraction in the matrix solution (no peaks were found in the chromatograms) suggest that the soluble CP1-Cu^{2+}-BSA complexes prepared in 0.154 M NaCl were transformed to the insoluble state by heating

while the insoluble complexes transformed to the soluble state. Therefore, water-soluble stable ternary complexes in salt solutions have a negative temperature solubility coefficient and show phase transition on heating. Dehydrated precipitate of ternary polycomplexes dissolved upon cooling, demonstrating their reversible properties.

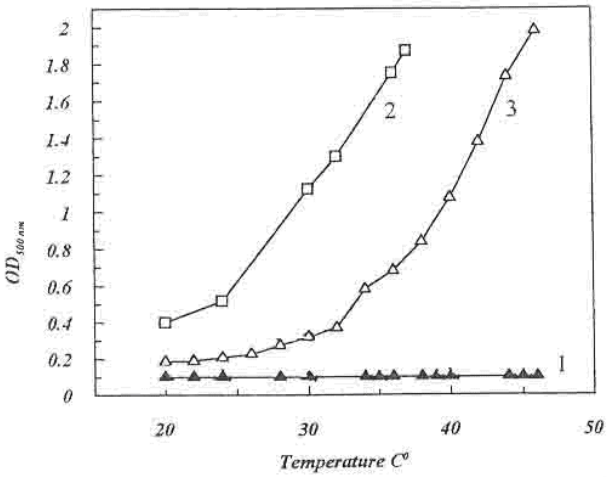


Figure 88. The temperature dependence of the optical density at 500 nm (OD_{500}) of CP-Cu^{2+}-BSA mixtures, prepared in water (1) and in 0.154 M NaCl: 2 (CP1-Cu^{2+}-BSA); 3 (CP2-Cu^{2+}-BSA); n_{Cu}/n_{AA}=0.2 (1,2) and 0.08 (3); pH 7.0; n_{BSA}/n_{CP}=1.0

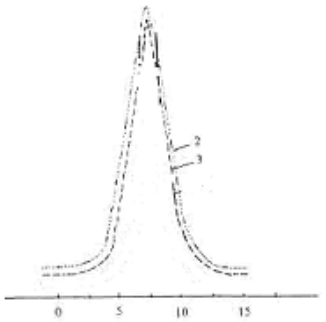


Figure 89. HPLC analysis of the soluble CPI-Cu^{2+}-BSA mixtures were prepared at different conditions: (1) ternary mixture prepared in water; (2) ternary mixture prepared in 0.154 M NaCl; (3) CPI-Cu^{2+}-BSA mixtures prepared in 0.154 M NaCl solution from the precipitate obtained after heating (37°C) the soluble ternary mixture in 0.154 M NaCl n_{cu}/n_{AA} = 0.2; n_{BSA}/n_{CP} = 1.0; pH = 7.0.

As seen in Figure 90, the UV spectrum of the ternary mixture in physiological salt solutions differed significantly from that of the polycomplexes in aqueous solution.

In the absorbance spectra of polycomplexes were prepared in physiological salt solution, two new supplementary peaks were observed at 324 and 364 nm. The intensity of these peaks increased and wavelength shifted from 360 to 376 nm with increased temperature. This alteration is essentially due to an exchange reaction, which is characterized by two processes. One is dehydration of the IPAAm/AA copolymers with increasing solution temperature. The other is a

cooperative hydrophobic interaction of dehydrated N-isopropyl groups, which increased the intensity upon addition of a low molecular weight salt to the system. These results suggest that the ---CONH— and ---COOH groups of the copolymers in salt solution may form ion-coordination complexes with copper ions which are observable in the UV at 320 and 360 nm.

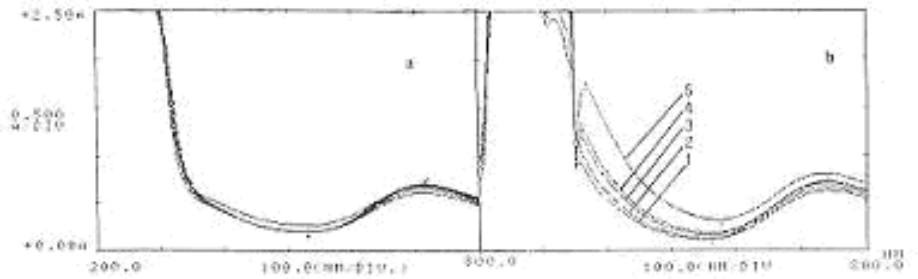


Figure 90. UV-Visible spectral results for the CP1-Cu^{2+}-BSA mixtures in water (a) and in 0.154 M NaCl (b) obtained at different temperatures °C: 15°(1), 18°(2), 30°(3), 35°(4), 45°(5), pH 7 n_{Cu}/n_{AA}=0.18; C_{BSA}/C_{CP}=1.0

Fluorescence Study. As it was mentioned above the formation of polycomplexes in the mixtures protein-metal-polyelectrolyte and their structure were intensively studied by titration, HPLC, electrophoretic, spectrophotometric (FT-IR, UV-Vis.), light scattering and hydrodynamic (viscosity and sedimentation) methods. These methods provide general information about the structure of polycomplex particles, while the information about the structure of protein globules in these particles is practically absent. Fluorescence techniques have recently been used to study protein-polyelectrolyte complexation (see above, section of protein-polyelectrolyte complexes). Previously we described the formation of water-soluble ternary polycomplexes between ovalbumin (OA) which was labeled with fluoresceinisotiocianate (F) (OAxF) and copolymers of acrylic acid with 2-methyl-5-vinylpyridine (CP) in the presence of copper ions [29]. Typical fluorescence spectra of pure OAxF and OAxF in mixtures with CP and CP-Cu(II) are given in Figure 91.

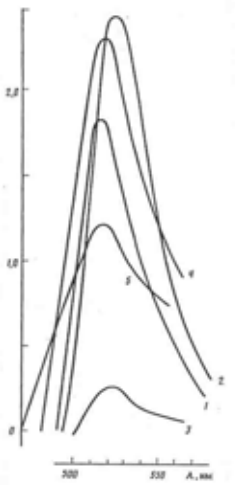


Figure 91. Fluorescence spectra of pure OAxF conjugate (I) and its complexes with PE:PVP(R_2, R_{16}) (2), PVP(R_2, R_{16}) (3), CP (4) and CP-Cu(II); β=4.6(2),13.2(3);

When CP solutions are added to solution OAxF at pH 7 the quantum yield of fluorescence of OAxF is increased that may testify to complex formation between protein and CP [29]. Hydrophobic interaction of protein molecules with methylvinylpyridine monomer units of copolymers and formation of hydrogen bonds between them provide the binding of the same (negative) charged macromolecules. The pattern is quite different the presence of copper ions; the fluorescence is quenched by adding of copper ions to solution of CP-OAxF mixtures. This results show to the including of copper ions into composition of ternary CP-Cu^{2+}-OAxF complexes.

It is known that from the fluorescent emission shift of tryptophan residues in proteins, it is possible to localize the interaction between proteins and polyelectrolyte at certain protein domains. Recently, BSA interactions with PAA, temperature sensitive poly(NIPAAm), and copolymers of acrylic acid with N-isopropylacrylamide in different composition at the presence of Cu^{2+} ions were investigated by fluorescence methods and HPLC analysis. The idea was to use Cu^{2+} ions both as an important component for the stabilization of the complexes and as a quencher. Effects of complexation conditions such as the sequence of mixing, metal/polymer and polymer/protein ratios, and hydrophobic-hydrophilic balance of polymers are discussed. Control experiments were made with a different quencher (succinimide, potassium iodide, and cesium chloride), which do not form complexes with the polymers used.

Poly(N-isopropylacrylamide) (PNIPAAm) and copolymers of *N-iso*propylacrylamide (NIPAAm) and acrylic acid (AA) (poly(NlPAAm-AA)) were prepared by a radical polymerization of NIP AAm and AA in distilled water in the presence of 2-oxoglutaric acid and UV irradiation (365 nm). The monomer compositions *(r)* of copolymers poly(NlPAAm-AA) used in this study were NlPAAm/AA = 3:1 (P25), 1:1 (P50), and 1:3 (P75), poly(NIPAAm) (PO), and PAA (PlOO) (Table 8).

Table 8. Characteristics of Polyelectrolytes

abbreviation	monomer composition NIPAAm:AA	η_{sp}/C,[a] dL/g	LCST, °C
P0	100:0	1.88	32
P25	75:25	2.95	32
P50	50:50	3.12	26
P75	25:75	13.25	34
P100	0:100	4.4	

[a] Reduced viscosity at 0.1 g/dL in PBS at 22 °C.

[Quencher]/[BSA] and [Cu^{2+}]/[BSA] ratios (n_q/n_{BSA}, n_{Cu}/n_{BSA}) were calculated using the equation $n = cN_A/M$, where n is the number of the molecules in 1 mL, M is the molecular weight of components, N_A is the Avogadro number, and c is the concentration in g/L. The heterogeneity of polymers and proteins and the fraction compositions of the mixtures were estimated by the HPLC system.

Fluorescence emission spectra were obtained using a Quanta Master spectrofluorimeter (Photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted to 2 or 3 nm. The excitation was at 280 nm. The fluorescence of proteins is widely used to study of their behavior depending on different influencing factors [129]. Binding of substrates, association reactions, denaturation, and interactions with other macromolecules may result in the changes of protein fluorescence spectra. In the present study we characterize them by the wavelength at the maximum of emission (A_{max}), fluorescence intensity in the maximum (I_{max}), and width at half-maximum level *(A)*.

From literature it is well-known that BSA interacts with Cu(II) in neutral water and water-soluble and insoluble complexes are formed [107]. However, these findings were obtained by the indirect methods. Therefore, in this investigation, the interactions between BSA and Cu(II)

were at first analyzed by HPLC, which allows study of the fraction composition at different metal/protein ratios. Addition of copper ions did not effect the solubility of BSA at pH 7 within a certain range of n_{Cu}/n_{BSA} values (Figure 92). The phase separation in the system BSA–Cu(II) occurred only at some critical metal concentration (n_{Cu}/n_{BSA} =10). Typical HPLC analysis of BSA and its soluble mixtures with Cu(II) ions at different ratios is given in Figure 93. Chromatograms of the mixture of BSA –Cu (II) are characterized by two peaks.

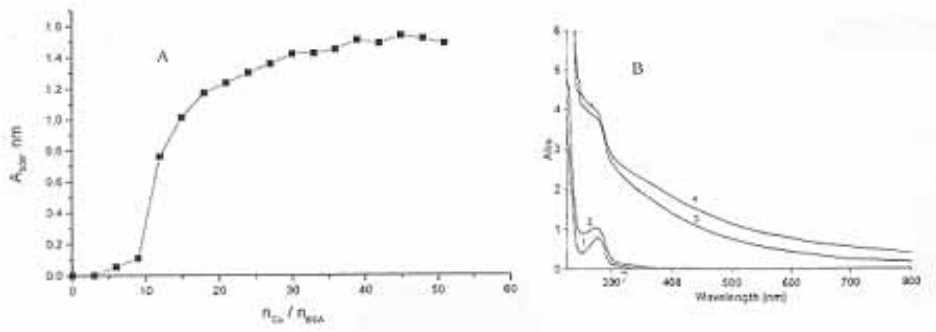


Figure 92. Turbidimetric titration of BSA solution with the solution of $CuSO_4$: (A) dependence of the optical density *(A_{500} nm)* of BSA-Cu^{2+} mixtures (n_{Cu}/n_{aSA}); (B) absorption spectra of BSA (1) and homogeneous solutions of BSA-Cu^{2+} mixtures at different n_{cu}/n_{asA} values (pH 7.0 and 25° C): 4 (2), 8 (3), and 40 (4). The concentration of BSA was 0.07 *g/dL*.

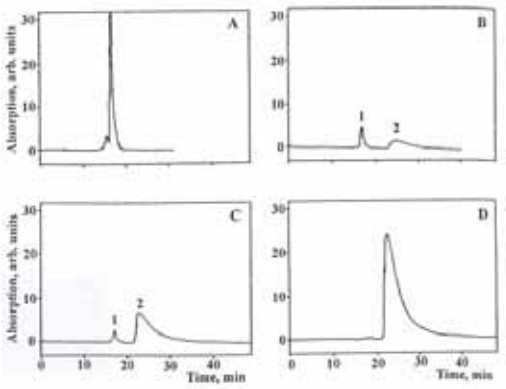


Figure 93. Gel filtration HPLC chromatograms of BSA (A) and of its mixtures with Cu^{2+} at different ratios of Cu^{2+} ions to BSA molecules (n_{Cu}/n_{aSA}): 4 (B); 8 (c); 40 (D).

The comparison of the retention time (RT) of the elution fractions corresponding to peaks 1 and 2 of the mixture and of the protein shows that the copper binding by BSA takes place: RT corresponding to peak 1 does not change and remains equal to that for free BSA, but RT of peak 2 differed from those values for a pure BSA peak. One may assume that peak 2 corresponds to the BSA–Cu(II) complex. HPLC analysis of homogeneous systems at different n_{Cu}/n_{BSA} permits the elucidation of some important features characterizing BSA–Cu(II) complex formation. Cu(II) increase in the mixture (at constant molar concentration of BSA) leads to a decrease of peak corresponding to free BSA molecules (peak 1) while the supposed peak of the complex (peak 2) increases (Figure 93 C). At the ratio [Cu(II)]:[BSA] = 10:1 the peak of free BSA disappears and there remains only the peak characterized by RT = 22.650 min. It is important to emphasize,

however, that free BSA still remains in the system over a rather wide range of the molar ratios [Cu(II)]/[BSA] even when n_{Cu}/n_{BSA} =10. These data indicate that the metal ions are unevenly distributed between the protein molecules: some protein globules may sorb the maximal quantity of Cu(II) ions, possible under given conditions, while the others remain practically unpopulated. Just this type of distribution was found previously at complexation of linear polyanionic and polycationic PE with Cu(II) ions [130,132]. The PE-Cu(II) complex exists as a chelate with carboxylate groups bound by copper ions. According to the literature [94] BSA has one specific binding site for Cu(II) at the NH_2-terminal tripeptide segment (Ala-Ala-His) involving the Asp-NH_2, His-N(l) imidazole, two deprotonated peptide nitrogens (Ala NH and His NH), and the Asp COO- group. At law concentrations of copper ions (n_{Cu}/n_{BSA} <10), the interaction can be considered to be only intramolecular: Cu(II) forms a complex with one protein globule. At high concentrations (n_{Cu}/n_{BSA} > 10) a phase separation takes place in the system (Figure 93 A). Analysis of the supernatant and sediment (by sediment dissolving in acetate buffer at pH 4.3) was carried out with HPLC gel filtration, UV-visible measurements, and a Zeeman atomic absorption spectrophotometer. The peaks with RT = 22.6 min, corresponding to the elution time of soluble BSA–Cu(II) complexes, were obtained in the chromatograms of the supernatants. The BSA fraction and Cu(II) ions coexist in sediment simultaneously. One can suggest that copper ions at n_{Cu}/n_{BSA} > 10 act more effectively as a crosslinking agent between two (or more) protein globules. This intermolecular interaction leads to the formation of soluble and insoluble protein-metal complexes with a complicated structure.

Solutions of mixtures of polyanions (PAA, poly(NIPAAm-AA) at different compositions) with BSA, in the absence and presence of Cu(II) ions, were analyzed recently by different physicochemical methods. It was shown that interaction between anionic PE and BSA at pH 7.0 at the absence of copper ions is weak, if not negligible. Stable complex formation took place, however, upon addition of copper ions. The extent of complex formation was dependent on the amount of Cu(II) added and nearly quantitative at n_{Cu}/n_{AA} = 0.2. Thus, under conditions where both PE and BSA have negative charges and are incapable of binding to one another, divalent Cu(II) ions act as "fasteners", promoting the formation of a fairly stable water-soluble ternary complex. The solubility, composition, and stability of these polycomplexes depend on composition of PE macromolecules and metal/PE (or protein) and protein/polymer ratios.

The values of optical density (A_{4OO}) did not change considerably over a wide range of metal concentrations ($0 < n_{Cu}/n_{AA} < 0.25$), when the system lost the homogeneity with formation of insoluble polycomplexes. At low concentrations of copper ions, their interactions may be considered as intramolecular (Cu(II) forms a complex with one polymer chain). At high concentrations the copper ion can act more effectively as a crosslinking agent between two (or more) polymer coils, which leads to the formation of soluble and insoluble ternary polycomplexes.

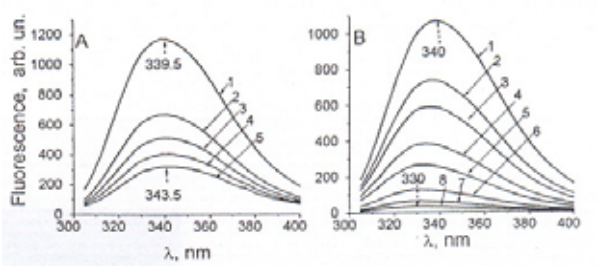


Figure 94. Fluorescence spectra of pure BSA (A) and BSA in mixtures of PE + BSA ([P50]/[BSA] = 3.52) (B) at different concentrations of Cu(II) ions in solution. BSA concentration was 0.71 mg/mL, phosphate buffer (pH 7). $CuSO_4$ concentration (in mM): 1, O; 2, 0.15; 3, 0.3; 4, 0.45; 5, 0.6; 6, 0.9; 7, 1.2; 8, 1.5.

Typical fluorescence spectra of pure BSA and BSA in mixtures PE (P50)-Cu(II)-BSA at different Cu(II) concentrations are given in Figure 94. It is well-known that tryptophan (Trp) fluorescence of proteins varies with their conformational changes resulting in changes of fluorescence parameters, such as the emission maximum (A_{max}), quantum yield, lifetime, an others. As shown in Figure 94 A the fluorescence intensity (I_{max}) of BSA at pH 7 and at the absence of polymer decreases (quenching) and A_{max} shows some red shift at Cu(II) concentration increase. BSA contains two Trp. One of them (spectral class 2 by Burstein with A_{max} = 340-342 nm and half width = 53-55 nm) is located on the bottom of BSA hydrophobic cleft. The second Trp of class 3 (A_{max} = 350-352 nm, half width = 59-61 nm) with law quantum yield (l/5 of the total BSA fluorescence) is superficial and completely accessible to aqueous solvent. The results in Figure 94 A indicate a much smaller efficiency of superficial Trp quenching at the background of very strong "cleft" Trp quenching, which suggests the presence of positively charged atoms in the vicinity of the former and negatively charged ones in the vicinity of the later. As a result at rather high [Cu^{2+}], the class 3 Trp becomes prevailing in BSA and the BSA emission spectrum shifts to longer wavelength. The situation is quite different at the same mainly conditions exert for polymer introduced into the solution. Figure 94 B shows that the BSA fluorescence spectrum, at the presence of polymer P50, shifts quite oppositely toward short wavelengths, which is indicative of the formation of a fairly stable water-soluble ternary polycomplex.

Study of a BSA tryptophan fluorescence for PE-Cu(II)-BSA mixtures in homogeneous systems at different component ratios and polymer compositions permits elucidation of some important features characterizing ternary polycomplex formation. Figures and present the dependence of A_{max} and l_{max} vs [Cu^{2+}] for BSA in the ternary mixture with different polymers at constant concentrations of the protein and different concentrations of polymers. These results clearly show that BSA fluorescence in mixtures depends on the chemical composition of polymers.

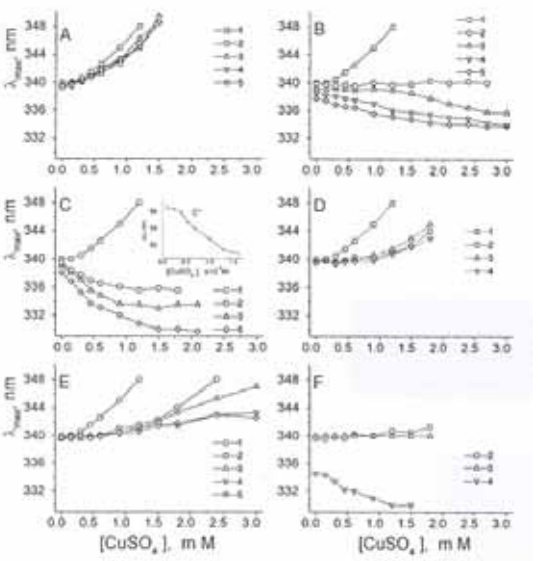


Figure 95. Position of fluorescence maximum of BSA in a mixture PE + Cu(II) + BSA vs Cu(II) concentration in solution (phosphate buffer, pH 7) at different polymers: A, O (PO); B, 25 (P25); C, 50 (P50); D,75 (P75); E, 100 (P100); F, the same as E but at pH 5. BSA concentration was 0.71 mg/mL. [P]/[BSA] (mg/mg) (A, B, C): 1, O; 2, 0.58; 3, 0.85; 4, 1.76; 5, 3.52. (D, E): 1, 0.58; 2, 0.85; 3, 1.76; 4, 3.52. Insert C*: half width of the spectrum vs [$CuSO_4$] for the data C, curve 5. F: 1, pH 7; 2, pH 5.8.

For the mixture PO + BSA the dependence of Amax vs [Cu(II)] for all PO concentrations is practically the same as that for pure BSA in solution (Figure 95 panel A), which witnesses for the absence of any interactions between BSA and PO through Cu(II) ions. This could be expected, because polymer PO contains no -COOH side groups; therefore the formation of coordination bonds between BSA and this polymer is impossible in principle.

The pattern is quite different with the presence of polymers P25 or P50: as the fluorescence is quenched by increasing [Cu(II)] (Figure 95, panels B and C), its maximum shifts toward the blue region (Figure 95, panels B and C). This indicates that BSA tryptophanyls become less accessible for water solution, which must be the result of increasingly tighter binding of the polymer through Cu(II) with the protein. Polymers P25 and P50 show some interactions with the protein even in the absence of copper in solution (panels B and C). Indeed at their maximal ratio to the BSA blue spectral shift of BSA fluorescence reaches 2 nm (from 340 to 338 nm), which suggests some screening of BSA tryptophanyls from water surrounding due to protein-polymers interactions. For other polymers we could not find such interactions with the absence of copper ions at neutral pH. But at pH 5 polymer P100 showed an even larger shift (about 5 nm) at a lesser polymer-to-protein ratio, R = 1.76 (Figure 95, panel F, curve 4).

The wavelength spectral shift is especially pronounced for BSA mixtures with P50. Indeed, even at a low ratio R = [P50]/[BSA] the increase of [Cu(II)] leads to the important blue spectral shift (Figure 95, panel C, curve 2).

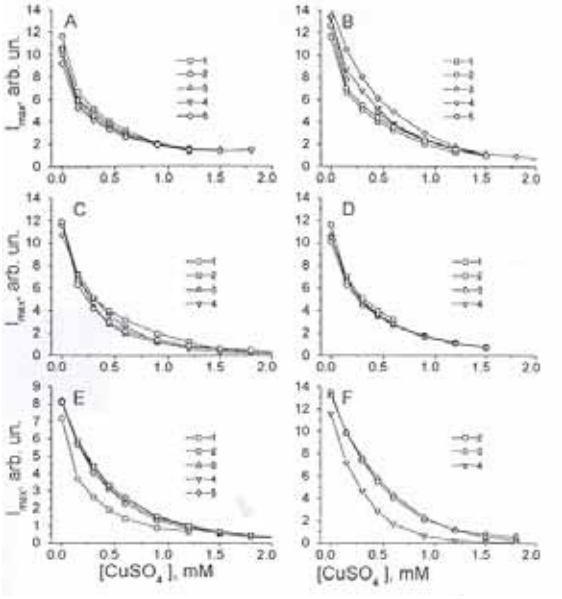


Figure 96. Intensity of BSA fluorescence in maxima for a mixture P + Cu(II) + BSA vs. Cu(II) concentration in solution (phosphate buffer, pH 7) at different polymers: A, O (PO); B, 25 (P25); C, 50 (P50); D, 75 (P75); E, 100 (P100); F, the same as E but at pH 5. BSA concentration was 0.71 mg/mL, [P]/[BSA] (mg/mg): 1,0.58; 2, 0.85; 3, 1.76; 4, 3.52.

At R = 3.52 (curve 5) this shift reaches a maximal value and the BSA fluorescence spectrum at [Cu(II)] = 1.5 mM becomes that of internal tryptophanyls, completely inaccessible for water surrounding (first spectral class) with A_{max} = 330 nm and half width of the spectrum 51 nm (see insert C*). This indicates that in the formed conjugate BSA + Cu(II) + P50 BSA tryptophanyls are completely isolated from water solution by the polymer, which is covering

seemingly all the BSA surface. It is worth noting that quenching of fluorescence for the system with P50 also reaches a maximal value compared with other polymers. Thus, at [Cu(II)] = 1.5 mM quenching for P50 is 20 times (Figure 96, panel C), when for other polymers it is about 10 times. This must indicate that in coordination bonds between BSA molecule and P50 there is utilized the largest quantity of copper ions as compared with other polymers used in the study.

In the case of P25 the spectral shifts are less pronounced (Figure 96, panel B). Maximal spectral shift at R = 3.52 and [Cu(II)] = 2.1 mM (B, curve 5) is 2-fold less (A_{max} = 334 nm) as compared with P50 (330 nm). So P25 covering of BSA in complex P25 + Cu(II) + BSA must be looser and, as a result, BSA tryptophanyls are not completely isolated from water solution (A_{max} = 334 nm is between the values characteristic for tryptophanyls of classes 1 and 2).

P75 and P100, which contain 75 and 100% of polar side chains, accordingly, must be a strong binder of Cu(II) ions. As one could see from Figure 96 at their presence in solution and [Cu(II)] increasing, the BSA spectrum shifts to longer wavelengths (panels D and E) as for pure BSA but less effectively. Comparison of curves (panels D and E) for pure BSA (curve 1) and BSA in mixtures with the polymers (curves 2-5) shows that there must be introduced into solution with the polymers 2.5 to 3 times higher concentration of Cu(II) ions to gain the same spectral shift as with pure BSA.

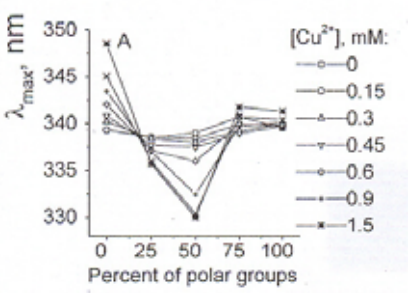


Figure 97. Position of fluorescence maximum (m) of BSA in mixtures P + BSA (1) and P + Cu(II) + BSA (2) vs percent of -COOH groups in the polyelectrolytes. [P]/[BSA] = 1.76; $[Cu^{2+}]$ = 0.5 mM.

This is the evidence that these polymers bind more then half of copper introduced into solution and therefore greatly decrease the concentration of free *Cu*(II) ions. It is their main effect. A *Cu(II)*-dependent red shift suggests that at neutral pH they do not form a covering around BSA molecules and the last behave mostly as those in pure BSA solution. But there is a peculiarity in this system behavior, which significantly distinguishes it from pure BSA solution. The matter is that the latter becomes turbid already at [Cu(II)] = 1.2 mM. So at this critical *Cu(II)* concentration coordinational bonds through *Cu(II)* ions are being established between BSA molecules, which leads to the formation of their aggregates. For the system BSA + Cu(II) + P100, the solution was transparent even at 3.9 mM *Cu(II)* , when the concentration of free *Cu(II)* in the solution was for certain higher than that of the critical one. This suggests that BSA molecules could not come together and form aggregates because they are anchored through Cu(II) ions on threads of P100.

The behavior of the system BSA + Cu(II)+P100 is quite different at low pH. As one can see from Figure 96 (panel F), polymer P100 at pH 5 and a rather high concentration even at the absence of *Cu(II)* ions produces a large blue shift (up to 335 nm) of BSA fluorescence, which points out its close interaction with the protein. At [Cu(II) increasing this shift reaches the value characteristic for first class tryptophanyls. Such a behavior of the system may be accounted for by the protonization of P 100 polar side group s (-COOH) and BSA amine groups at low pH, which leads to the formation of salt bonds between the protein and polymer. Further tightening of the formed cover is reached by the coordination bonds through Cu(II) ions.

To elucidate some important features characterizing the polymer-BSA complex

formation, we studied quenching of BSA fluorescence with standard quenchers that do not form coordination bonds with polymers or proteins. As such there were used [137,138] nonpolar succinimide, positively charged Cs^+ (CsCl), and negatively charged I^- (KI). Figure 98 shows dependence of I_{max} and A_{max} for pure BSA and BSA in mixtures P50- BSA and P AA - BSA vs. concentrations of added quenchers. As one can see, the quenchers used showed the same trends as Cu(II) ions in quenching BSA fluorescence but their effectiveness in the region we used for Cu(II) concentrations was about 10 times less.

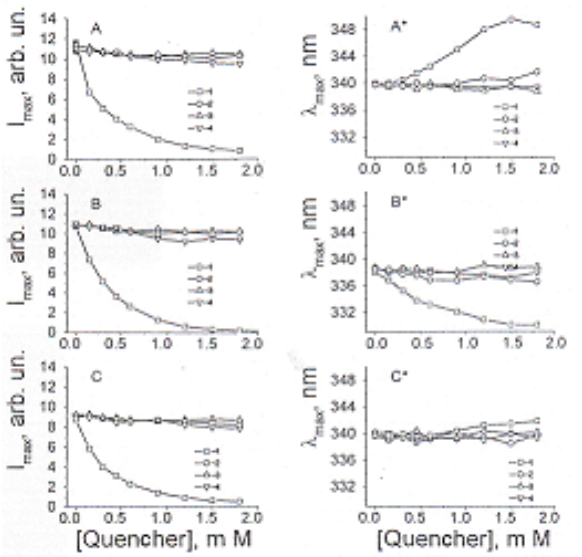


Figure 98. Intensity of fluorescence in maximum and position of the maximum for BSA solutions (0.71 mg/mL, phosphate buffer, pH 7) vs concentration of a quencher: A, A* for BSA solution without polymer; B, B*, at the addition of 2.5 mg/mL of P50 (copolymer 1:1 of acrylic acid with N-isopropylacrilamide); C, C*, at the addition of 2.5 mg/mL of P100 (polymer of acrylic acid). Fluorescence quenchers used: 1, $CuSO_4$; 2, CsCI; 3, succinimide; 4, KI.

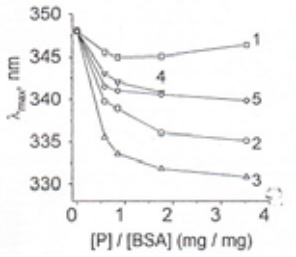


Figure 99. Florescence parameter λ_{max} for P + BSA mixtures at the presence of Cu(II) ions (1.2 mM) vs. ratio [P]/[BSA] (phosphate buffer, pH 7). P is polymer with percent of polar residues: 1, 0 (P0); 2, 25 (P25); 3, 50 (P50); 4, (P75); 5, 100 (P100).

We observed a small decrease in BSA fluorescence in the region (somewhat larger with the presence 75 of I^- ions) but no noticeable spectral shifts, which could be expected for such small quencher concentrations. These findings suggest that besides one site of Cu(II) binding , described in the literature [137] there is virtually an other one located in the vicinity of the "cleft"

Trp. *Cu(II)* binding in the former site, which is remote from both trpyptophanyls, has no influence on their fluorescence, but the emission of the latter Trp is strongly quenched at *Cu(II)* binding. At the absence of polymer this leads to prevailing in total fluorescence of Trp with A_{max} = 350 and low quantum yield. Just this Cu(II) bound in the BSA cleft must take part in coordination bond formation with polymers.

Figure 99, which presents data from Figures 98 with another scale on abscissa, shows some peculiarities of polymer-protein interactions. As can be seen from this figure, at rather high [Cu(II)] the maximal interaction of BSA with polymer, testified by spectral shift and quenching, is achieved already at rather low ratios;[P]/[BSA] (0.5-1), which indicates the maximal quantity of the polymer that can bind with the protein.

In this interval, the quenching in complex BSA + Cu(II)+ P50 reaches the maximal level and does not change with increasing the ratio of components, which suggests that in the formed tight cover the quenching is mainly static. Figure 98 shows that the ratio about 1:1 between polar and hydrophobic groups in the polymer is optimal for formation of a dense cover around protein. It seems that for smaller amounts of polar groups the polymer exists in the water solution as a coil, whereas at higher amounts the prevailing form is threadlike.

Kinetics of Complex Formation. To study transition at mixing to the equilibrium and the role of the sequence of different component mixing, the kinetics of fluorescence quenching was studied. The mixing was realized in such ways: (I) addition of Cu(II) in given concentration to the mixture of PE and BSA solutions (I), (2) addition of PE solution to the mixture BSA – Cu(II), or (3) addition of BSA solution to the mixture PE-Cu(II). Solutions were permanently stirred and the last component mixing took about 20 s. After that registration of fluorescence began immediately and continued for 15-20 min.

The kinetics curves of the PE + Cu(II) + BSA mixtures, prepared with different sequences of mixing for different polyelectrolytes are shown in Figure . One can see that the fluorescence quenching after 20 s reaches limiting values independent of the ways of mixing (1, 2, or 3) and chemical composition of the copolymers used. Somewhat lower quenching was achieved (5-7%) when Cu(II) ions were introduced into the mixture PE-BSA. An important conclusion, which follows from the data presented in Figure 100, is that the reaction of Cu(II)-induced ternary complex (PE –Cu(II) - BSA) formation is an equilibrium.

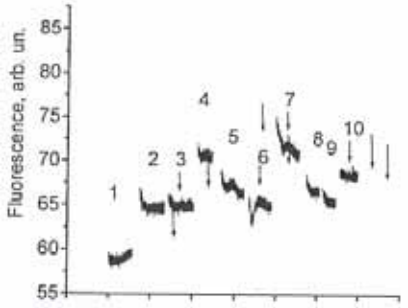


Figure 100. Quenching of BSA fluorescence by Cu(II) ions at the presence in solution of polymers depending on the order of different component mixing (polymers with percent of polar groups, 25 (P25), 50 (P50), and 75 (P75): 1, BSA + Cu(II) (control, after Cu(II) addition BSA fluorescence decreased 2-fold); 2, (P50 + Cu(II)) + BSA; 3, (BSA + Cu(II)) + P50; 4, (BSA + P50) + Cu(II); 5, (P75 + Cu(II)) + BSA; 6, (BSA + Cu(II)) + P75; 7, (P75 + BSA + Cu(II); 8, (P25 + Cu(II)) + BSA; 9, (BSA + Cu(II)) + P25; 10, (BSA + P25) + Cu(II). Fluorescence registration began immediately after mixing of the last component, which took about 20 s, and continued for 15-20 min. The concentrations of polymer, BSA, and Cu(II) in the resulting mixture were 2.5 mg/mL, 0.71 mg/mL, and 0.3 mM, accordingly.

Thus, the structure of ternary PE-Cu(II)-BSA complex at pH 7.0 depends on the monomer composition of copolymers. At low amounts of hydrophobic monomer ([COOH]/ [NIPAAm Upon] = 3/1) the forming polycomplex particles have friable structures with protein molecules practically exposed to the solution. Upon increase of hydrophobic monomer in the composition of copolymer, protein molecules in the ternary structure become densely covered as a shell by polymer chains and practically "fenced off' from the water environment. At low polymer concentrations, an intrapolymer complex is formed. This intrapolymer complex aggregates to interpolymer species upon increase in the polymer concentration.

Cu(II) ions quenches Trp fluorescence in the PE-Cu(II)-BSA complex by a static mechanism, suggesting that polymer-metal complexes interact preferentially with BSA tryptophan sites.

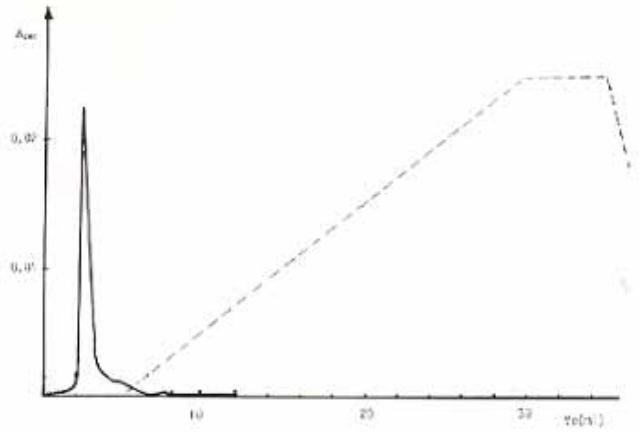


Figure 101. Anion-exchange chromatography of the matrix solution of the ternary mixture BSA-Cu^{2+}-PAA. (C_{PAA} = 0.1 g/dL): n_{Cu}/n_{AA} = 0.30 n_{BSA}/n_{pAA} = 1.0; pH = 7; 25°C. Broken line corresponds to (NaCl) concentration gradient.

3.2. Mechanism of Complex Formation

Our results indicate that water-soluble and insoluble stable ternary PAA-Cu^{2+} -protein complexes are formed at neutral pH. The preexisting electrostatic repulsive forces between PAA and proteins do not prevent the formation of polycomplexes in the presence of Cu^{2+} ions. Comparing these results with the corresponding results of copper-binding properties of proteins, we suggest that copper ions lead to the formation of chelate units. From what is known in the literature, [133-135,139,140,] the native sequence tripeptides, Asp-Ala-His-, Asp-Thr-His-, sequences represent the actual Cu^{2+} ions binding sites of HSA and BSA. NMR and, in particular, the ^{13}C technique results suggest that, in addition to the four nitrogen ligands (one amino, two peptides, and one imidazole nitrogen), the carboxyl side chain of aspartyl residue is involved in a pentacoordinated structure of the protein- Cu^{2+} complexes. Therefore, the carboxyl groups of PAA may compete with carboxyl group of aspartic residue and involve in Cu^{2+} binding (see Figure 101).

Hb-Cu^{2+}-PAA and Gl-Cu^{2+}-PAA systems at pH= 7 gives rise predominantly to insoluble ternary polycomplexes and the binding of protein molecules to a polymer is of a cooperative character, for instance, such binding lead to an irregular distribution of the protein between the macromolecules. The complex formation with BSA takes place in an analogous manner in relatively high concentration of Cu^{2+} to hemoglobin and globin, although, in some ratio n_{BSA}/n_{PAA}, in parallel to insoluble complexes, soluble triple complexes are simultaneously formed. Figure 102 A schematic presentation of the formation of chelate units between the functional

groups of the PAA and the protein globules with participation of copper ions (b) and structure of ternary PAA-Cu^{2+} -protein polycomplexes (a).

According to refs. [34,35], the reason for the demonstrated disturbance of the randomness of the distribution in the metal-containing triple systems Hb-Cu^{2+}-PAA and Gl-Cu^{2+}-PAA is probably a positive interaction of the protein globules adsorbed by one chain. In other words, the formation of contacts between protein globules "condensed" on the same polymer macromolecules results in an additional decrease of the free energy exceeding a free energy increase caused by the disturbance of the randomness of the distribution.

In our case, the interaction in the ternary mixtures was investigated at pH 7, which corresponds to isoelectric points of Hb and Gl. Therefore; these proteins at pH 7 show higher ability for intermolecular association in aqueous solutions.

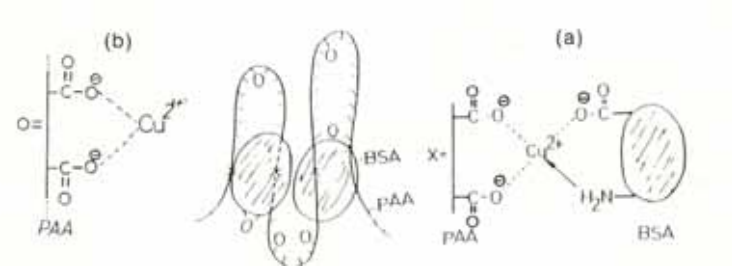


Figure 102. A schematic presentation of the formation of chelate units between the functional groups of the PAA and the protein globules with participation of copper ions (b) and structure of ternary PAA-Cu^{2+}-protein polycomplexes (a).

The pH of the reaction in the cases BSA-Cu^{2+}-PAA, HSA-Cu^{2+}-PAA and Tr-Cu^{2+}-PAA mixtures corresponds to the condition pH > pI The globules of these proteins being in this case negatively charged, their aggregation ability is low. One can see from the titration data as that these mixtures remain in a wide range of n_{pr}/n_{PAA} soluble. Phase separation in this system occurs at relatively high concentrations of metal ions. The results of physicochemical studies led us to propose a hypothetical structural scheme of ternary water soluble protein-metal-polyanion complexes (Figure 103).

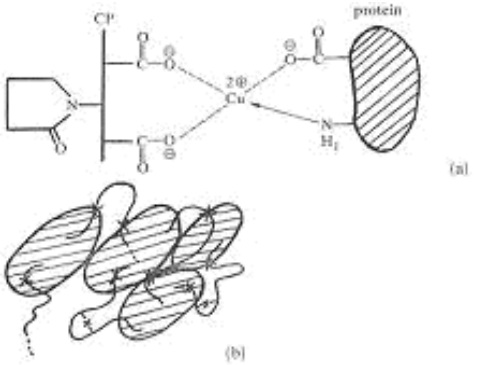


Figure 103. Schematic illustration of (a) the formation of chelate units between the functional groups of the copolymer (CP) and the protein antigen with participation of copper ions, and (b) the hypothetical structure of the triple polymer-metal complex of the protein antigen (crosses demote copper ions).

When PMC solution is titrated with protein solution ($n_{pr}/n_{PAA} < 1$), the protein globules are crosslinked with a linear polyion via copper ions. Some of the copper ions form intramolecular crosslinks in the free sections of polyions and, thus, stabilize the structure as a whole. The regions/sections of the polyion not directly involved in the complex formation (both with metal ions and without metal ions) exist in the form of free loops "dissolved" in water. The form of chelate units between protein globules and PAA and in the free sections of polyions leads to a change of charges of particles of ternary complex in dependence of the concentration of protein molecules. Therefore, as can be seen from the results in Figure 104, these substances migrate in electrophoretic field more slowly than the free protein molecules. On further increase in n_{pr}/n_{PAA}, the electronegativity increases and then attains a limiting value. Under this condition, the values of the electrophoretic mobilities of the triple complexes and free protein molecules are fairly close. The formation of nonstoichiometric polycomplex and redistribution of copper ions from the free sections to protein bound sections of PAA may contribute to this latter process. [We cannot analyze the distribution of protein molecules between polymer coils in soluble mixture because free PAA or (PMC) do not separate from polymer-protein complex in HPLC.] The formation of the water-soluble aggregates in the protein-PMC systems at the higher concentration of the Cu^{2+} ions is shown in Figure 103. At low concentration of copper ions, the interaction can be considered to be intramolecular only as Cu^{2+} forms a complex with one polymer chain. At high concentration one can speculate that the copper ion can act more effectively as a crosslinking agent between two (or more) polymer coils. This intermolecular coil interaction in the case of ternary systems leads to the formation of soluble and insoluble polycomplexes with a complicated structure, for instance, the existence of Cu^{2+}-induced crosslinking self-assembly of polycomplexes.

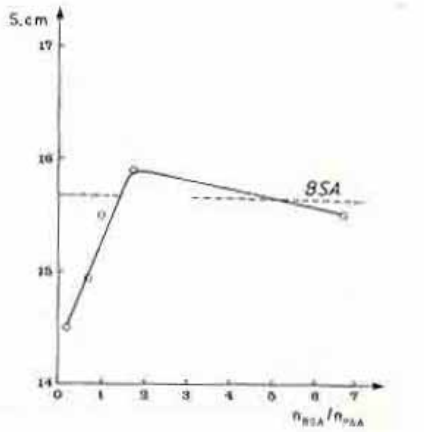


Figure 104. Dependence of the electrophoretic mobilities (distance-S) of the products of solution of the ternary mixture BSA-CU^{2+}-PAA on n_{esA}/n_{fAA}; C_{PAA} = 0.1 g/dL n_{cu}/n_{AA} = 0.25; pH = 7; 25°C. Broken line represents free BSA.

We can propose that in this case with the increase in concentration of protein molecules in ternary mixture, the mechanism (2) becomes more probable, resulting in phase separation and transformation of insoluble polycomplexes to a soluble state in the system.

3.3. Polycation-Metal-Protein Systems

The interaction between poly-4-vinylpyridine and bovine serum albumin with the participation of bivalent copper ions in acidic medium has been studied by Mustafaev [61,101].It is known that both P4VP and BSA (pI=4.9) carry the similar (positive) electrical charge in acidic media and

cannot react with each other without some intermediates. Interaction between cationic P4VP and BSA at pH=4.3 at the absence of copper ions is weak, if not negligible. At this pH the electrostatic repulsion forces between positive macromolecules prevent a stable complex formation. However, complexes were formed following the addition of copper ions. The ternary P4VP-Cu-BSA mixture was prepared by adding protein solutions to the polymer-metal complex (PMC) solution at pH 4.3. To produce a P4VP-Cu^{2+} complex, different concentrations of the $CuSO_4x5H_2O$ solution were added to P4VP solutions at pH 4.3. The ratio of copper ions per pyridine (n_{Cu}/n_{Py}) was 1:4, i.e. one Cu^{2+} ions per 4 pyridine groups. These numbers correspond to maximum capacity of P4VP to bind of copper ions. The fraction of P4VP with degree of polymerization (P) 950 (PVP-1), 2150 (PVP-2), 7400 (PVP-3) were used for the investigation. The mixing of P4VP and Cu^{2+} ions at this conditions results in formation of insoluble polymer-metal complexes with intensive blue color. The whole P4VP and Cu^{2+} were including into composition of insoluble complex. However, the situation changes completely in the presence of BSA molecules in this mixture, which is accompanied by partial (or full) elimination of the phase separation process depending on the protein concentration in the reaction mixture. The ultracentrifugation measurements of matrix solution of the PVP-Cu^{2+}-BSA mixtures show that only one peak with the sedimentation coefficients 20-30 sved and 50-80 sved. in the case of PVP-1 and PVP-2, respectively was observed on sedimentograms of mixtures (Figure 105).

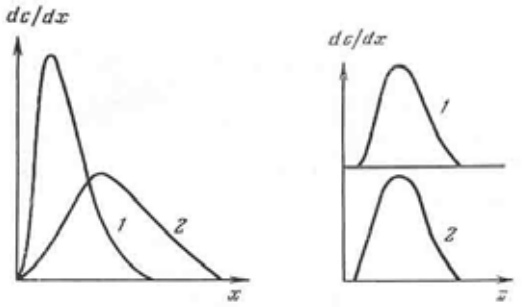


Figure 105. Sedimentograms of mixtures BSA-Cu(II)-PVPI (1) and BSA-Cu(II)-PVPII (2) at n_{BSA}/n_{PVP}=4(1),10(2);$P_w=10^3$ (PVPI) and $7.4x10^3$(PVPII) t=12 min. ώ =26000 min^{-1}.Right sedimentograms corresponds to BSA(1) and BSA-Cu(II) (2),t=60 min. ώ=56000 min^{-1}

From the results that the sedimentation coefficients of the reaction products were characterized by rather large value than Sc for free BSA (Sc for BSA equal 4.4 sved), and for BSA-Cu^{2+} complexes and the PVP-Cu^{2+} complex is not soluble at this reaction conditions, we can consider that these peaks corresponds to the soluble products of the binding of BSA with polycation. Thus, under conditions where both P4VP and BSA are positively charged and incapable of binding to one another that the divalent Cu^{2+} ions act as "fasteners", promoting the formation of relatively stables water soluble and insoluble ternary complexes.

Studies of solubility of ternary mixtures at different molecular weight of P4VP and protein concentrations permits to elucidate some important features characterizing Cu^{2+}-mediated protein-polycation complex formation. An increase of the number of protein molecules in the mixture (the weight concentration and Cu^{2+}/PVP ratio of PMC is kept constant) leads to a decrease of the amount of precipitate corresponding to free PMC insoluble complexes while the area of the peak of the water-soluble complex increases. When the BSA/PVP ratio (the minimum amount of BSA per one gram of PVP which is necessary for full prevention of phase separation in mixtures) is 2.8g BSA/1g PVP, the precipitate of PMC disappears and only soluble product remains. The described situation is typical for all studied fractions of PVP and equal 2.8g BSA 71 g PVP. At the same time the ratio of numbers of macromolecules at which one observes a

disappearance of the free PMC precipitate, depends on the degree of P4VP polymerization. From the sedimentation data obtained it fallows that the free protein is absent in the system over the whole studied range of the ratios n_{BSA}/n_{PMC}, i.e. all added BSA is strongly bound by the polycation-metal complexes. It was suggest that the complex formation is not dependence on the molecular weight of polycation in the range of degree of polymerization P_v =1000-7500. Taking into account the above proved fact of the quantitive binding of BSA with PMC one may consider that $\lim(n_{BSA}/n_{PMC}) = N_i$, when amount of precipitate "m" is 0. This limit equals the number of the protein molecules bound by polycation-metal complexes of a given degree of polymerization under given conditions. It follows from the results that the higher the degree of polymerization of PVP, the higher N_i is. Within the experimental error this dependence is linear. It means that the average the site of the polycation chain of the approximately constant definite length "l" is used for such globule binding. The average value of "l" is about 250 monomer units.

The molecular weight of the particle of soluble ternary BSA-Cu^{2+}-P4VP complex with the composition $n_{BSA}/n_{PMC} = N_i$ were determined by light scattering measurements (Figure 106). As seen from the sight scattering results the dependence of $K.c/R_0$ on the concentration was linear, indicating the absence of dissociation of the polycomplexes at dilution. In other words, the studied soluble ternary BSA-Cu^{2+}-PVP polycomplexes are stable over a wide range of the solution concentrations

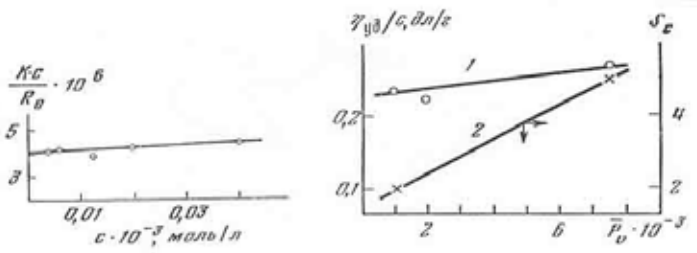
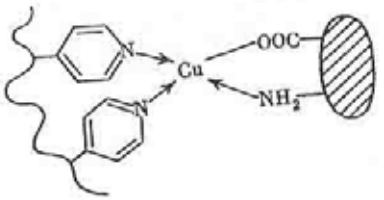


Figure 106. Dependence of $K.c/R_o$ on the concentration of BSA-Cu(II)-PVPI complexes, pH 4.25. Dependence of inherent viscosity (1) and sedimentation coefficient (Sc) of BSA-Cu(II)-PVP complexes at n_{BSA}/n_{PVP} corresponding to full homogenization of the systems on the degree of polymerization of PVP.

The average value of molecular weight (Mw) obtained from this dependence for the complexes of BSA with PVP-1 copper complexes is about 2.5x10. At the same time, the characteristic composition N_i for the polycomplexes in the case with PVP-1 was equal to 4 (N_i=4). The average value of molecular weight for this complexes calculated from $M_{sd} = M_w$ (PVP-1) + N_ixM(BSA) with taking into account of amount of copper ions was equal to $3.8x10^5$. Therefore the real particle of the triple polycomplex is an associate, which includes 6-7 polycations binded with protein globules via copper ions. One can assume that the contacts of protein and polycation are carrying out via chelate nodes in which the copper ions play a part as a central atom:



The further definition of the ternary BSA-PMC complex structure is obtained from the comparison of the inherent viscosities and sedimentation coefficients for these complexes formed

by the polycations with different length when $n_{BSA}/n_{PMC} = N_i$. It is seen in Figure 106 that the sedimentation coefficient of ternary complexes sharply increases with increasing degree of polymerization of the polycation. At the same time in this range of degree of polymerization of PVP the viscosity of the solutions was changed insignificantly. Remarkable that the values of η_{sp}/c were equal only 0.25 for such higher molecular weights of polycomplexes. The relation of this kind may exist only for particles with sufficiently compact structure. Thus, it is established the high compactness of particles of soluble triple complex by light scattering, sedimentation and viscosimetry methods.

In conclusion, the presented results show a wide variety to prepare polymer-protein complexes with desired physicochemical properties. A considerable interest exists for the establishment of the correlation between the structure of the polymer complexes of antigens and their immunological activity. Comparison of these results with the formation of complexes involving biopolymers, in particular, polynucleotides and nucleic acids, will contribute to the investigations on the roles of the multivalent ions in the regulation of these processes. Moreover, studies of the mechanisms of cooperative binding of proteins to synthetic PE will be of interest for the elucidation of the mechanism of action of PE in the organism, for example, in immobilization of enzymes and specific sorption of proteins on surfaces. In addition, such reactions may simulate, for instance, antigen-antibody reactions and processes of self-organization in biological systems.

4. COVALENT COMPLEXES (CONJUGATES)

Synthetic polyelectrolytes (PE) have been widely used to modify proteins via covalent attachment, increasing (or reducing) the immunoreactivity and/or immunogenecity of original antigenic proteins [1-11,141-149], and improving their in vivo stability with prolonged clearance times [19]. Besides, the PE conjugates with individual microbe antigens develop strong protective properties [9,9a] and they can be considered as a new generation of vaccinating compounds.

It is known that conventional methods of synthesis of protein-protein and protein-linear PE covalent conjugates are based on reactions between activated functional groups of macromolecules in aqueous solutions [150]. A whole series of well-known reactions can be used to activate functional groups of a polymer carrier or a protein molecule and to link them together. However, such reactions between macromolecules, carried out in solutions, meet some inherent difficu1ties and limitations with respect to their direction and yield control.

The basic reaction mechanism of carbodiimide-mediated modification of carboxyl group includes a two-step reaction sequence of condensation between carboxyl groups of polymer and amino groups of proteins. During the first step, the carboxyl group is activated by the carbodiimide an o-acylisourea intermediate, which can react in further with an amino group from a protein molecule. Reaction with an amino group from a protein will lead to a cross-link between the polymer and protein components. One can assume, that the formation of conjugates can involve several sequential steps. In our opinion, the first and the most important step is primary complex formation between the polymer activated by carbodiimide and the protein by electrostatic interactions and strengthening of the structure by hydrophobic interactions. Covalent cross-linking reaction is limited by structure formation and occurs in a slower time scale.

As it was mentioned above the complex formation between proteins and PE have been the subjects of numerous research efforts. Soluble polymer-protein complexes were studied by a wide range of methods that well known in colloid and polymer chemistry. The mode of binding proteins to PE has been found to depend on the ratio of components. The system exhibits characteristics of cooperative binding, so that the protein molecules are in homogeneously distributed among the polymer chains, and free PE molecules coexist with complex. Further increase in r leads to a secondary binding process along with an increase in the amount of free protein. In this approach however, the systematic analysis of the covalent binding mode in the

polymer-protein mixture is stilI absent and the mechanism is not completely understood. Fundamental questions, concerning the magnitude of the binding constant, the cooperativity of the binding and the effect of bulk mass ratio of protein to polymer on the binding mechanism, still remain to be answered.

Poly(N-isopropylacrylamide) (PNIPAAm) is a well known water-soluble polymer showing unique, reversible hydration-dehydration changes in response to small changes in temperature [151-153]. An aqueous solution of PNIPAAm demonstrates phase separation and the polymer precipitates at a certain temperature, which is called a lower critical solution temperature (LCST).

The objective of the present study is to examine the covalent binding mechanism of poly(N-isopropylacrylamide-co-acrylic acid) (poly(NIP AAm)) copolymers with BSA molecules depending upon the weight concentration ratio *(r)* of BSA to poly(NIP AAm-AA). The fraction composition of conjugates, degree of binding, thermal collapse property as revealed through a combination of HPLC, electrophoretic, fluorescence, UV - Visible spectroscopy and viscosity measurements are reported [153].

Temperature-responsive (N-isopropylacrylamide-coacrylic acid) (poly(NIPAAm-AA)) copolymers (CP) was synthesized from N-isopropylacrylamide (NIPAAm) and acrylic acid (AA) [153]. Compositions from mol feed ratios [NIPAAm]/[AA] = I/I were prepared. The viscosity (sp/c) in 0.154M NaCl solutions of the copolymers was 0.4434 dl/g. The aqueous polymer solutions were characterized by transformation from hydrophilic to hydrophobic states (LCST) at 26°C. Bovine serum albumin (BSA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from the Sigma Chemical Company, Sİ. Louis (USA) and used as received.

Activation of polymer acid group was carried out in water (pH 6.0) in a molar ratio [EDC]/[AA] = 1: 2; CP (50 mg) was dissolved in water, stirred at 4^oC and EDC (20 mg) was added. After 1 h, the pH values of mixture were adjusted to with 1 M NaOH and different concentrations of BSA (C_{BSA}/C_{CP} = 0.28; 0.59; 1.14; 1.70; C_{BSA} and C_{CP} weight concentration of BSA and CP; concentration of CP was kept constant and C_{CP} = 0.25 g/dl) were added to the reaction mixture and stirred 12 h. After removal of o-acylisourea intermediate by dialysis, the sample was lyophilized. The purified conjugates were redissolved in 50mM PBS at 4°C for the physico-chemical analysis.

The compositions of the soluble mixtures were estimated by gel filtration chromatography using a Bio-Sil Sec 250 column (7.8 x 30 cm). A Waters Model 501 HPLC was run with a 10 mM potassium phosphate buffer, pH 6.8 and 100mM NaCl at flow rate 1.0 ml/min at room temperature. The eluate was monitored by absorption at 278 nm. The standards used to calibrate the column were thyroglobulin (670kDa), immunoglobulin G (155 kDa), myoglobin (16.9 kDa), and vitamin B12(1.35 kDa).

Spectrophotometric titrations were performed both with a WTW 537 type pH meter and VV-Visible (Shimadzu VV-160 A) spectrometer with temperature control attachment. Reactions were monitored between 200 and 1100 nm. Optical transmittance of CP and conjugates in aqueous solution at various temperatures was measured at 500 nm using a spectrophotometer (Shimadzu VV-160 A). The cell was thermostated by a Cary Temperature Controller.
The viscosity measurements were carried out with an Ubbelohde-type viscometer. The temperature of the sample was controlled to 0.02^oC.

BSA and polymer-BSA mixtures were analyzed by polyacrylamide gel electrophoresis (PAGE) [154] and SDS-polyacrylamide gel electrophoresis [155]. Protein gels were detected by Coomassie Blue staining. The band densities were determined using a Hoefer Scientific Instruments Scanning Densitometer. SDS-polyacrylamide gel electrophoresis experiments were carried out in the presence of strongly anionic detergent, sodium dodecyl sulfate (SDS) in combination with a reducing agent and heat to dissociate the proteins before they are applied on the gel as already described [155]. The denatured proteins (polypeptides) bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the

molecular weight of the polypeptide, SDS-polypeptide complexes migrate through polyacrylamid gels in accordance with the size of the polypeptide.

Fluorescence emission spectra were obtained by using the Quanta Master spectrofluorometer (photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted to 2 or 3 nm. The excitation wavelength was 280 nm.

For the determination of amino groups of BSA, fluorescent measurements were performed in the presence of fluorescamine at the wavelengths of excitation 390 nm and fluorescence 475 nm. Conjugates and BSA were prepared in boric buffer with pH = 9. The boric buffer was made by dissolving the boric acid in water at concentration 1.24 g/100 ml. Fluorescamine was dissolved in acetonitrile and was added in the ratio one molecule of fluorescamine to one amino group of BSA.

Figure 107 presents the viscosity of reaction products and CP-BSA mixtures as a function of ratio of components, $r = C_{BSA}/C_{CP}$, in neutral water solution (pH = 7). The viscosity of solution of simple polymer-protein mixture practically does not change by the titration of polymer solution with BSA and is characterized by viscosity of polymer components because of the absence of stable complex formation between anionic polyions and similarly charged protein globules. In contrast to these mixtures, the viscosity behavior of the solution of the products of the reaction of carbodiimide activated CP with BSA depend strongly on the initial protein concentration in the mixture. By increasing of protein amount, viscosity of the systems first rises sharply ($C_{BSA}/C_{CP} < 1$) and then attains a maximum value ($C_{BSA}/C_{CP}=1$).

At this ratio, the viscosity of the modified CP-BSA mixtures is about three times of magnitude higher than the viscosity of the free CP or simple CP-BSA mixtures. As it follows from these viscosity results, the formation of the covalent conjugate CP-BSA occurs in these systems. It is important that the reaction products which are prepared over a wide range of the [BSA]/[CP] ratios are water-soluble.

The typical HPLC chromatograms of bioconjugates which were synthesized at different initial ratios (C_{BSA}/C_{CP}) of their macromolecule concentrations in the solution are given in Figure 108a. As it follows from this figure, the system is characterized by a bimodal distribution of elution components. A comparison of the values of the retention times (RT) corresponding to the peaks 1 and 2 of the solution of reaction mixtures and of the individual components shows that the rapidly eluting substance (peak 1) corresponds to a free CP and peak 2 corresponds to the polymer-protein conjugate.

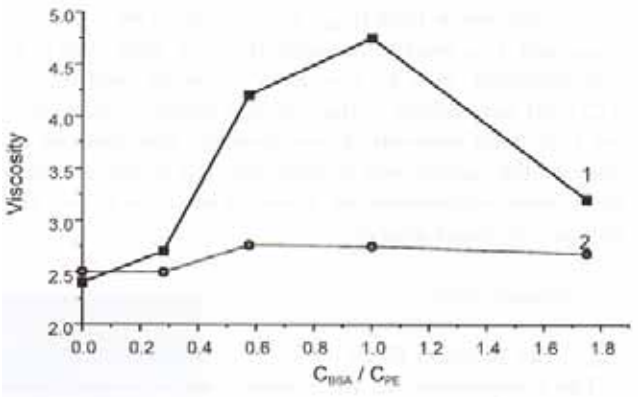


Figure 107. Dependence of viscosity (η_{sp}/C, dl/g) of reaction products (CP-EDC-BSA) (1) and mixtures (CP-BSA) (2) on the initial ratio BSA/CP in neutral water solution (PBS, pH 7). Molar ratio of [EDC]/[AA] = 1: 2; different BSA concentration; 23°C.

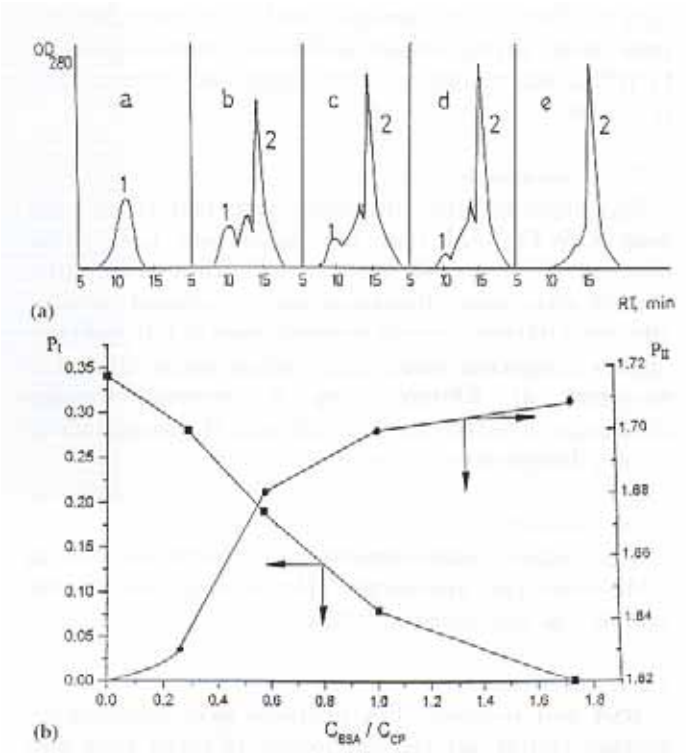


Figure 108. (a) Gel filtration HPLC chromatograms of free CP (1) and reaction products of CP-EDC-BSA prepared at different BSA/CP: 0.28 (a); 0.59 (b); 1.14 (c); 1.70 (d); free BSA (e) CBSA = 0.1 g/dl; different polymer concentration; 25°c. pH 7.2. RT- retention times. Diagrams represent normalized OD280 values. (b) Dependence of the chromatogram peak area (Po) of free CP (1 peaks I) and reaction products (2, peaks 2) (PII) on the BSA/CP'

The solution of the reaction products is characterized by one peak on the e1ectrophoregrams, obtained by the native and SDS-electrophoresis methods (Figure 109). The electrophoretic mobilities of the reaction products depend on the initial ratio of reaction components (Table 9).

The results obtained at the $C_{BSA}/C_{CP} < 1$ convinces the protein binding by CP and the absence of pure BSA in solution of the reaction products. Therefore, the peaks (2) in HPLC chromatograms (Figure 108) correspond to soluble covalent bioconjugates of CP-BSA. The character of the dependence of the studied parameters of the reaction products on the ratios C_{BSA}/C_{CP} shows that the mechanism of the binding and the composition (and structure) of the bioconjugates polyion-protein depends on the initial ratios of components.

It is remarkable that the above-described results, obtained by the analysis of the overall physico-chemical measurement data is confirmed by the data of fluorescence analysis. These conjugates, which had been prepared at different *r,* were studied by fluoremetric method. Figure 110 shows fluorescence spectra for solutions of free BSA and of conjugates with varying initial concentrations of CP. These results shows that the maximum yields of binding of BSA molecules to polymer chains occur at about $r = 0.28$ because of the maximum reduction of tryptophan fluorescence intensity. On the other hand, conjugation at $r < 1.0$ induces a marked blue shift of Amax (Table 9).

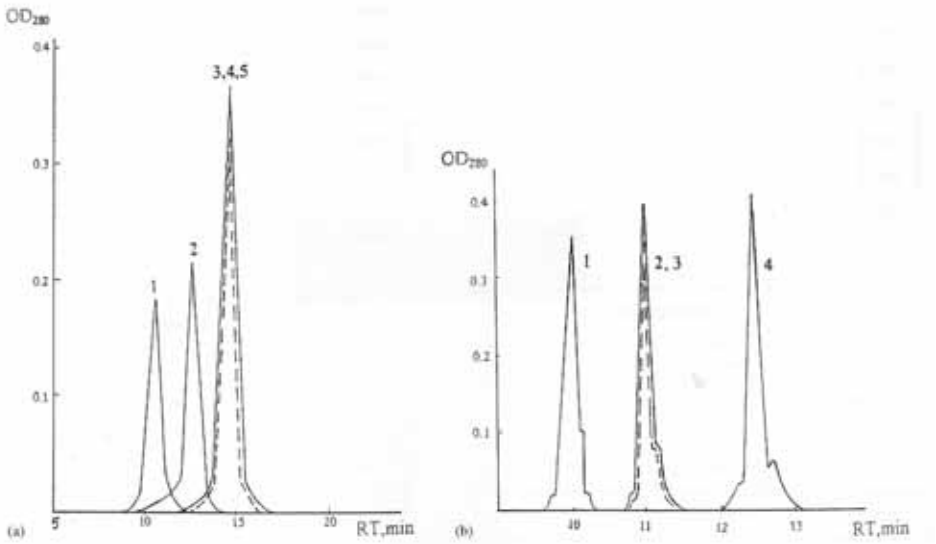


Figure 109. Electrophoregrams of BSA and reaction products of CP-EDC-BSA mixtures obtained at different BSA/CP ratios by two methods: native (a) and SDS-polyacrylamide gel electrophoresis (b). BSA/CP: 0.28 (1); 0.59 (2); 1.14 (3); 1.70. (4); free BSA (5 - native, 4 - SDS). Different polymer concentrations, RT - retention time. Diagrams represent normalized ODZ80 values.

Table 9. Characteristics of BSA-poly(NIPAAm-AA) conjugates

CBSA/CPE	β	RTn (min)	RTsDs (min)	in	Amax (nm)
0.28	48	10.5	10	4.4	338.0
0.59	39	13	11	6.5	339.9
1.14	35	15	11	9.2	340.9
1.70	35	15		9.5	341.0
BSA		15	12.5	10	341.0

aRTn and RTsDs - retention times in native and SDS-electrophoresis; in - intensity of fluorescence in maxima, Amax - robition of fluorescence maximum; β - average number of binding NHz -groups.

This indicates that in the CP-BSA conjugates formed, BSA tryptophanyls are complete1y isolated from water solution by the polymer, which cover apparently all the BSA surface. By increasing *r,* the position of the maximum of the spectrum approaches max of free BSA. Thus at $r > 1$ the polymer chains do not form covering around BSA molecules and the formed conjugate particles possess more friable structures in which protein molecules are practically open for the solution.

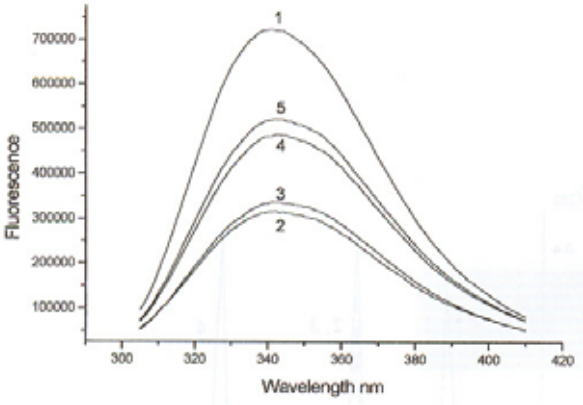


Figure 110. Fluorescence spectra of pure BSA (1) and conjugates, preparing at different initial BSA/CP ratios: 0.28 (2); 0.59 (3); 1.14 (4); 1.7 (5); BSA concentration 0.71 mg/ml, phosphate buffer (pH 7.2). Different polymer concentrations; 23 C.

The yield of conjugation should be directly proportional to the concentration of ε-aminolysil groups of protein molecules, assuming that they are all accessible for the reaction. The ε-aminolysil contents of the BSA and polymer-protein conjugates studied by fluorescamine interact with the primary amino groups of the samples. Figure 111 shows the fluorescence spectra of fluorescamine in its mixtures with BSA and conjugates, at different concentrations of fluorescamine *(*N_F/N_{BSA}). As the fluorescamine concentration in the solution of free BSA is increased, emission intensity of solution increases and reaches a maximum value at $N_F/N_{BSA} = 60$, which corresponds to total number of free ε -amino groups in one protein molecules. The fluorescence intensity in the bioconjugate solutions under the same conditions is lower than that in free BSA solutions and depends on the initial conjugation ratio *(r)* of components. Therefore, by analyzing the content of a free amino group in the reaction mixture, one can determine the number of covalent bonds formed by a protein globule with a polyelectrolyte macromolecule. The ε -aminolysil contents of BSA and the reaction products studied here are shown in Table. It is seen that an increase in BSA content in the mixture results at first, in an essential decrease in the number of free aminogroups *(Ni* = 12, *r* = 0.28). According to the scheme A (Figure 111), it shows an increase in the overall covalent bond number between the protein globules and the polyions. At $r > 0.3$ the number of covalent bonds between protein and polymer components make up about $N = 21\text{-}25$.

Thermal collapse property. In Figure 112 the reduced viscosity of the samples as a function of temperature is plotted. The reduced viscosity of solution of free polymer as well as bioconjugate solution decreases sharply with temperature and finally the reduced viscosity value becomes minimal. It seems that analogous to the pure polymer molecules, the bioconjugate coils collapse as the temperature increases buffer (pH 7.2) above the lower critical solution temperature (LCST) of the polymer, LCST = 24°C, i.e. in all the cases the coil-globule transition occurs. Nevertheless, between this, some differences are observed between these conjugates. The temperature which corresponds to minimal viscosity values of bioconjugates depends on the BSA/polymer ratio *(r)* and is decreased with increasing *r*. Figure 113 shows the temperature dependence of OD_{500} values for the solutions of CP and CP-BSA conjugates prepared at different concentrations.

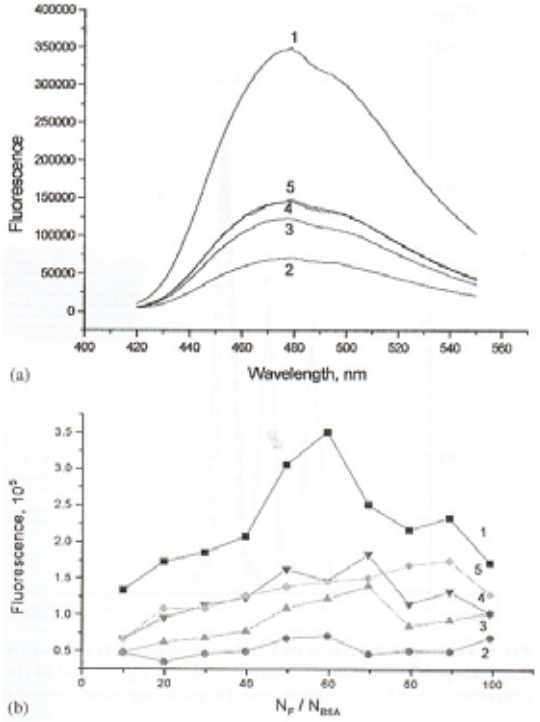


Figure 111. (a) Fluorescence spectra of pure BSA (1) and conjugates in the mixture with fluorescamine at different C_{BSA}/C_{CP}: 0.28(2); 0.59(3); 1.14 (4); 1.70 (5) C_{BSA} = 0.07 mg/ml; C_F= 1.8 x 10 -2 mg/ml; different polymer concentrations. (b) Intensity (1 max) in the second excitation maximum of fluorescamine bound to BSA (1) and reaction products (2-5), preparing at different initial ratios vs. concentration of fluorescamine C_{BSA}/C_{CP}: 0.28 (2); 0.59 (3); 1.14 (4); 1.70 (5); CBSA = 0.71 mg/ml; different polymer concentration.

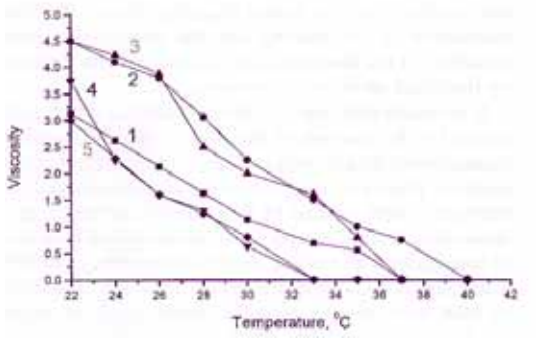


Figure 112. Dependence of viscosity (sp/c, dı/ı) of solution of pure CP (1) and conjugates on temperature, preparing at different ratios: 0.28 (2); 0.59 (3); 1.14 (4); 1.70 (5) Ccp = 0.25 ı/dı, different protein concentrations. Phosphate

It was found that the phase state of CP-BSA conjugates at low concentrations was

independent of temperature rise, remaining soluble in water. On the contrary, more concentrated solutions of CP-protein conjugates as free CP solutions retain a cloud point around 26°C in the phosphate buffer (pH 7.2). Therefore, it can be concluded that protein conjugation does not significantly influence the cloud point or the thermally induced precipitation of poly(NIPAAm-co-AA). These findings agree with the results of the study by Chen et al. [156], who found that the conjugation of proteins with poly(NIPAAm) did not change the LCST of the polymer. Besides, Cole et al. [157] did not actually see the precipitation (i.e. the cloud point) of the poly(NIP AAm)-monoclonal antibody conjugate, probably because the concentration they use was too low. What they observed was the collapse of individual polymer chains using laser light scattering in the dilute solution.

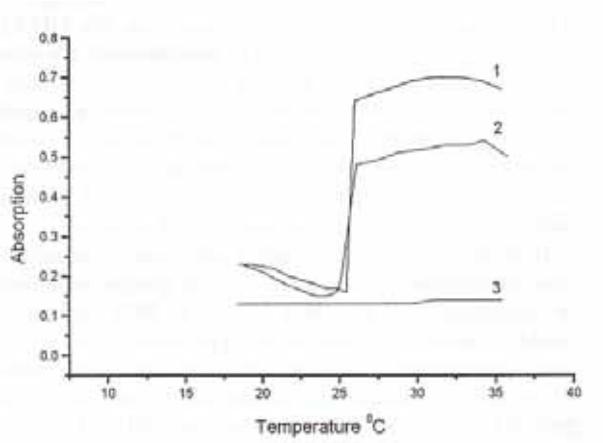
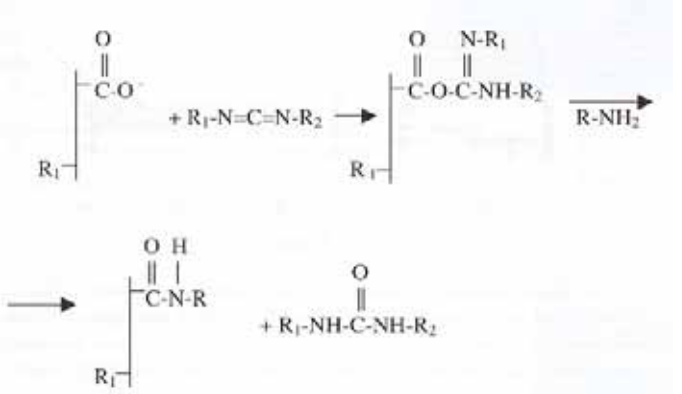


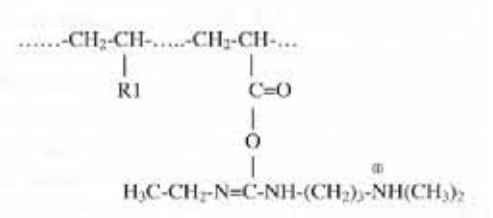
Figure 113. Temperature dependence of OD_{500} values for the solutions of CP (1) and CP-BSA conjugates at different concentrations: 1.0 g/dl (2), 0.25 g/dl (3); phosphate buffer (pH 7.2); $C_{BSA}/C_{CP} = 0.28$.

The most important chemical modification reactions of carboxyl groups utilize the carbodiimide-mediated process. In the presence of an amine, carbodiimides promote the formation of an amide bond in two steps. In the initial reaction, the carboxyl group adds to the carbodiimide to form an o-acylisourea intermediate with an amine for the polymeric carboxylic acids, yields the corresponding amide:



Water-soluble carbodiimide- 1 -ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) CH_3CH_2-N-C=N-CH_2CH_rNH+ ($CH_3h(R_1$-N=C=N-R_2) contains two radicals, one of them is

positively charged (R_2) and at the activation of anionic polyelectrolytes by these carbodiimide the carboxyl group of polymer molecules transform to positively charged ester derivatives and we practically have to deal with cationic polymers. In our case, the chemical structure of copolymers activated with EDC is likely the following:



where $R_1 = CONHCH(CH_3)_2$

When BSA solutions are added to activated CP (CP-EDC) solutions, one can asstime that first the formation of the electrostatic (and hydrophobic) interpolymer-protein complexes occurs as a most rapidly proceeding process and then the condensation between carboxyl and amino groups of components (covalent conjugation) occurs.

HPLC, electrophoretic, fluorescence and viscosimetric results suggest that the mode of binding of BSA to activated polyions depends upon the weight concentration ratio *(r)* of protein molecules to CP. When *r* is smaller *(r* < 1), the free and bound CP (conjugate with BSA) is coexistent and free BSA in these solutions is not detectible. These results suggest that for $r < 1$ values, the CP molecules are either covalently bound with BSA or free, an indication of a cooperative binding mechanism. A similar nonuniform distribution of BSA among polyelectrolyte (hosts) in electrostatic complexes was observed earlier by Kabanov and Mustafaev [34,35] and lately in [153]. Cooperative binding has also been observed in complexes of oppositely charged synthetic polyelectrolytes [34] and native calf thymus DNA with cationic polypeptides [73]. We show that the values of binding amino groups in composition of bioconjugate molecules (degree of binding, β) depend on the *r* conditions, have a maximum values at $r < 1$, then decreased and remains essentially constant for *r* 1 values. This means that each incremental addition increase in the mass of bioconjugate. Because of the constant degree of binding it can be assumed that at $r >$ 1, the structure of the conjugate is constant and the concentrations of bound BSA will increase proportionately as well. This process is consistent with cooperative binding. Although the present results do not provide any direct information on the structure of polymer-protein conjugates, some speculations concerning conjugate structure can be made. When BSA complexes with oppositely charged activated CP is formed, charge neutralization makes the bound protein more hydrophobic. Consequently, hydrophobic interactions between adjacent bound proteins can be a driving force for the cooperative behavior proposed in this study. However, after condensation reaction within the polycomplex particles, and removal of positively charged carbodiimide derivative from the solution by dialysis the volume and the asymmetry of conjugate particles increase because of occurrence of repulsive interactions the between negative charges of bound protein molecules and carboxylic groups of polyions and bound protein molecules.

Figure 114 shows the schematic representation of the carbodiimide-induced conjugation of BSA with polyanions and hypothetical structures of the CP-BSA conjugates. The character of the binding depends on the [BSA]/ [polymer] ratio and two types of bioconjugate particles are formed: at $r < 1$, the protein molecules in the structure of conjugate particles are densely covered as a shell by polymer chains and practically "fenced off" from water environment; at $r > 1.0$ forming conjugate particles possess more friable structures in which protein molecules are practically exposed to the solution.

It is reasonable to conclude from these observations that the negatively charged carboxylic groups remaining in composition of poly(NIPAAm-AA)-BSA conjugates could eventually prevent phase separation if the conjugate concentration is very low. At the same time,

when the temperature is raised above the LCST of poly(NIPAAm), the precipitating poly(NIPAAm) segments of the conjugates will flocculate together if the concentration is high enough, similar to free poly(NIPAAm-AA). In this study, we see the thermally induced precipitation and inoculation of conjugates in all studied C_{BSA}/C_{CP} ratios since we used more concentrated solutions.

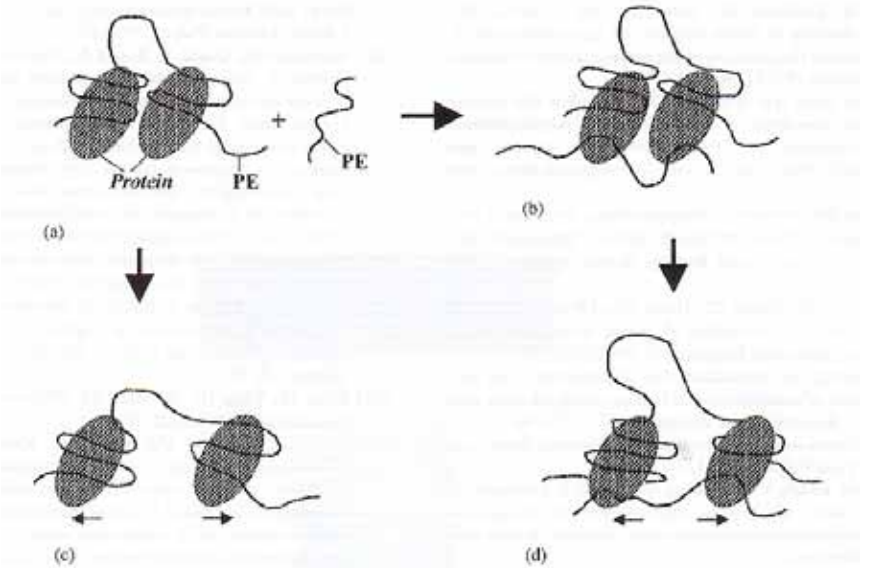


Figure 114. Models of possible organization of CP-BSA conjugates and structure of conjugates: polycomplex particles of CP-EDC-BSA *(a,r* > 0.3), *(b,r* < 1); conjugates after condensation reaction *(c,r* < 1), *(d,r* > 1).

A single immunization with these conjugates increases specific immune responses to BSA, whereas, the level of the antibody titers does not depend on the composition of soluble conjugate molecules. In the aqueous solutions, poly(NIPAAm-AA) transforms from hydrophilic to a hydrophobic state at 36.5°C (body temperature). The precipitation of conjugate molecules leads to "leveling off" of the effect of conjugate composition on their immunogenicity and the hydrophobic aggregation site on the surface of poly(NIPAAm-AA)-BSA molecules would increase the absorptive capacity of BSA (antigenic determinants) on the immunocompetent cells. The efficiency of such "forced" interactions of conjugate aggregates (high epitope density) is high enough for the immune response (see below).

5. BIOPOLYMER SYSTEMS IN IMMUNOLOGY

The principle of development of vaccines discovered by Pasteur in 1881 still serves as a basis for the whole immunology practice. Pasteur's vaccine is a suspension of attenuated or killed microbes which do not provoke a disease but can cause the protective reaction of the organism, i.e. more or less stable immunity to living pathogens. Special proteins, antibodies, which are one of the products of the immune reaction, bind specifically to antigens, foreign proteins and polysaccharides of the pathogen and thus, block them. The immune response strength is characterized by the number of antibodies or antibody-forming cells (AFC) produced by the organism in response to introduction of the antigen.

For the last 100 years the development of practical immunology, which provided

humanity with powerful weapons against many destructive infections, was mainly based on the development and improvement of the Pasteur method of vaccination. But this method though being of fundamental importance and great productivity has, at least, two restrictions.

The first restriction is as follows. The whole or partly destroyed microbes, which are introduced into the organism, are multi-component complex systems. Alongside with the production of antibodies necessary to destroy the infection, they inevitably inflict the production of multiple varieties of ballast antibodies. The immune system here works idIe to a great extend and this often causes undesirable side effects. The attempts to overcome this drawback by vaccination with previously extracted individual microbe antigens (pure proteins and polysaccharides) are not successful: individual antigens do not ensure strong enough immune protection.

The second restriction is stilI more serious. The strength of the organism protective reaction to this or that antigen is genetically programmed and controlled by special immune response genes (Ir-genes). Such genetic control means that depending on the structure of its genome-, the species can produce high or low response to the introduction of particular antigens or even cannot respond at all. That is, apparently, why it is so difficult to prepare effective vaccines against some dangerous infections by way of inactivation of corresponding viruses or bacteria.

Thus, to overcome these restrictions, one must find the way of the enhancement of immunogenicity of individual antigens (or their active fragments - haptens) independent from genotype of the organism, i.e. avoiding the genetic control. Then, low responder species would be converted into high-responder ones. In recent years certain achievements in solving this problem were made by a joint effort of a group of immunologists and immunogeneticists headed by R.V.Petrov and R. M. Khaitov and a group of polymer chemists represented by V.A. Kabanov and M.I. Mustafaev [158-186]. Cells of two types - B-lymphocytes and T-lymphocytes are known to be the most important components of an animal and human immune system. The former are formed from the stem cells in the mammal bone marrow. The final maturation of B-cells occurs both in bone marrow and in the spleen. The latter are formed in the thymus and also originate from the stem cells of bone marrow. The surface protein receptors of B- and T-lymphocytes "recognize" antigens introduced into the organism. B-lymphocytes are smaIl antibody producing factories. Its own B-lymphocyte clone corresponds to each antibody variety. The set of B-lymphocytes, which can be formed in the organism, is wide enough to react to all natural antigens and produce corresponding antibodies against them. But in majority of cases the signal of T-lymphocytes helpers is also necessary to initiate the "production". In order to send such mitogenic signal, T-lymphocyte should also "recognize" the antigen. But unlike B-lymphocytes, T-lymphocytes set of each species is individual, limited and genetically determined. That is to say, the genetic control over the immune reaction strength is realized by means of T-lymphocytes. Thus, to avoid this, one should first find the way of activating B-lymphocytes without T-helpers participation.

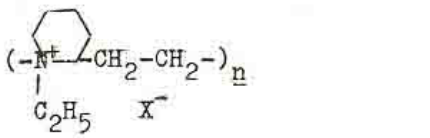
5.1. Polyelectrolyte Immunomodulators

Pilot studies of the adjuvant activity of non-natural (synthetic) polyelectrolytes demonstrated that some high molecular weight compounds of polyelectrolyte origin, e.g., macromolecules bearing positive and negative charges or capable of being charged at physiological values of the ionic strength and pH (polybases, polyacids and their copolymers), despite the differences in their electric charge or chemical structure (or even in the chemical nature of their constituent components) displayed a similar immunostimulating activity. At the same time neutral and uncharged polymers as well as polymers incapable of acquiring an electric charge in aqueous media (for example, poly-N-vinylpyrrolidone, polyethylene glycol, dextran, etc.) had no effect on the immune response to classical antigen-sheep red blood cells (SRBC) and thus exhibited no

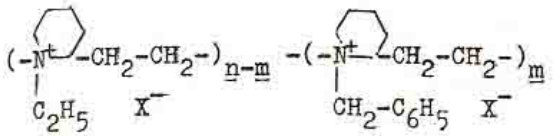
adjuvant activity. Monomeric derivatives of polymeric adjuvant components were also devoid of immunostimulating activity. Quite an opposite effect was observed when copolymers of acrylic acid with methylvinylpyridine whose polymeric chains contain both acidic and basic groups produced immunosuppressor effects and inhibited the immune response by inducing selective elimination of T-B interactions. This finding led the authors to suppose that the action mechanism of polyelectrolyte adjuvants is not directly linked with the fine peculiarities of their chemical structure (nonspecificity towards the monomeric link of PE) and that their immunomodulating activity is due to some common properties that are conferred on them by their polymeric origin.

Polyelectrolyte Immunostimulants. The activation of the immune system is known to be achieved with the help of great variety of different polyelectrolytes (PE) both of polycationic and polyanionic nature [9,9a,187-195]. Synthetic PE structural analogues of which do not exist in nature are of particular interest because they are not immunogens, i.e. they do not cause the production of unnecessary antibodies against themselves.

The list of synthetic PE immunostimulants (adjuvants) used by Kabanov and Mustafaev includes polyacrylic acid (PAA), copolymers of acrylic acid with vinylpyrrolidone of various compositions, poly-4-vinylpyridine (PVP) and its quaternary salt s (PVP-R), poly-2-methyl-5-vinylpyridine (PMVP), quaternary polyconidine salts (PC-R), in particular:



(PC-C_2H_5)



(PC-C_2H_5, -CH_2-C_6H_5)

m/n = 0.2

Despite of considerable differences in structure and even in the very chemical nature of the repeating monomer units, PE adjuvant activity does not differ much if their chains are long enough. They all increase the number of AFC by several times (usually not more than by a factor of 10). PE monomeric analogues (propionic acid, ethylpyridine etc.) are not active.

The establishment of ability of different PE immunostimulants to substitute, at least partly, T-helpers function [196,197] represented the important stage in solving the problem discussed above. So-called B-mice, i.e. the mice that do not have T-cells, T-helpers in particular, were used in these experiments. The immune response strength was determined by the number of AFC produced in mouse spleen in response to the joint introduction of a standard dose of sheep erythrocytes (SE) as antigen and PE. As it follows from Table I, B-mice cannot practically develop the immune response to SE, what can be accounted for by T-helpers deficiency. However, the introduction of the antigen together with PE causes a considerable immune response.

Thus, the presence of PE compensates for the deficiency of T-helpers, i.e. the immune response becomes T-independent (here and further terms "T-dependence" or "T-independence" mean only the presence or absence of a considerable immune response in the absence of T-lymphocytes). It is important that PE, when introduced into the organism, induce, at the same time, the transformation of many various B-lymphocytes clones into AFC (specific in relation to SE, donkey and horse erythrocytes, trinitrophenol on serum albumin etc). In other words, PEs are nonspecific polyclonal activators of B-lymphocytes [198,199].

The data presented above as well as some additional immunological data on which we cannot dwell here (for details, see review [228] lead to the conclusion that PE can affect the immune system avoiding T-helpers.

Table 10. Influence of Polyelectrolytes on B-mice antibody Genesis (SE dose $I0^7$ per mouse, polymer dose 50 mg/kg

Polymer	M.W.	Number of mice	Number of antibodies in the spleen
-	-	I2	30 ± I0
PVP	50000	I5	500 ± 80
PAA	80000	I3	800 ± I50
C-AA•VPD (55:45)	I00000	9	400 ± 500

Even in the early-published papers [191] we assumed the determining role of cooperative PE adsorption on outside membranes of immune cells in immunogenesis stimulation. This assumption is in agreement with recently published data on dependency of immunostimulation activity of different PE upon their degree of polymerization (n) [172]. As it follows from Figure 115 the adjuvant effect appears only when “n” exceeds some "critical" value", sharply increases and, then, practically reaches the limit. The similar dependencies were observed for polymerhomologous series of PAA, PVP and PVD-C_2H_5. Sensitivity of the effect to the structure of PE monomer unit manifests in "critical" value of “n” characteristic for each PE.

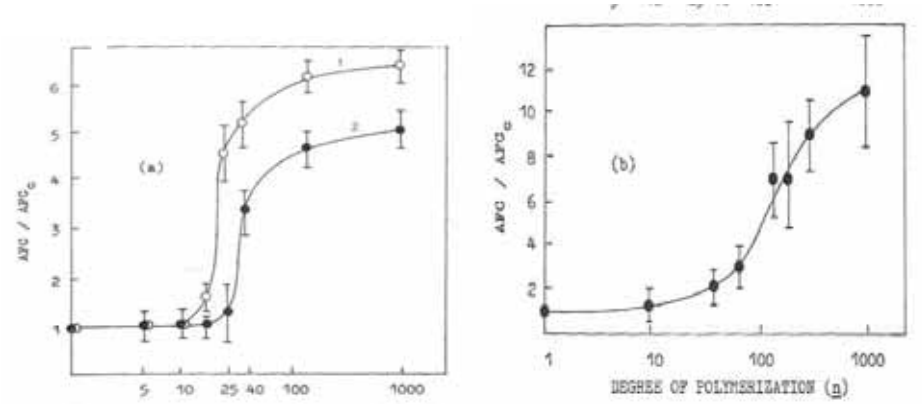


Figure 115. Dependencies of the relative number of antibody-forming cells (AFC) in the B-mouse spleen on the degree of polymerization of quaternary polyconidine salts (a) and PAA (b): 1- (PC-C_2H_5); 2- (PC-C_2H_5, -$CH_2C_6H_5$). The dose of the PE immunostimulants is 50 mg/kg mice were immunized by SE in dose of 5.10^6. AFC_C is the number of AFC in the blank experiments

At the same time, ”n” was found to depend on the conformation peculiarity of PE, and it decreased during the transition from flexible-chain PE (PAA,PVP,PVI) to rigid-chain ones (PC). Macromolecules of PC and its derivatives were less labile due to the presence in the main chain of their macromolecules of cyclic fragments, which start to “operate” at much lower degrees of the macromolecule polymerization.

A detailed investigation of structure and immunostimulating properties of some water-soluble complexes of different PE with low-molecular weight “additives” (hydrophobic lateral

radicals, transient metal ions and surface active substances-sodium dodecyl sulfate (SDS) was carrying out by Mustafaev [9,9a,26,39,85].

Figure 116 shows the immunomodulating properties of PVP(Ro,R_n) polycations relative to the number of lateral cetyl radicals, β, and to the length of side alkyl radicals, correspondingly (see section 1).

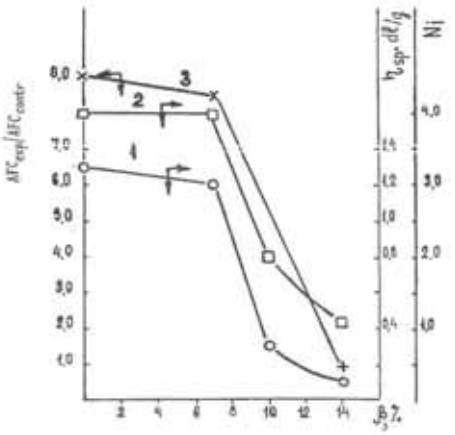


Figure 116. The dependence of the relative number of AFC in the spleen cells of SRBC-immunized mice on the number of lateral cetyl radicals (β) in the PVP(R_0,R_{16}) molecule (3). 1 - dependence of viscosities (hydrodynamic sizes) of PVP(R_0,R_{16}), on β 2 - dependence of the number of protein molecules (N_i) bound to one PE molecule, on β .

PVP(Ro,R_8) and PVP(Ro,R_6) injected to SE-pre-immunized mice stimulated the AFC production in practically the same degree as PVP. However, PVP(Ro,R_{10}) had a far more pronounced stimulating effect on antibody genesis than PVP(Ro,Rn) with $n \leq 8$.With a transition from R_{10} to R_{16} the degree of intramolecular compactization of PVP increased and ,as can be seen from the results depicted in Figure 116,the AFC level in PVP(Ro,R_{16}) injected animals was decreased in comparison with PVP(Ro,R_{10}) injected mice. It can be seen from the data in Figure 116 that the adjuvant properties of the polycation chains depend on the β and are correlated with the conformational transition from the coil to the compact structure in the polyelectrolyte molecule. The analogous behaviors were obtained also for the PE-Me and PE-SDS systems [9,183-186]. Data from physico-chemical analysis of the PE behavior in aqueous solutions containing low-molecular weight complex-forming "additives" as well as immunological data provide compelling evidence that in all systems under study the mechanisms underlying the immunomodulating effect of polyelectrolyte complex are universal, being coupled with the structural and chemical transitions and conformational state of PEC.

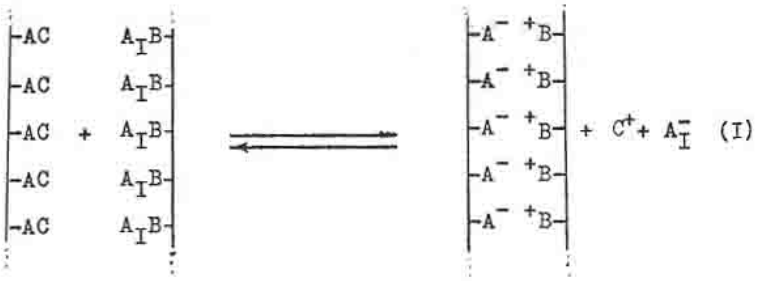
It is known that the constant of cooperative binding of macromolecules is known to be an exponential function of "n" and in the first approximation can be expressed as follows:

$$K=\exp(-\Delta G_m .n/(RT))$$

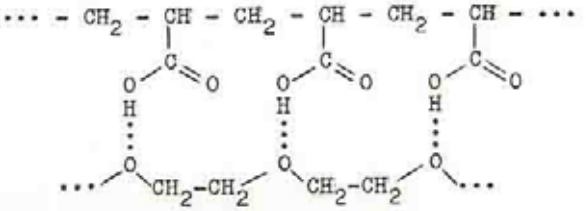
Where ΔG - Gibbs free energy per one unit of the chain. Correspondingly, the degree of binding as a function of n sharply increases from a value, which is close to 0 up to 1 in a rather narrow range of "n". So, the phenomenon appears to resemble a critical one. Therefore, the establishment of the critical character of the dependence of the immunostimulating effect of PE on the chain length has a decisive significance in evaluation of reliability of the above expressed hypothesis.

Physico-chemical foundations of this hypothesis spring from the studies of cooperative reactions between complementary charging PE and PE with proteins, which result in formation of

interpolyelectrolyte and interpolyelectrolyte-protein complexes. Oppositely charged polyions and proteins in such complexes bind with each other by means of multisite sequence of salt and hydrogen bonds, hydrophobic interaction and etc. [200,201]. Interpolyelectrolyte reaction can be represented in the scheme:

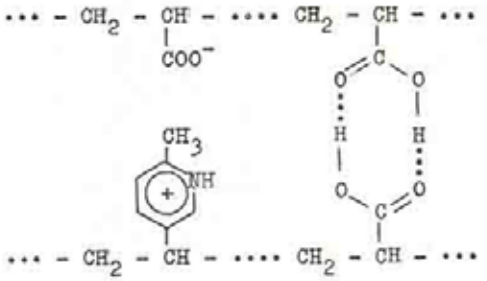


where: -AC is polyacid (or protein) or its salt unit, $-BA_I$ is polybase (or protein) or its salt unit, C is cation (in particular case - proton), A_I is anion (in particular case - hydroxyl ion). In some polymer complexes the multisite interaction of components is realized due to formation of hydrogen bonds, for example, in PAA (or polymethacrylic acid)-polyethylene oxide complex:



PAA (or polymethacrylic acid)-PVPD complexes, etc. (see [202]).

Likewise, interpolymeric complexes are also known in which the multisite system of interacting units includes both salt and hydrogen bonds such as, for example, a complex of PAA with acrylic acid and 2-methyl-5-vinylpyridine copolymer [203]:



In the given case, the macromolecule of the copolymer in the polycomplex plays a role of a heterofunctional cooperative partner with respect to PAA homopolymer.

In recent years there was a detailed investigation of structure and properties of some water-soluble complexes of synthetic linear PEs both of cationic and anionic nature with globular proteins and, in particular, with blood serum proteins (albumin, gamma globulin (see section of "Polyelectrolyte-Protein Complexes" in this review) [33-39,60-66,204-207]. Distinctly expressed cooperative character of protein binding with polyions was shown. A role of a cooperative partner, with respect to a linear polymer is played by the totality of functional group*s* at the surface of a protein globule. In terms of multisite cooperative interaction, the surface of a protein globule is a heterofunctional sorbent. Bovine serum albumin (BSA), for example, in the neutral

pH region, forms complexes with polycations, also with some polyanions and with some amphoteric linear polyelectrolytes. Complexing of BSA with PVP(C_2H_5) at pH=7 is the reaction of type I. It goes with formation of salt bonds.

However, depending on the nature and chemical composition of the chain of the linear partner, in conjunction with the salt bonds essential contribution in stabilization of complexes can further be brought about by hydrogen bonds and by hydrophobic interaction. Complexing of proteins with linear PEs, in fact, consists of a "gluing" of more or less extended molecular chains to the surface of protein globules. Depending on the degree of polymerization in otherwise equal conditions, one molecule can “glue” itself to one or to several globules. In the last case, a complex particle represents an agglomerate of protein globules entangled in linear polyion. In this case, protein molecules can link up with each other. It is important to stress, that a linear PE chain forming a complex with protein molecules does not bind with them in all of its units. A complex particle stilI contains sufficiently long fragments of a linear chain preserved in the form of loops or free ends, which secure the potential possibility of additional multisite binding with other complementary species.

A multisite adsorption of PEs at the surface of colloidal particles is also related to a category of cooperative phenomena, which in their general features are analogous to the formation of interpolymeric complexes [172]. It is known, in particular, that the polycations strongly adsorb on the negatively charged surfaces of colloidal particles. The same applies to the polyanions in their relation to the positively charged colloids.

The dimensions of the cells of the immune system are in the order of 10 μm, that is to say, they are in the dimensional region of particles of typical organic and inorganic sols. The portions of polymeric chains can be adsorbed on the outcoming membrane proteins as well as on polysaccharides fragments of glycolipids and glycoproteins, which represent a considerable part of the overall membrane surface. The polar "heads" of lipids, which form an outer surfaces of a double layer, contain anionic and cationic groups as well as the groups which are capable of hydrogen bond formation, and which can also serve as the centers of adsorption. Certain phenomena of agglutination (sticking) of erythrocytes [208,209] and thrombocytes [210-213] by polybases in terms of their physicochemical aspect are fully analogous to flocculation of ordinary "nonliving" sols by PEs

Thus, the surface of cells formed by outer membranes, is, generally speaking, a universal multisite heterofunctional sorbent for PEs.

The assumption of the nonspecific activation of B-lymphocytes by adsorbed linear PEs agrees with the obtained experimental data. In the *in vivo* experiments, it was shown, that, PAA or PVP introduced in a spleen cell culture of intact mice, increase by 2,5-3,5 times the velocity of the cell division (with respect to the velocity of incorporation of H–thymidine in the DNA composition [214]. It was found, in particular, that PAA selectively activates fraction of the mouse spleen cells, strongly enriched with B-lyphocytes and does not show a mitogenic effect on T -cells. The activation of DNA synthesis in B-lymphocytes begins within 24 hours after the introduction of PAA polyanions [215]. Polycations, on the contrary, are characterized by greater universality of mitogenic effect (i.e. by less selectivity).

What does the mechanism of activation of the immune system cells by ionogenic macromolecules consist of?

Macromolecules of PE entering the blood within the composition of multicomponent biological mixtures interact, primarily, with blood plasma proteins, polysaccharides and nucleic acids as well as with the surface of blood sells; therefore their immunological activity may be manifested in different ways.

One of putative mechanisms of the adjuvant effect of linear PE is the adhesion of immunocompetent cells via the multipoint adsorbtion of linear macromolecules on the cell membrane surface. Evidence in favour of this hypothesis can be derived from the fact that cooperative reactions of the macromolecular substitution type facilitate the interactions of every

macromolecule circulating in the blood and coming into contact with many other molecules before it reaches a certain local thermodynamic equilibrium.

Kabanov assumed [216] that the segments of the chains adsorbed on the cell membrane can considerably change its properties such as permeability for ions and other low-molecular substances, characteristics of lipid matrix fluidity and even induce a phase transition in lipid layer. Any of such change may serve as a source for mitogenic signal.

What is the mechanism of ionic permeability formation? Generally speaking, the potential centers of cooperative sorption of ionogenic macromolecules can exist either on the lipid bilayer surface or on outcoming membrane proteins. In fact, model lipid membrane, which does not contain proteins (lecithin black films) adsorbes polyions. An indication of that is the change of membrane electric potential when the PE solution is added [216]. However, their adsorption in this case is not followed by appearing of ionic permeability of the bilayer. Therefore membrane proteins distributed in the lipid matrix are most likely to participate in formation of nonspecific ionic channels when interacting with polymer. It was suggest, that the adsorption of the polyions on real membrane results in formation of protein globules clusters like it happens in case of complexing in solution [34]. Electronic microphotograph of longitudinal sections of the membrane previously treated with polycations confirm this hypothesis (Figure 117). The process of protein cluster self-assembling is schematically presented in Figure 118. The channels responsible for ionic permeability are very likely to appear on the boundaries of joint protein globules.

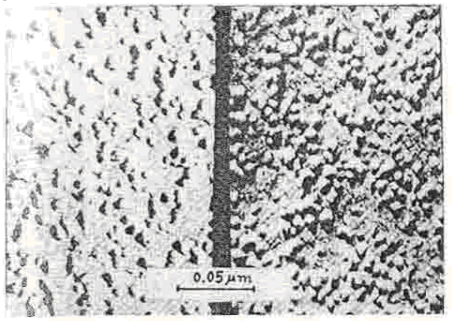


Figure 117. Electron micrographs of longitudinal section of the lymphocyte membrane before (left side) and after (right side) PAA solution treatment

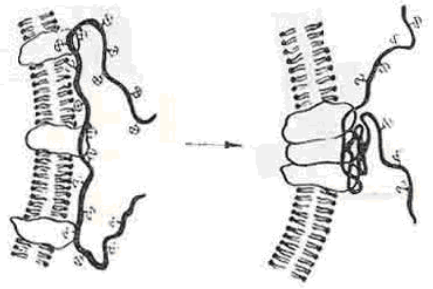


Figure 118. Hypothetical scheme of PE-membrane proteins interaction

How does the lymphocyte react to the presence of nonspecific PE - ionophore on its surface? Taking the obtained data into consideration authors suggest that PE adsorbed on plasmatic membrane acts as a trigger in Iaunching the mechanism of cell division. The adsorption goes with formation of nonspecific transmembrane ionic channels and emergence of corresponding transmembrane ionic flux. The disturbance of a cell homeostasis leads, in its turn, to switching on of enzyme ionic pumps (ATPases) to compensate for the K^+ deficiency and Na^+ and Ca^+ excess inside the cell. These are very likely the first stages of succession of molecular events, which, in the long run, lead to the accomplishment of the whole mitotic cycle. It is significant that the dependencies of ionophore and immunostimulant activity of PE on its degree of polymerization perfectly correlate with each other. Macromolecules obtain the capability of induction of transmembrane ionic flux and activating of immune cells only if their length exceeds a certain "critical" value (Figure.119, compare with Figure 115).

The described above mechanism of cell activation as applied to B-lymphocytes, can probably explain the ability of PE adjuvants to substitute the T-helpers function in the immune

response to typical T-dependent antigens. B-lymphocyte receives a necessary nonspecific mitogenic signal from polyion and thus, does not need a mediator, which is usually produced by a corresponding T-helper only after it "recognizes" the antigen.

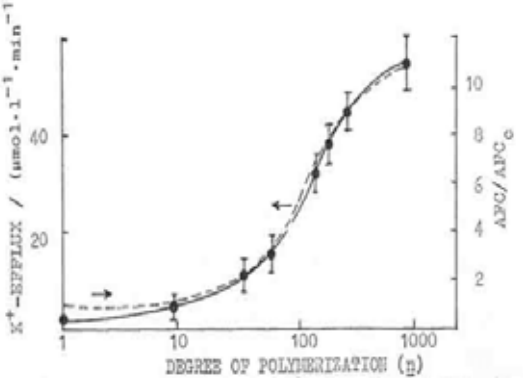


Figure 119. Dependencies of K^+ efflux through B-lymphocyte membranes in vitro and relative strength of the immune response in vivo on PAA degree of polymerization. Immunization of mice by SE

We proceeded from that an essential role in the manifestation of immunological activity of PE belongs to a proteinoceous factor, since the PE molecules entering the blood predominantly interact with plasma proteins that are present there in large excess. The PE-protein complexes formed thereby may further interact with the surface of different cells, be adsorbed by macrophages, etc. According to this hypothesis, the triggering mechanism of the PE action depends on the mode of PE interaction with blood proteins as well as on the nature of complexes formed as a result of this interaction. Noteworthy that the “adhesiveness” of PE macromolecules within the composition of PEC which reflects the structural integrity of free sites of PE (loop-like structures, free ends) may differ considerably from that of the original macromolecule because in many cases, especially in the case of copolymers-polyampholytes, the complex formation is accompanied by the involvement of essential groups of PE in their interaction with proteins (selective fractionation of essential groups). Therefore, the mode of binding of individual PE and their complexes to cell surface membranes may be quite different. This difference obviates the need for a thorough analysis of mechanisms underlying the interaction of such protein-polymer complexes with the surface of immunocompetent cells.

This hypothesis is consistent with earlier reported data and with the results described herein. As it was mentioned above the length of the polymeric chain determines the composition of protein-polyelectrolyte complexes, and under identical conditions the number of bound protein globules attached to one PE macromolecule increases linearly in proportion to the elongation of the PE chain. As can be seen from Figure 120 there is a direct correlation between the adjuvant activity of PE and the number of protein globules bound to one PE macromolecule, i.e., the composition of the polycomplex. Similar results were obtained during the analysis of PE complexes with SDS and transient metal ions. PE or their complexes with low molecular weight ligands having a more unfolded structure and showing the ability to form nonstoichiometric complexes with proteins due to the high epitope density of their antigenic determinants “operate” as immunostimulators, whereas PE or PEC whose macromolecules have a compact structure and which form stoichiometric complexes in aqueous solutions are either inactive or produce a strong immunosuppressor effect. Consequently, the behavior of polymeric adjuvants in the blood flow depends on the supramolecular structure of their constituent macromolecules; the mode of the interaction with proteins (plasma or membrane proteins, etc.) predetermines the future fate of

PEC in the organism. Typical interactions of this kind are schematically presented in Figure 120.

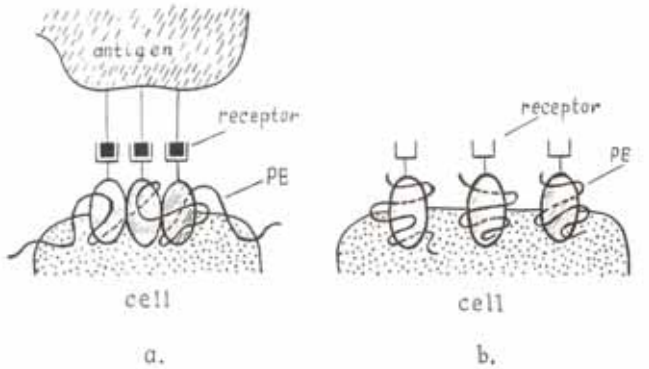


Figure 120. A schematic presentation of the interaction between linear (a, immunostimulators) and compact (b, immunosuppressor) macromolecules of PE having degree of polymerization with the antigen and the cell surface

During the formation of type "a" structures the local concentration of antigenic (or recaptures) determinants increases in parallel with the increase of the constants reflecting the multipoint interactions between the receptors and antigens; hence the high immunostimulating activity of such PE. Polyelectrolytes having a compact structure show a tendency to form complexes of the "b" type. In the latter case the initial local concentration of free receptors (or antigenic determinants) does not change, such PE either have no effect on the immune response or display an immunosuppressor effect by screening the receptor (or antigen) molecules.

These results provide compelling evidence in favour of the previously observed inhibiting effect on the immune response of polyampholytes containing both acidic (AA) and basic (MVP) groups [164]. Most probably, the suppressor effect of such polyampholytes reflects their structural peculiarities in aqueous solutions rather than a cooperative interaction as was believed earlier. Such polyampholytes have a compact structure, which is due to the intramolecular hydrophobic interactions of their methylpyridinium links. Their interactions with proteins result in the formation of stoichiometric complexes, which seems to be the main reason for their immunosuppressor activity.

Recently, the possibility of such migration was directly proved on the system, which models a cell suspension in aqueous medium (by Kabanov) [136,217-220]. The particles of practically monodispersed polystyrene latex of 0.5μm dimension, covered with chemically bound carboxyl groups (in average one COOH group per $25A^2$) served as cell models. The particles were labelled by means of chemical binding of fluoresceinisothiocyanate (FITC). PVP-C_2H_5 ($n \approx 10^3$) served as PE. Polycation units are capable of quenching of FITC luminescence. The curves of latex luminescence quenching by adding of PE at different pH are presented in Figure 121. The fact of quenching and the increase of quenching efficiency when the negative charge of latex particles is increased (with pH increase) indicate polycations adsorption on their surface. Ultra centrifuging with scanning in UV-adsorption range of PVP-C_2H_5 (250 nm) reveals that at pH=9 practically all PE are adsorbed on the latex particle surface. When a new portion of the same but nonlabelled latex is added to the system, the intensity of fluorescence sharply increases (Figure 122).

This unambiguously means that some fraction of PE macromolecules migrated to nonlabelled latex particles. It is probable that antigen-PE conjugate migrates, in a similar way, from cell to cell in search of antigen specific receptors. The search stops when the antigens find on B-lymphocyte surface the complementary receptors and bind with them. A minimum of free

energy corresponds to such state (Figure 123). In other words, the immunostimulant effect of PE is as if focused on certain cells. In the series of experiments PE conjugates with T-dependent (T,G-AL) polypeptide antigen of high specificity (polylysine graft copolymer with copolymer of alanine, glutamic acid and tyrosine [221,222,223] were used for mice of pure low-(CBA) and high-responder (C57/BL) strains These experiments revealed that the binding of antigen with synthetic PE not only increases the immune response to this antigen but also makes it T-independent and noncontrolled by Ir-genes [224].

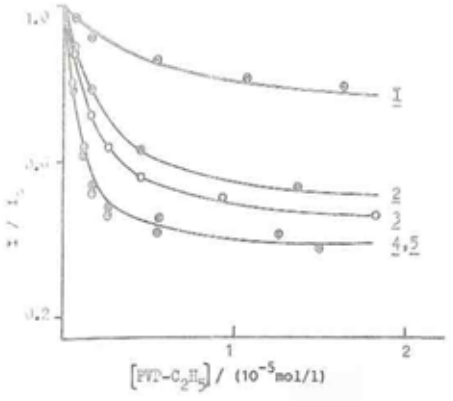


Figure 121. The dependencies of the fluorescence intensity of FITC-labelled carboxylated latex on PVP-C_2H_5 concentration, pH=1-6, 2-7, 3-8, 4-9, 5-10. Latex concentration c=1,3.10^{-8} cm^{-3}; [$NA_2B_4O_7$]=0,01 mol/l, 25^{0}C

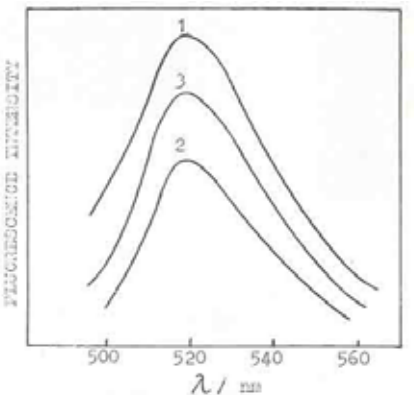


Figure 122. The fluorescence spectra of the model latex systems. I-FITC-labelled latex (c=1,3.10^{-8} cm^{-3}), 2- FITC-labelled latex + PVP-C_2H_5 (10^{-6} M), 3- FITC-labeled latex + PVP-C_2H_5 after adding of equal amount of nonlabelled latex (c=1,3.10^{-8} cm^{-3}), pH=9. The turbidity spectra are subtracted

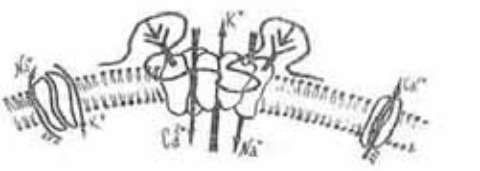


Figure 123. The hypothetic scheme of the interaction of an antigen-PE conjugate with B-lymphocyte membrane

5.2. Model Artificial Immunogens

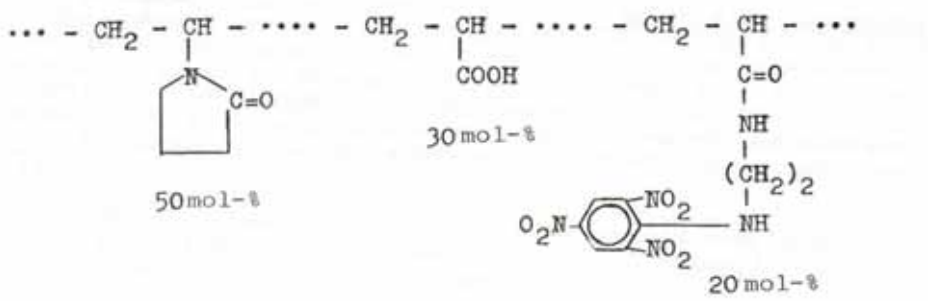
Nevertheless, B-lymphocyte activation by synthetic polyelectrolyte immunostimulants without help of T-lymphocytes is still not a solution of the whole problem. Firstly, the effect *in vivo* itself turns out to be not high enough: the number of antibody forming cells increases only by several times; secondly one and the same polyion acts nonspecifically: it can activate B-lymphocytes of various clones (polyclonal activation), i.e. inflicts the production of many mostly unnecessary antibody varieties [198,199].

Quite another result can be achieved if individual antigen or its active fragment–hapten is attached to the synthetic membrane active polyelectrolyte chain. Then, the strength of the T-independent immune response increases by dozens and hundreds of times. The immune response

becomes highly specific, i.e. antibodies against the antigen (or hapten) included into complex are produced.

Hapten or antigen attachment to polymeric immunostimulant can be achieved by means of either chemical or strong enough adsorbtion bonds, which cannot be destroyed in the organism for the time sufficient for switching on the immune system.

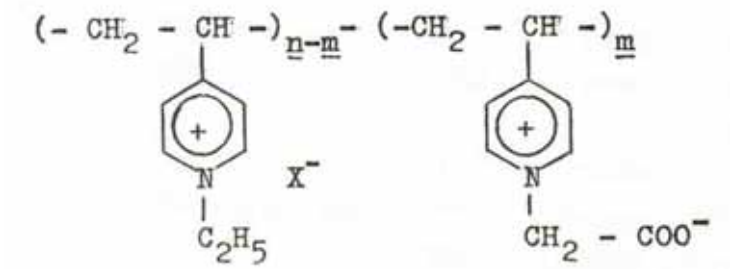
The first artificial antigen constructed on the basis of a synthetic PE is the electrostatic complex (EC) of trinitrophenol (TNP) with poly-2-methyl-5-vinylpyridine (PMVP) and covalent conjugates of TNP with copolymers of acrylic acid and N-vinylpyrrolidone [169,221]. The fragments of the artificial immunogen structures are presented on the following scheme:



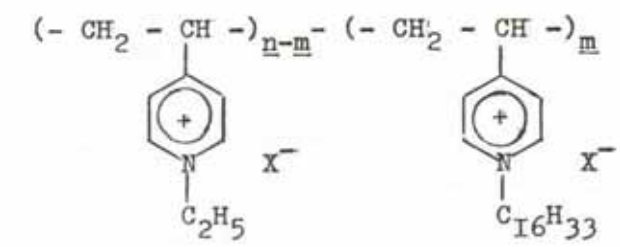
One distinctive feature of these antigens is that it not only elicits immune responses without the help of adjuvants but also is also able to stimulate the antibody production even in low responder athymic animals. It is evident that in contrast to TNP or its conjugates with protein carrier (bovine serum albumin). TNP-PE conjugate causes a very strong T-independent immune response, especially after secondary immunization. AFC specific to TNP are produced.

Recently, the possibility construction of such complete synthetic immunogens was demonstrated by the directly conjugation of steroid hormones (estradiol and progesterone), anticancer betuline and functionally polypeptides (Hepatite B surface antigen and Foot-and-Mouth Disease Virus VP1 protein epitopes) with different polyelectrolytes (see below).

Another group of artificial immunogens based on nonnatural PE carriers includes polycomplexes of protein antigens that are stabilized by cooperative electrostatic and hydrophobic interactions (Kabanov and Mustafaev, 1978) and covalent conjugates of PE with protein antigens (Kabanov and Mustafaev, 1978,1979). BSA covalently binding with copolymer:



where P=1000,m/n=0.4, or strong adsorbtion of BSA, BGG and some other proteins on copolymer:
where $P_n=10^3$,n/m = 0.1, resulted in the formation of the artificial antigens which cause much stronger immune response than the corresponding pure proteins.



It is noteworthy, that not only normal mice develop strong immune response (genetic strain C57BL/6(+/+)) but the thymusless as well (genetic etrain nude (nu/nu)). Consequently, the presence of linear PE fragments in artificial immunogen actually ensures the switching on of the immune reaction without the involvement of T-cells [26,158], i.e. these immunogens are able to stimulate the production of protein-specific antibodies all by themselves and "operate" independence of the control of the thymus or of the Ir-gene control of the immune response. In the series of experiments PE conjugates with T-dependent (T, G-AL) polypeptide antigen of high specificity (polylysine graft copolymer with copolymer of alanine, glutamic acid and tyrosine [222,223]) were used for mice of pure low-(CBA) and high-responder (C57/BL) strains. These experiments revealed that the binding of antigen with synthetic PE not only increases the immune response to this antigen but also makes it T-independent and noncontrolled by Ir-genes [224].

The principle of conversion of relatively weak T-dependent antigens into very strong T-independent by their attachment to nonimmunogenic synthetic polyelectrolyte immunostimulants appears to be quite universal. It was confirmed by the examples of more than dozen of model, microbe protein and polysaccharide antigens (including BSA, BGG, OA, cancer protein antigen-alphafetoprotein, tuberculin, surface antigens of the influenza virus-hemagglutinin and neuraminidase, or the protein fraction of BCG (TPF), fraction F of the plague microbe, polysaccharide of salmonella O-antigen etc.) [9,9a,18,18a,26,39,85,105,118,158-186]. The antigen or hapten included into the polycomplex particle addresses the complex macromolecule as a whole, i.e. the artificial immunogen, to the antigen specific B-Lymphosite. The search of the B-lymphosite of the proper clone is realized by trial-and-error method by means of conjugate migration from sell to sell according to the mechanism of macromolecular substitution and exchange. This type of migration was discovered and studied by Kabanov [201,202] on the examples of interpolymer complexes and by Mustafaev on the examples of protein-polyelectrolyte complexes [38,60]. Recently, the possibility of such migration was directly proved on the system, which models a cell suspension in aqueous medium [218-220].

In this chapter we shall consider some physico-chemical criteria that are normally taken into consideration during the construction of artificial immunogens on the basis of PEC with special reference to the structural characteristics of PEC (stability, charge, size, conformation, etc.) as well as their immunological activity. A structural model of a polymer-subunit immunogen will also be described. These data shed additional light on the triggering mechanism of PEC. Besides they rationalize the selection of and outline the approaches to the directed synthesis of PEC with predetermined immunological activity.

Highly immunogenic PEC. The results of experiments aimed at the elucidation of immunogenic properties of soluble mixtures consisting of a model protein (bovine serum albumin, BSA) and PE, e.g., PVP and its derivatives, PAA, sodium polystyrene sulfonate (PSSNa) and their copolymers, are shown in Figure 124. It can be seen from these data that the immunogenic activity of the tested mixtures depends on the chemical nature of PE, although their immunostimulating activity is nearly identical.

What is the reason for this phenomenon? A detailed analysis of physico-chemical properties of PE-BSA mixtures revealed that the chemical structure of PE strongly affects their interaction with the protein antigen and, correspondingly, the stability of the polymer-protein

complexes formed thereby. It was found that under these conditions PVP acquires a weak positive charge and thus becomes unable to form complexes with BSA. The results of immunological studies suggest that the level of AFC production in BSA-PVP immunized mice is very low. However after the loading of PVB with lateral hydrophobic cetyl radicals (PVP-Rn) the former acquire the ability to form complexes with BSA.

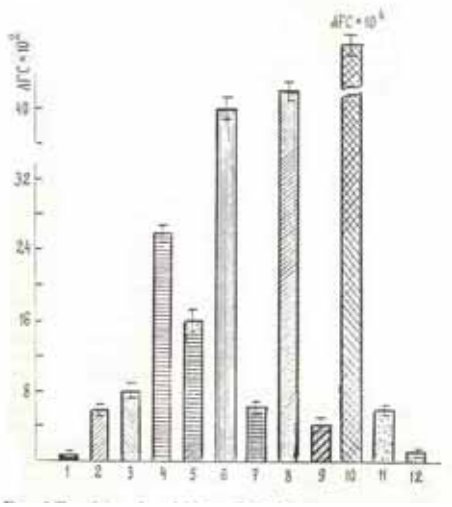


Figure 124. The relative values of BSA-specific AFC in the spleen cells of mice immunized with PE-BSA mixtures. 1- pure BSA; 2- PVP+BSA; 3- PVP-R_{16}+BSA; 4- (PVP-R_2+BSA) in H_2O; 5- (PVP-R_2+BSA) + 0,15 M NaCl; 6- PVP(R_2,R_{16}) + BSA; 7- PVP(R_0,R_{ac}) + BSA; 8- PVP(R_0,R_{ac}) + BSA + carbodiimide; 9- PAA + BSA; 10- (PAA + BSA) + carbodiimide; 11- PSSNa + BSA; 12- CP(AA + MVP) + BSA.

The complex-forming capacity of PE molecules is different and depends on the number of alkyl radicals, ß, introduced into the PVP molecule. Polycations of PVP-Rn whose cetyl radicals are abundant enough to induce the formation of PVP-BSA complexes but which are insoluble in neutral aqueous media can form stable electrostatic and hydrophobic complexes with BSA in acid aqueous solutions (pH 4.3). However, at physiological values of the ionic strength and pH such complexes lose, to a certain extent, their stability: part of the protein molecules dissociate from the main complex to form an insoluble pellet, in which one polyionic chain corresponds to one protein globule (so-called stoichiometric complexes). With an increase in the ionic strength the newly formed water-soluble PSSNa-BSA complexes also lose part of their BSA molecules and at physiological concentrations of the low molecular weight salt acquires a stoichiometric composition. One may infer from these data that the same phenomenon will take place after injection of these complexes into the blood. Both complexes are characterized by a relatively low immunological activity, although they are more active than mechanical protein-PVP mixtures.

In solution PVP(R_2)-BSA complex particles are stabilized due to the electrostatic interactions of oppositely charged PVP(R_2) and BSA molecules. Under physiological conditions the EC formed in such mixtures are less stable and partly decomposed, as a result of which their immunogenicity markedly diminishes (Figure 124). In contrast with other systems, the formation of PVP(R_2,R_{16})-BSA complexes is provided for by cooperative electrostatic (salt) and hydrophobic interactions. Such complexes are rather stable and do not dissociate under physiological conditions. As can be seen from Figure 125, these complexes display a much higher immunogenic activity as compared with other known PEC. Speaking differently, among other

protein PE complexes used for immunization of animals the highest immunogenic activity is manifested by stable polyionic complexes that are resistant to physiological values of the ionic strength and pH. The structure of such complexes is depicted in Figure 125. The protein globules in each complex molecule seem to be in close contact with one another, being "entwined" by the polycationic carrier. Some hydrophobic cetyl radicals of PE are bound to the hydrophobic regions of the protein; while others interact with one another, thus promoting the stabilization of the overall structure. Fragments of the polycation containing no cetyl groups can form H_2O-accessible free loops or salt bridges with the negatively charged groups situated on the surface of the protein globules.

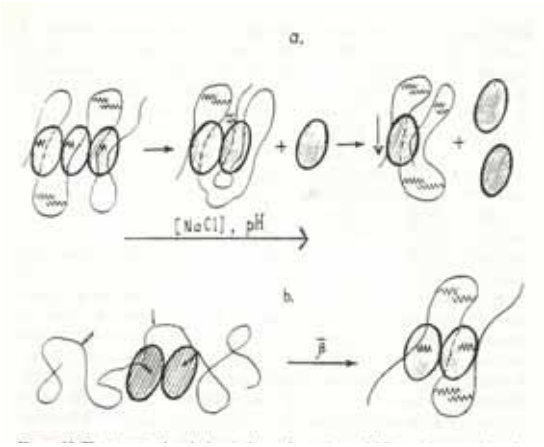


Figure 125. The structural and chemical transformations of PE-protein complexes in different reaction media. (a) BSA-PVP(R_0,R_{16}) pH 4.3 (b) BSA-PVP(R_2,R_{16}). For explanations see text

What is the functional significance of the covalent chemical bonds in this particular case? Its estimation is very important, both theoretically and practically, for all those whose ultimate goal is the construction of conjugates possessing maximal stability. In our studies this task was accomplished through the covalent attachment of the free carboxyl groups of PAA and 4-vinylpyridine copolymer with 4-vinyl-N-acetylpyridinium bromide [PVP(R_o,R_{ac})] to the amino groups of BSA via their activation by carbodiimide (Mustafaev *et al*., 1986):

BSA via their activation by carbodiimide (Mustafaev *et al.*, 1986):

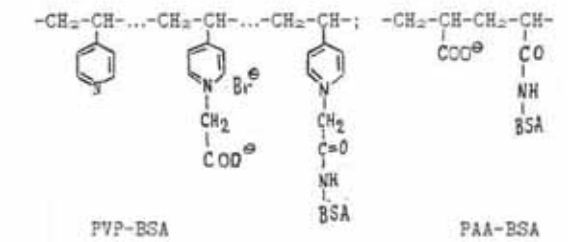


Figure 124 shows the results of immunological studies, in which the number of anti-BSA AFC in the spleens of mice immunized with polymer-protein conjugates is given relative to control. It can be seen from these data that the AFC number in the spleen cells of mice immunized with BSA covalently bound both to the polyacid and the polybases markedly exceeds that formed in the spleen cells of mice injected with an equal amount of the protein and PE that are chemically unrelated to each other.

The dependence of the mode of SRBC binding to PE on the immunogenicity of the PEC

formed thereby was especially apparent during the analysis of immunostimulating properties of PE able to form covalent complexes with corpuscular antigens (SRBC). In the previous chapter we emphasized the crucial role of the PAA chain length in the adjuvant activity of PE. In this case the immunostimulating activity of PE and SRBC was practically independent of the mode of their administration (separate or combined), being approximately at the same level. The stability of the PAA-SRBC complexes was provided for by the weak electrostatic ("mobile") bonds formed by the carboxylic groups of the polyacid and the corresponding sites situated on the heterofunctional surface of immunocompetent cells.

Naturally a question arises as to how the introduction of functionally important groups possessing the ability to form covalent bonds with SRBC influences the immunostimulating activity of PE.

Activation of PAA was induced by the routine carbodiimide method. This reaction is normally accompanied by the formation of anhydride bonds of the polyacid capable of forming covalent (amide) bonds with the amino groups of BSA as well as with the surface of SRBC. The relative values of AFC determined in the spleens of PAA-immunized mice at varying lengths of the PAA chain and at different numbers of their constituent anhydride bonds are shown in Table 10. These data suggest that the appearance in the polymeric molecule of anhydride bonds possessing the ability to form covalent complexes markedly increases the immunostimulating activity of PAA. With an increase in the number of such macromolecular bonds and, as a matter of consequence, with the elongation of the polymeric chain the adjuvant effect linearly increases. In this case the immunostimulating activity of PAA depends on whether it is injected alone or in combination with SRBC; its effect on immunogenesis is especially apparent after immunization of mice with ready-to-use solutions of covalent polymer-SRBC complexes.

Table 11. The effects of PAA and its anhydride derivatives on the AFC production in the spleen cells of SRBC-immunized mice. $AFC_{exp.}$ and AFC_{contr} – AFC levels in experimental and control mice. AFC_{contr} =4700. PAA and PAA* - original PAA and anhydride derivatives of PAA. β-number of anhydride bonds in the PAA molecule

mode of injection of PAA and SRBC	PAA $AFC_{exp.}/AFC_{contr.}$		AFC_{exp}/AFC_{contr} (PAA*)		
	$\bar{P}_z = 570$	$\bar{P}_z = 1100$	$\bar{P}_z = 570$ $\beta = 10\%$	$\beta = 20\%$	$\bar{P}_z = 1100$ $\beta = 10\%$
Separate	2.0	3.30	4.0	5.0	4.8
Combined	1.4	2.35	3.0	7.25	9.2

These results suggest that under *in vivo* conditions the high stability of polymer-protein complexes is a necessary prerequisite for the manifestation of their high immunogenic activity.

In our further studies design ed to investigate the immunogenic activity of PE-protein complexes in more detail we used two types of stable complexes, namely, PVP(R_2,R_{I6})-BSA and the covalent conjugates, PAA-BSA and PVP-BSA. The kinetics of BSA-specific AFC accumulation in the spleen cells of mice immunized with PEC and CC constructed on the basis of polybases is shown in Figure 126. The experimental plots are characterized by the extreme; the peak of the immune response is observed on the 10th post-immunization day.

The dynamics of this reaction is irrelevant to the nature of the immunizing antigen and is manifested only in the increased amplitude of the immune response. In additional experimental series we studied the dose dependence of the immune response in the spleen cells of mice immunized with PVP(R_2,R_{I6})-BSA. The increase in the conjugate dose caused a proportional increase in the AFC level in mouse splenocytes.

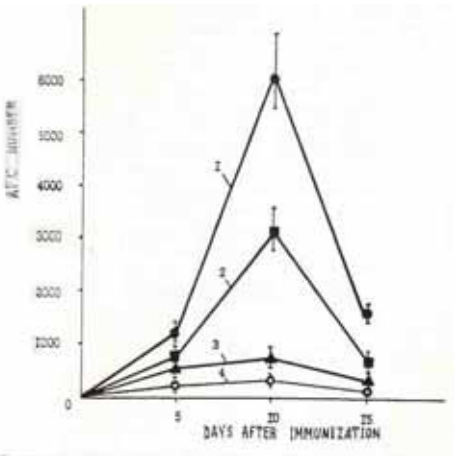


Figure 126. The kinetics of BSA-specific AFC accumulation in the spleen cells of mice immunized with BSA-PE complexes. 1-BSA-PVP(R_0,R_{ac}); 2-BSA-PVP(R_2,R_{16}); 3- BSA-PVP-R_2; 4-pure BSA. The BSA dose was the same in all cases

Specially designed experiments revealed that the AFC present in the spleens of mice immunized with such PEC were able to generate BSA-specific antibodies. The AFC accumulated in the spleens of PVP(R_2,R_{16})-BSA immunized mice could be detected only when BSA-SRBC (but not OVA-SRBC) was used as the test antigen. The AFC activity was fully eliminated by the BSA added to the agar.

Immunization of mice with BSA covalently bound to polymeric carriers having a different chemical composition (polyacid polyanions) also induced a high immune response whose magnitude was time-dependent (Table 12). Antibodies to BSA were detected in the blood sera of immunized animals already on the 4th postimmunization day; the peak of the immune response was observed on the 10th day with a subsequent decline on the 14th day. These AFC appeared to be specific towards BSA: an addition to the titration wells of pure BSA (final concentration *500,ug/ml)* caused a practically complete inhibition of the hemagglutinin reaction, whereas bovine γ-globulin (BGG) had no such effect. Therefore it seemed very enticing to investigate the effect of complete Freund's adjuvant (CFA) routinely used in immunological studies on the strength of the immune response elicited by a new artificial antigen. The conjugate was injected intraperitoneally with saline or in a water-in-oil emulsion with CFA at a 1:1 ratio (0.2 ml of the conjugate + 0.2 ml of CFA). The results of these experiments are depicted in Table 11. It can be seen from these data that the immunogenic activity of BSA-PAA conjugates markedly exceeded that of BSA + CFA mixtures, although the immune response to this antigen was not manifested immediately. A drastic increase in the immune response could be attained through a simultaneous administration of the conjugate and CFA: the AFC number was 30-40 times as high as that in BSA-PAA immunized mice and 100-130 times as high as that in BSA + CFA immunized mice. The latter finding points to a synergy of the "directed" effect of PAA and the stimulating effect of CFA on the BSA-specific immune response. It may be inferred from these data that the formation of ternary complexes in the conjugate-CFA system promotes the increase in the "adhesiveness" of the conjugate and thus stimulates the immune response to the protein antigen (see below).

It follows, therefore, that the combination of weak protein antigens with polymeric carriers markedly increases their immunogenicity irrespective of their chemical structure and the charge of their constituent macromolecules.

Similar results were obtained in experiments, in which PE were conjugated with other

proteins (model antigens, microbial and bacterial antigens). By illustration, complexes of BGG and the purified protein derivative of tuberculin (PPD) with quaternated PVP displayed a high immunogenic activity (Table 13). At the same time, complexes of the H-antigen of *Salmonella,* complete influenza antigen, meningococcal microbial B-polysaccharide and α-fetoprotein (oncofetal antigens) were also found to possess an exceedingly high immunological activity in comparison with the original antigens (see below). Hence, the observed phenomenon is unrelated to the nature of the protein antigen or the polymeric carrier and is more universal.

Table 12. The titers of BSA-specific antibodies in the blood sera of mice immunized with pure BSA., BSA-PAA, BSA-PAA in CFA + pure BSA and BSA-adjuvant. *-number of AFC formed in the spleen cells of mice immunized with BSA-PAA after addition of pure BSA (500μg/ml) to the titration plates

preparation	antibody titers (log_2), M ± m			
	4th day	7th day	10th day	14th day
BSA	0.5 ± 0.1	0.5 ± 0.12	0.5 ± 0.1	0.5 ± 0.12
BSA + PAA	0.5 ± 0.12	0.5 ± 0.15	1.0 ± 0.05	3.0 ± 0.14
BSA-PAA	0.5 ± 0.1	3.5 ± 0.20	5.0 ± 0.8	3.2 ± 0.15
BSA-PAA*	0.5 ± 0.1	0.2 ± 0.05	0.3 ± 0.05	0.3 ± 0.06
BSA-PAA + CFA	—	8.5 ± 0.19	11.0 ± 0.21	8.7 ± 0.20
BSA + CFA	2.0 ± 0.2	2.2 ± 0.15	3.0 ± 0.14	3.0 ± 0.15
BSA – PAA	0.5 ± 0.1	4.0 ± 0.30	5.0 ± 0.25	4.0 ± 0.25

Table 13. The AFC titers in the spleen cells of mice immunized with pure BSA, PPD and BGG-PPD complexes with quaternated Pvp loaded with lateral cetyl groups

substance	AFC titers	number of animals
BGG	5000 ± 5.35	18
PVP(R_2, R_{16})-BGG	100000 ± 15000	18
PPD	25 ± 5.0	18
PVP(R_2, R_{16})-PPD	25000 ± 2000	18

As above, pure PE carriers enhance the helper signal of T-Iymphocytes (Table 14), induce polyclonal activation of B-cells and promote the Ir-independence of the immune response to separately injected antigens. But can a protein conjugate with a polycation acquire the properties of a T-independent antigen? To answer this question we used as immunogens the polycationic complexes loaded with lateral hydrophobic groups combined with weak (BSA) and strong (BGG) protein antigens. The immunogenic activity of PVP(R_2,R_{I6})-BSA and PVP(R_2,R_{I6})-BGG complexes was studied on C57BL[6(+ / +)] mice as well as on athymic mice homozygous at the "nude" gene. The animals received intraperitoneal injections of the pure protein and an equal amount of the protein-PE complexes. The results of these studies are depicted in Table 14. It was found that in "nude" mice the immune response to pure BGG was much lower than in normal mice due to the T-helper deficiency: no specific AFC to pure BSA could be generated in these animals. However, injections of mice with BSA or BGG complexes with PE not only restored the original level of T-helper lymphocytes but also induced an additional synthesis of antigen-specific AFC.

Table 14. The effects of synthetic PE on antibody genesis in SRBC-immunized B-mice

PE	M_r	no. of animals	AFC number
—	—	12	30 ± 10
PAA	80000	13	800 ± 150
PVP	50000	15	500 ± 80
CP(AA-VPD)	100000	9	4000 ± 500

Thus, conjugation of standard T-dependent protein antigens, both strong and weak ones, with T-independent polymeric stimulators allows one to obtain strong T-independent artificial immunogens and thus promotes the T-independence of the immune response to T-independent antigens within the composition of PEC. In practical terms, the ability of PEC based on protein antigens to elicit secondary (anamnestetic) responses to booster doses of the antigen and, correspondingly, to stimulate the immunological memory, presents special interest. In our studies mice were immunized with PVP(R_2,R_{I6})-BSA and, one month thereafter, with the pure protein antigen, after which the number of specific IgM- and IgG-synthesizing antibodies was counted in their spleens. The data depicted in Table 16 illustrate the levels of IgM- and IgG specific AFC determined in the spleens of experiment al mice on the 7th postimmunization day. After the primary (or secondary) immunization (M±Ip, $P \leq 0.05$) were by several orders of magnitude higher than in control animals. In these mice injections of the protein complex with the polycation stimulated the formation of stable immunological memory already after a single immunization, which made it possible to avoid the additional use of adjuvants.

Table15. The immunogenic activities of BSA. BGG and Their complexes with quaternated PVP loaded with lateral hydrophobie groups in the spleen cells of athymic and intact mice.

genotype	immunogen	AFC	number of animals
C57BL/6(+/+)	BSA	25 ± 10	5
C57BL/6(+/+)	BSA-PVP(R_2, R_{16})	2500 ± 400	6
Nude (nu/nu)	BSA	—	3
Nude (nu/nu)	BSA-PVP(R_2, R_{16})	600 ± 100	5
C57BL/6(+/+)	BGG	2500 ± 400	6
C57BL/6(+/+)	BGG-PBP(R_2, R_{16})	3500 ± 200	6
Nude (nu/nu)	BGG	100 ± 20	5
Nude (nu/nu)	BGG-PVP(R_2, R_{16})	20000 ± 2500	5

Table 16. The secondary immune response to BSA in mice immunized with pure BSA and a BSA complex with quaternated PVP loaded with lateral hydrophobic groups [PVP(R_2, R_{16})]

immunization			AFC	
1st	2nd	number of animals	IgM	IgG
BSA	—	18	23.5 ± 1	5.5 ± 2
BSA	BSA	11	20.0 ± 4	6.0 ± 2
PVP(R_2, R_{16})-BSA	—	15	3840 ± 470	3990 ± 300
PVP(R_2, R_{16})-BSA	BSA	14	5920 ± 720	7450 ± 680

PEC with lowered immunogenicity

As can be seen from Figure 125, in some cases tight binding of protein antigens to PE, e.g., PVP-

R_n and PSSNa, causes no appreciable changes in their immunogenic activity despite rather a high immunostimulating activity of these polyelectrolyte carriers. Conjugation of BSA with CP(AA-MVP) also fails to increase the immunogenic activity; in this case CP eliminates the cooperative interactions of T- and B-Iymphocytes. Therefore in the next series of our experiments we carried out a detailed analysis of physico-chemical properties and immunological activities of several protein antigens with carbo-chain linear polyampholytes *(*Petrov *et al.,* 1982). These AA and MVP copolymers (CP) contained 66 (CP-1), 50 (CP-2) or 34 (CP-3) mol% links of MVP. Analysis of interactions of two model proteins, BSA and BGG, with these polyampholytes revealed that the tightness of their binding to each other depended critically on the number of MVP bonds, the most stable complexes being formed in the case of CP-1. The decisive role in the binding of likecharged molecules belongs to the hydrogen bonds and the nonpolar interactions of the protein globules with the hydrocarbon fragments of the polyampholyte chains. A typical structure of a CP-protein complex is shown in Figure 127.

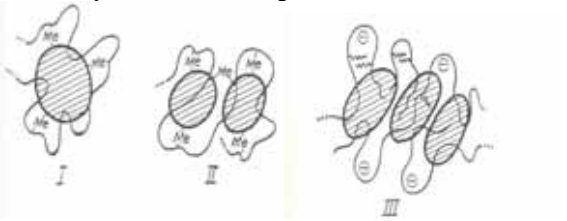


Figure 127. A schematic presentation of the structure of protein complexes with CP (III), PMC-1 (I) and PMC-2 (II). For explanations sec text.

The number of protein-specific AFC was determined after immunization of mice with the soluble polycomplexes, BSA-CP and BGG-CP. it appeared that the BSA complexes with these polyampholytes (Cp-1, CP-2 and CP-3) were practically devoid of immunogenic activity. In mice immunized with this PEC the levels of BSA-specific antibodies did not practically differ from "background" values.

Figure 128 shows the AFC titers in the spleens of mice immunized with pure BGG and its complexes with CP-1, CP-2 and CP-3. Within the composition of PEC BGG, it being a strong natural antigen, displays a higher immunogenic activity than in the free state. At the same time, in animals immunized with BGG-PEC mixtures having the same protein content the number of splenic AFC appeared to be different. For example, in the spleen cells of mice immunized with BGG + CP-3 the number of BGG-specific AFC was the same as in mice immunized with an equal dose of the pure protein antigen.

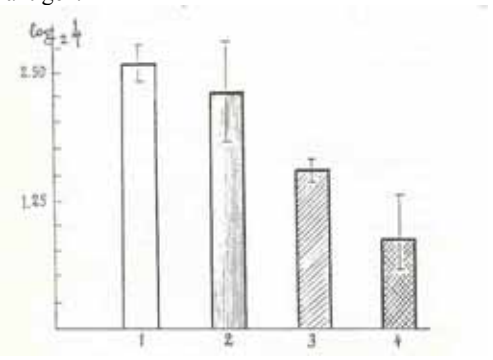


Figure 128. The immune response to BGG in the spleen cells of mice immunized with pure BGG (1), BGG + CP-3 (2). BGG + CP-2 (3) and BGG + CP-1 (4). Ordinate titers of BGG-specific AFC

Thus, MVP and AA copolymers, which form stable complexes with BGG suppress, whereas PE stimulate the immune response to the protein component of PEC. Marked inhibition of BGG-specific AFC production was seen when this polyampholyte was injected to mice together with *Clostridium perfringens* α-anatoxin [228]. It might be expected from these data that the observed differences in the immunogenic activity of proteins conjugated with polycations and polyampholytes is due to their different effects on the immunogenesis of polymeric carriers within the composition of PEC. In contrast with polycations possessing a high immunostimulating activity, copolymers of MVP and AA inhibit the cooperative interactions of T- and B-lymphocytes during the induction of immune responses to SRBC.

In this context the physico-chemical mechanism of this phenomenon can be interpreted in terms of the ability of polycations and polyampholytes to form complexes with structurally different proteins, which, in contrast with polyampholytes having a linear unfolded structure and a considerable chain length in aqueous solutions, possess rather a compact structure due to intramolecular hydrophobic interactions between their MVP bonds. This hypothesis has been open to criticism because the polyampholytes in question are heterogeneous both in respect of MMD and their chemical composition. Therefore, in our recent studies we used PMC of narrow fractions of a homopolymer (PVI), which differed in their molecular structure in aqueous media, i.e., PMC having a compact (PMC-1) and a crosslinked (more folded) conformation (PMC-2). As above, PMC-2 can form complexes both with BSA and BGG via transient metal ions, which play the role of crosslinking agents between the corresponding functional groups of the complex components. Noteworthy that PMC-1 exhibits an immunosuppressor, whereas PMC-2 - an immunostimulating activity (Mustafaev *et al.,* 1990). In neutral aqueous media these conjugates form complexes both with BSA and BGG at the expense of transient metal ions, which play the role of crosslinking agents between the appropriate functional groups of the complex components. In terms of their composition PMC-2 have an equimolar, where as PMC-2 - a nonstoichiometric structure (Figure 127, structures I and II) that reflects the differences in the structure of the original PMC. The ternary polycomplexes, BSA + PMC-1, BSA + PMC-2, BGG + PMC-1 and BGG + PMC-2 were used for immunization of mice, after which the levels of BSA- and BGG-specific AFC were determined in mouse spleens. The results of these experiments are depicted in Table 17. It can be seen from these data that the immunogenic activity of BSA + PMC-1 does not differ from background values, whereas that of BGG + PMC- is markedly decreased. The use of PMC-2 as carrier promoted a strong proteinspecific immune response in both systems.

It may thus be concluded that PMC-1 possessing a high immunosuppressor activity, rather a compact structure and the ability to form stable stoichiometric complexes with proteins suppresses the immune response, whereas PMC-2 which exhibits immunostimulating properties, has an unfolded (crosslinked) structure and the ability to form stable nonstoichiometric complexes strongly stimulates it.

It is very probable that PEC carrying a great number of protein molecules and having a larger molecular size in comparison with the original proteins circulate in the blood for longer periods of time and thus display a higher immunogenic activity. At the same time, PEC whose loose polymeric envelope surrounding the protein globule protects it from the deleterious effects of external factors, are characterized by a lowered immunogenicity.

These findings gave a strong impetus to our systemic studies aimed at the elucidation of relationships between the immunogenic activity of PEC and their chemical composition.

Table 17. The levels of protein-specific Afc in the spleen cells of mice immunized with pure BSA and BGG or their complexes with PVI (data from R. I. Gadzhiev), n.d. – not determined.

immunogen	PMC dose, mg	protein dose, mg	number of animals	AFC
BSA	—	0.5	16	35
PVI-Cu^{2+}-BSA (PMC-1) + BSA	0.01	0.5	18	n.d.
BGG	—	0.5	17	4400 ± 430
PVI-Cu^{2+}-BGG (PMC-2 + BGG)	0.01	0.5	16	340 ± 30
PVI-Cu^{2+}-BSA (PMC-2 + BSA)	1.0	0.4	18	5000 ± 740
PVI-Cu^{2+}-BGG (PMC-2 + BGG)	1.0	0.5	18	28000 ± 6500

5.3. The composition of PEC

We prepared three solutions of BSA + PAA at equal (w/w) concentrations of BSA and PAA: I- BSA + $(PAA)_1$, II - BSA + $(PAA)_2$ and III - BSA + $(PAA)_3$ having molecular masses (M_w) of 3, 40 and 80 kDa, respectively. All the solutions were prepared under conditions of the polyacid activation by carbodiimide by using the previously described procedure (Mustafaev *et al.,* 1986). Our experiments showed that in mice immunized with these mixtures AFC could be detected in the blood sera already on the 4th postimmunization day, the peak of the immune response being observed on the 10th day with a further decline on the 14th day. The titers of BSA-specific antibodies showed a great scatter in values depending on the solution used. The highest AFC titers were seen in mice immunized with complexes I and III, whereas in mice injected with solution II the titers of BSA-specific antibodies were minimal. It is noteworthy that the complexes used in this study differed from one another only by the molecular mass of the polyacid. Therefore the relative values of AFC and the titers of BSA-specific antibodies in the spleen cells of mice immunized with BSA-PAA mixtures (Figures 130A-130B) are given relative to M_r of PAA. These data indicate that the AFC plots linearly depend on M_r of PAA and are characterized by a minimum.

The dependence of antibody production on M_r led us to suppose that the observed effect is due to the structural peculiarities of the BSA-PAA-carbodiimide complex molecules. A detailed physicochemical analysis of the experimental mixtures revealed that in all the cases the carboxyl groups of the newly formed conjugates were covalently bound to the amino groups of the protein via amide bonds (Mustafaev, 1986, 1989).

However, the mechanisms of the conjugate molecule formation in the above mixtures differ essentially from one another depending on the molecular mass of PAA. According to the ratio of the linear sizes of the polyacid and protein globules, such binding results in the formation of conjugates whose composition and, correspondingly, chemical structure also show substantial differences. Figure 130B shows the composition of conjugates obtained at different values of M_w for PAA. At $M_{w(PAA)} \geq 40$ kDa the number of bound BSA globules increases linearly with the increase in the length of the PAA chain. In the case of $(PAA)_2$ the conjugate has a stoichiometric composition (1:1) (structure II), where as that of $(PAA)_3$ is nonstoichiometric, for one macromolecule of PAA appears to be bound to three molecules of BSA (structure I). In the case of the PAA complex with a relatively low molecular weight polymer the situation is quite different. At M_w = 3 kDa the mode of binding changes: the protein globules now play the role of the carrier. This may lead to the formation of two structural types: i) conjugates formed by interacting BSA globules that are crosslinked by multiple short chains of the linear polyacid

(structure IIIb) and, ii) conjugates in which each protein globule is bound to several PAA molecules. Such conjugates are not crosslinked and form a "hedgehog"-like branched structure (IIIa).

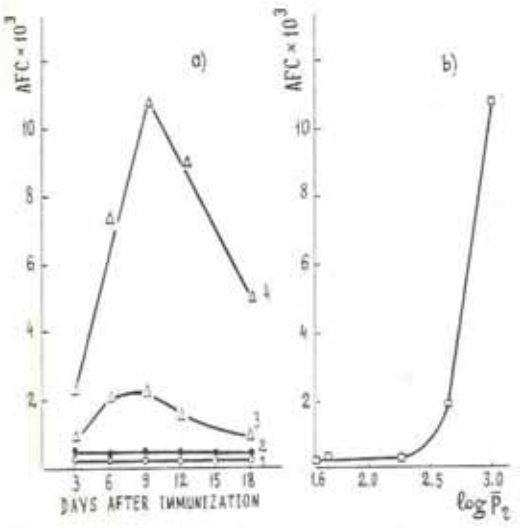


Figure 129. The dynamics of AFC production in the spleen cells of mice immunized with covalent conjugates (BSA-PAA) at different lengths of the PAA chain (n). 1- Pure BSA; 2- BSA-PAA (n=43); 3- BSA-PAA (n=550); 4- BSA-PAA (n=1140). At n=43 the structure of BSA-PAA was the same as that of IIIa (Figure 129). (b) The dependence of the AFC number on the degree of polymerization (β) of PAA within the composition of the PAA-BSA conjugate

A comparative study of BSA-PAA mixtures and analysis of their immunogenic activity revealed that the conjugates "enriched" with protein molecules exhibit the highest immunogenic activity (Figure 130A). The high epitope density of the protein antigens within the composition of the polymeric carrier confers on such PEC a high immunogenic activity. As already mentioned, the conjugates having an equimolar composition are characterized by the lowest immunogenicity. However, in these studies we succeeded in obtaining conjugates of different composition by changing the molecular mass of PE. It follows, therefore, that the above conclusion postulating a relationship between the composition of CC and their immunogenic activity may be rather premature.

Therefore in the next series of experiments we performed a chemical synthesis of PEC in which one macromolecule of the polymeric adjuvant irrespective of its molecular mass was attached to an equimolar amount of the protein antigen, after which the immunogenic activity of the complexes was estimated (Mustafaev and Norimov, 1988). The structure of such synthetic PEC is shown in Figure 131.

In our work we used polycations carrying lateral cetyl radicals. Each macromolecule irrespective of its molecular mass was bound to no more than two protein molecules. In all the eases the binding was achieved through the formation of a multipoint system of electrostatic salt contacts stabilize d by the embedment of hydrophobic cetyl radicals into the hydrophobic regions of the protein globules, thus promoting the stability of the complexes at physiological values of the ionic strength.

The water-soluble complexes thus prepared were used for immunization of intact mice, after which the levels of protein-specific AFC were determined in their spleens. As can be seen from Table 18, in all the eases studied the strength of the immune response increased significantly

in comparison with the control group. The relative values of AFC did not practically depend on the molecular mass of the polycationic carrier, being approximately at the same level. These findings led us to conclude that the immunogenicity of a polycomplex is correlated with the number of bound antigenic molecules, i.e., with their epitope density, and (at least within the studied range of polymerization degrees) it does not depend on the length of the polymeric chain.

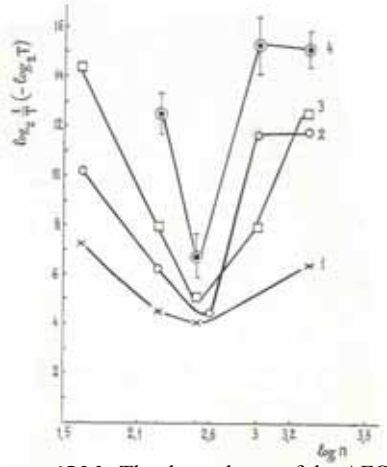


Figure 130A. The dependence of the AFC titers ($-\log_2 T$) in the blood sera of mice immunized with PAA-BSA conjugates on the length of the polymeric chain (n). 1-primary immune response (13th day); 2, 3, 4-secondary immune response: 7th day (2), 11th day (3) and 10th day (4). At n = 43 the structure of CC was the same as that of IIIb

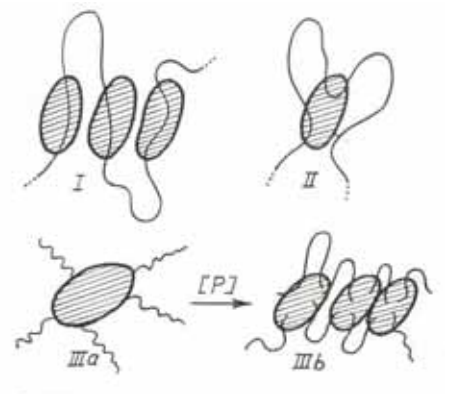


Figure 130B. A schematic presentation of the structure of covalent conjugates (PAA-BSA) at different lengths of the polymeric chain (degree of polymerization), n: 1140 (I), 550 (II) and 43 (III). At n=43 two types of structures were formed depending on the BSA/PAA ratio. For explanations see text

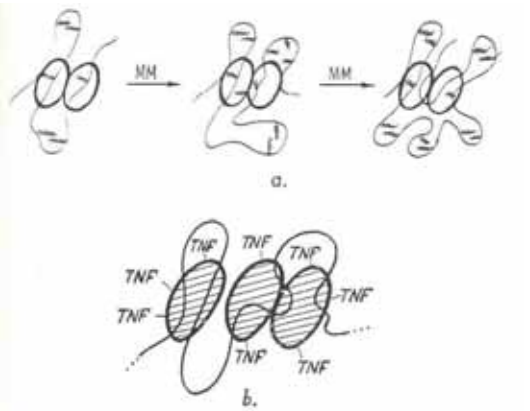


Figure 131. The dependence of the structure of water-soluble complexes of BSA with PVP(R_2,R_{16}) on the degree of PE polymerization (n): 103 (a), 2.15 x10^3 (b) and 7.4 x 10^3 (c). Degree of quaternization by: cetyl bromide-7-9 mol%, ethyl bromide- 90 mol%. (b) A schematic presentation of ternary water-soluble complexes, PAA-(BSA-TNP)

Table 18. The levels of protein-specific AFC in the spleen cells of mice immunized with pure BSA and BSA complexes with quaternated PVP loaded with lateral hydrophobic groups at different degrees of polymerization (P) of PE

immunogen	P, 10^3	AFC	number of animals
BSA	—	25 ± 5.0	18
PVP(R_2, R_{16})-BSA	1.0	2800 ± 250	17
	2.15	2400 ± 200	16
	4.5	1900 ± 180	18
	7.5	2000 ± 222	16
	12.0	2100 ± 200	18

The dependence of the composition of PEC on their immunogenic activity becomes especially apparent in the cage of microbial antigens, e.g., B-polysaccharide (PS) that represents a purified meningococcal antigen. The AFC values and antibody titers relative to the PS/PE ratio (w/w) are given in Table 19. It can be seen from these data that the highest titers of protein-specific antibodies (including AFC) are characteristic of PS-enriched complexes.

Table 19. The immunogenic activity of the pure meningococcal B-prolysaccharide and the antigen-polymer complex at different w/w ratios of the antigen-polymer mixture. PS – polysaccharide. PE concentration was varied at a constant concentration of PS

PS/PVP(R_2), w/w	AFC	antibody titers (log_2)
Control (PS)	30 ± 5	0.3 ± 0.05
2	80 ± 7	0.6 ± 0.1
5	150 ± 11	1.4 ± 0.12
7	170 ± 15	1.2 ± 0.1

Thus, the composition of artificial thymus-independent antigens is another factor, which determines, to a large extent, their immunological activity.

One necessary prerequisite to the induction of high immune responses is the binding of more than one molecule of the protein antigen to one macromolecule of the polymeric carrier. However, the role of bound protein molecules and their number in the immunogenic activity of protein-polymer conjugates will finally be clarified only after the chemical synthesis of conjugates whose polymeric chains carry different numbers of protein molecules at an equal degree of polymerization (macromolecular size) and compactization of their coils. Studies in the field are currently under way. It is conceivable, therefore, that the strength of the immune response to haptens included in the composition of PEC depends on their epitope density.

The first artificial antigen, MVP-TNP, by reason of its low solubility in aqueous media cannot be regarded as a convenient model in physico-chemical studies. The impossibility to control the composition and, correspondingly, the epitope density of the hapten determinants of the polymeric carrier is a considerable obstacle to the wide-scale application of such complexes in immunological studies. In complete contrast, polymer-protein complexes are highly soluble in neutral aqueous media; the composition and size of their constituent molecules are readily controllable. The protein fragment of PEC retains both its, native conformation and accessibility to other molecules. Therefore in further studies we prepared water-soluble PEC with a different content of TNP and tested these complexes for immunological activity towards the immobilized hapten (Mustafaev *et al.,* 1989). TNP was at first bound to BSA in order to achieve the incorporation of the TNP-groups into PEC, after which the TNP-BSA conjugates were mixed with PAA in neutral aqueous solutions. Under these conditions both PAA and pure BSA acquired

the like negative charge, which made impossible their further involvement in the complex formation (Mustafaev, 1981). The situation was quite different when aqueous solutions of TNP-BSA were mixed with PAA. BSA, which contains only five TNP-groups, was also unable to interact with the polyacid to form stable PEC. However, a further increase in the number of TNP groups led to the formation of stable ternary water-soluble complexes on the BSA molecule, PAA(TNP_{17}-BSA) and PAA(TNP_{26}-BSA).

The structure of these complexes carrying hapten groups is schematically presented in Figure 131; immunological data are shown in Figure 132. After a single immunization of mice with TNP-BSA conjugates the latter were unable to stimulate the production of TNP-specific antibodies: the immune response to them was manifested only in the presence of CFA, whereas the relative values of AFC were increased 3.5-5-fold.

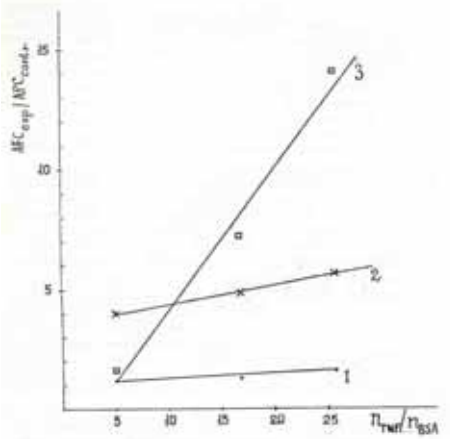


Figure 132. The dependence of the relative values of TNP-specific AFC (AFC_{exp}/AFC_{contr}) (ordinate) after immunization of mice with TNP-BSA (1), (TNP-BSA). CFA (2) and (TNP-BSA)-PAA (3) on the number of TNP groups in the conjugate mixtures (abscissa). AFC_{exp} and AFC_{contr}-AFC levels in the experimental and control groups, respectively.

It should be noted that in this cage the strength of the immune response did not practically depend on the number of TNP molecules bound to the protein carrier. However, in mice immunized with PAA- (TNP_x-BSA a strong immune response to TNP was elicited: its magnitude varied considerably depending on the number of hapten groups in the PEC molecule. The relative values of AFC increased linearly in parallel with the increase in the number of TNP groups. At x = 5, as above, neither PAA nor TNP-BSA, being immunologically inert, were able to form such complexes. In complete contrast, in their mixtures an augmented synthesis of protein-specific antibodies concomitant with the formation of stable complexes took place.

It follows from these data that immobilization of the hapten molecules on stable water-soluble PEC markedly increases (10-15 fold) the immune response to the given hapten. In contrast with water-in-oil mixtures (e.g., CFA), in PAA-(TNP-BSA) one macromolecule of PAA binds several molecules of the TNP-BSA conjugate, as a result of which the epitope density of the regularly organize d TNP groups within the complex molecule linearly increases which, in turn, causes a linear increase of the AFC titers relative to the number of the TNP groups included into PEC.

5.4.Immunogenicity of PEC in the presence of competitive PE

Within living organisms polycomplexes of protein antigens can interact with blood proteins, cell

surface as well as with other strongly charged macromolecules, such as nucleic acids and acid polysaccharides that are present in large amounts in the animal blood. It seemed, therefore, important to investigate the immunogenic activity of polymer-antigen complexes in the presence of competitive PE.

To this end we selected two types of artificial antigens [168,174,175], one of which represented an electrostatic complex (EC) of BSA with poly-4-vinyl-N-ethylpyridinium bromide, whereas the other one was a covalent conjugate (CC) of BSA with carboxymethylated PVP. These studies revealed that EC and CC differently interacted with heparin, a polyanion characterized by a high charge density *in vitro* (Mustafaev and Kabanov, 1980). An addition of heparin to EC led to the complete displacement of BSA, which remained in solution, whereas the stoichiometric heparin-PE complex formed an insoluble pellet (Figure 133).

The situation was quite different when heparin was added to CC. In this case the polyanion did not displace BSA from the complex, because the covalent bonds formed between the protein and PE prevented the macromolecular substitution but facilitated the formation of a soluble complex between heparin and CC.

Immunological studies were conducted on mice injected with definite doses of heparin immediately after their immunization with EC or CC or 30 min, 2 hours and 24 hours thereafter. The AFC titers were determined at the peak of the immune response, i.e., on the 9th post-injection day.

The relative values of AFC in the spleen cells of mice measured at different periods after heparin injection are shown in Table 19, it can be see n from the se data that heparin injected immediately after EC or 30 min after immunization of animals fully prevented the immune response to the artificial antigen. In this way heparin "encompassed" EC within the organism and caused its destruction by binding to the polycation and thus displacing the protein before the immune response could be triggered on. Being injected 2 hours after immunization, heparin was still able to elicit the immune response, although in this case its amplitude was lower than in the control group (in the absence of heparin). Heparin injected 24 hours after EC had no effect: the magnitude of the immune response was the same as in the control group.

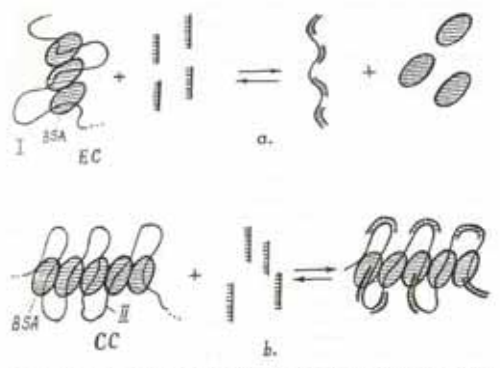


Figure 133. A schematic presentation of EC (a) and CC (b) interactions with heparin (c). For explanatations see text

In animals immunized with CC heparin did not practically affect the strength of the immune response irrespective of the time of its administration. It is noteworthy that in this case heparin was unable to prevent the CC interaction with the membranes of appropriate immunocompetent cells even if it could "encompass" the conjugate within the organism.

These data are consistent with the behavior of EC and CC in the presence of heparin under *in vitro* conditions. The results of these studies present substantial interest from two

viewpoints. First, they provide a perfect model of exchange reactions occurring in living organisms. Second, they make it possible to calculate the minimal time needed for the immune response to an artificial T-independent antigen to be triggered on. Both considerations are very important for the practical solution of many fundamental problems of modern day immunology.

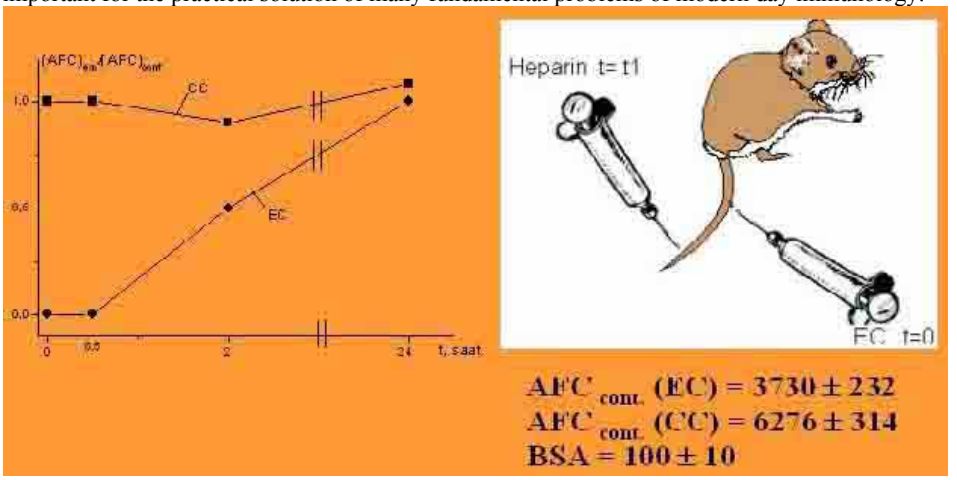


Figure 134. The relative values of AFC ($AFC_{exp.}/AFC_{contr.}$) in the spleen cells of mice immunized with EC or CC at different times after heparin injection. -$AFC_{contr.}$ After immunization of mice with EC and CC were 3730±232 and 6276±314, respectively.

The loop-like structure and "adhesiveness" of PEC. Needless to say that the further fate of PEC, CC or free PE entering the organism depends, primarily, on the mode of their interaction with plasma proteins, cells and other charged constituents of the blood. Therefore model studies of antigen interactions with proteins and cells may provide valuable information for all those who are engaged in the study of the structure and immunological activity of such antigens. These studies are all the more important, because they allow one to discover previously unknown factors, which play a crucial role in the manifestation of the high immunogenic activity of artificial immunogens.

It is known that macromolecules of polymer-protein complexes contain flexible free sites of PE (Ioop-like structures, free ends) that are not directly involved in the interaction with the protein globules. These free sites form an extensive hydrophobic area along the surface of the PEC molecule, which, in turn, promotes their high solubility. In solutions containing other charged macromolecules the physicochemical properties of PEC (interactions with proteins, surface adsorption, etc.) depend on their chemical nature as well as on the structure of their free polymeric fragments.

Data from physico-chemical analyses of PEC interactions with other proteins suggest that according to the mode of their binding PEC can be divided into two groups. The first group includes PEC, in which the free sites of the polymeric carrier can effectively bind protein molecules in model systems in the presence of blood serum proteins. In this case the binding is provided for by cooperative electrostatic or hydrophobic nonpolar interactions; their combination promotes a high level of PEC binding to the protein. As a result of PEC interactions with polycations the net positive charge of the PEC molecules decreases; a further rise in the protein concentration leads to their aggregation (Figure 135). Noteworthy that such PEC displays a high immunogenic activity. This group of PEC also comprises complexes (or conjugates) with negatively charged polymeric carriers, including protein conjugates with a polyacid (e.g., PAA).

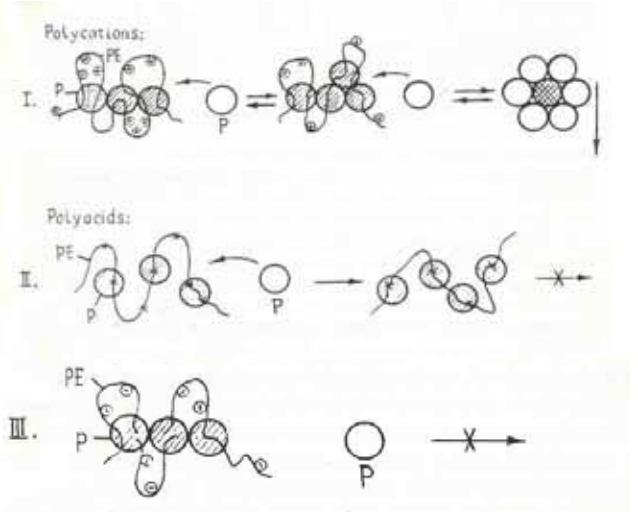


Figure 135. A schematic presentation of the interaction between polymer-protein complexes (conjugates) having a characteristic composition with free addional protein molecules. For explanations see text.

Such PEC is also endowed with the ability to bind additional protein molecules via multipoint electrostatic interactions between their carboxyl groups situated in the loops of the polycomplex. However, in contrast with PE complexes with polycations, under these conditions the rise in the protein concentration is unaccompanied by the aggregation of the conjugate molecules or the formation of an insoluble pellet. The excess protein remains in solution in an unbound state (Figure 135, structure II). The immunogenic activity of such complexes is also rather high.

The second group comprises PE, in which the free sites of the polymeric carrier are devoid of re active (complex-forming) functional groups and are thus unable to bin d additional protein molecules. Protein complexes with linear polyampholytes provide an illustrative example of such PEC. The critical role in the complex formation belongs to hydrogen bonds and nonpolar interactions between the methylpyridinium links and the negatively charged protein globules bearing the like negative charge. This leads to the formation of strands between the interacting protein globules that are linked together by linear polyampholyte chains. The negatively charged functional groups situated in the free sites of such polyampholytes are not directly involved in the protein binding, as a result of which the complex particles acquire a surplus negative charge. For this very reason such PEC are unable to form complexes with extrinsic proteins. The immunological data suggest also that these PEC differ from those described in the previous chapters in that they display a much lower immunogenic activity in comparison with the original highly immunogenic protein antigen.

Hence a question arises as to how the "closure" of the loops in the structure of PEC induced by protein antigens affects the immunogenicity of these complexes. An answer to this question came from the analysis of PEC constructed on the basis of polycations and a model protein (fraction F_1 of the plague microbe [229]. In contrast with serumal proteins, protein F_1 shows the ability in .a strong intermolecular association in aqueous solutions. For this very reason the mechanism of its, binding to polycations differs essentially from those of BSA, BGG" etc. The initial step of the complex formation in the F_1-PE mixture is the uneven distribution of protein molecules between the adsorbing polycations. Such a system is made up of two fractions: a free polycation and the Ft-PE complex. A similar mechanism of the complex formation was

observed during the interaction of PE with the, protein antigen of the plague microbe having rather a high molecular mass (ca. 1.2 x 10^6*)* (Figure 137, scheme III). Quite a different situation was seen after a further increase in the M_r of the antigen. By reason of the strong ability of F_1 to associate after its addition to the mixture at a concentration, which exceeded a certain critical level, i.e., the protein/PE ratio, the complex formation was accompanied by the binding of the protein molecules to the already formed molecules of PEC (but not to free PE). Such a mechanism promoted the binding of additional F_1 molecules in the free loops of PE within PEC as well as the formation of molecular associate s of limited solubility. The structure of such PEC is characterized by the lack of free polycationic sites (Figure 135). Immunological studies revealed that the highest immune responses in delayed type hypersensitivity (DTH) and phytohemagglutinin (PHA) tests were elicited after immunization of animals with mixtures composed of a free polycation and a PEC having a loop-like structure. With an increase in the F_1 concentration in the solution of the polymeric carrier the free sites (loops) disappeared, and the mixtures containing such PEC became inactive. The stimulation indexes (SI) were in both cases dose to unity.

Immunization of animals with PVP(R_2,R_{I6})-OVA provides an illustrative example of how the free sites of PE influence the immunogenicity of PEC. The results of immunological studies in which mice were immunized with a single dose of water-soluble PEC-OVA complexes prepared at different [OVA]/[PE] molar ratios at a constant concentration of OVA and at varying concentrations of PE are listed in Table 20. It can be seen from these data that the titers of IgE antibodies exceeded the control values in those cases when PEC contained free sites of PE noninvolved in the binding with the protein ($n_{OVA}/n_{PE} \leq 4$) *(*Mustafaev *et al.,* 1990). An increase in the protein concentration in the PE-OVA mixture caused a drastic reduction of the number of IgE AFC down to the control level.

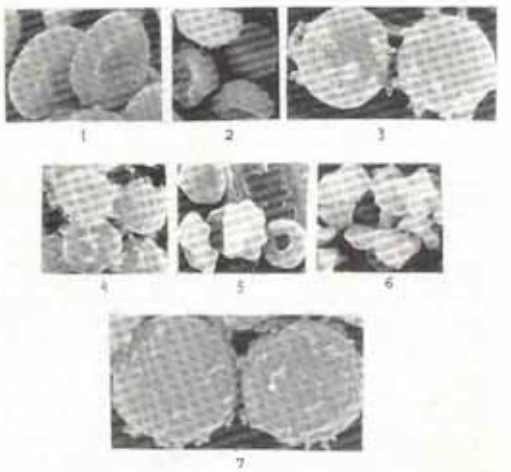


Figure 136. A scanning electron microscopic view of normal SRBC (1, x 6300); SRBC + PAA (2, x 2400); SRBC + PVP(R_2,R_{16}) (3, x 10800); SRBC + BSA- PVP(R_2,R_{16}) (4, x 10800); SRBC + BGG-CP (AA-MVP) (6, x 4200) and SRBC + (PAA-BSA) (7, x 1500). PVP(R_2,R_{16}) (5, x 12800) was added to SRBC + BGG. [230].

The different "adhesiveness" of PEC molecules in the protein globules, which was found to depend on the chemical nature of the polymeric carrier, was also manifested during their interaction with SRBC and spleen cells (SC) of intact mice [230,231]. Figure 136 shows a scanning electron micrograph of normal and washed from contaminant proteins SRBC and their mixtures with PE, PEC and SC. It can be seen from the se data that the shape and size of SRBC

changed considerably after the addition of PE and BSA-PVP(R_2,R_{I6}) which testifies to a strong interaction of different cell fragments with PE. One can visualize large associate s formed by surface proteins of SRBC and PE. After mixing of PE with SRBC in the presence of a specially added protein the mode of PE binding to SRBC did not change: the characteristic alterations in the shape and structure of the cell surface can be seen in the micrographs.

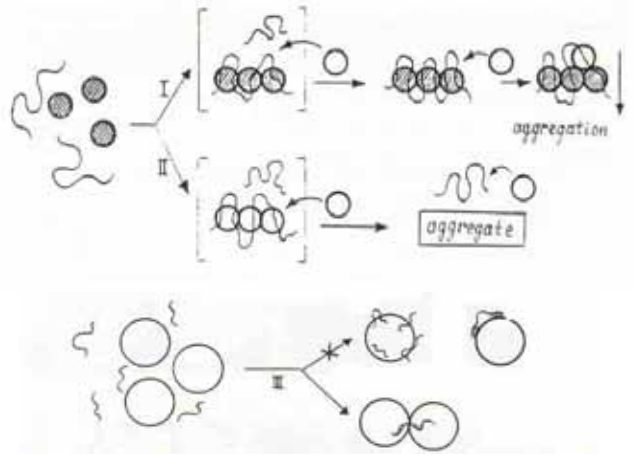


Figure 137. A schematic presentation of the PE binding to blood serum proteins (I) and plague microbe fractions F_1: 120 kDa (II) and 1200 kDa (III)

In contrast with pure PE, the interaction of PE molecules with SRBC was accompanied by the appearance on the cell surface of a large number of vesicles and finger-like projections called microvilli (cones). Incubation of polycations and their protein complexes with SRBC resulted in the clusterization (aggregation) of these molecules as a result of nonspecific interactions of SRBC with the polycation. In contrast with PE, incubation of SRBC with the negatively charged PAA, CP(AA-MVP--BGG and PAA-BSA did not induce any appreciable alterations in SRBC, although there were some changes in the cell shape. The surface of SRBC remained smooth; there were no signs of intercellular clusterization.

Table 20. The titers of OVA-specific IgE antibodies after a single immunization of mice with pure OVA or OVA-PEC at different values of the [OVA]/[PEC] ratio (Popov, unpublished data).
* - The AFC titer for the IgE response to pure OVA was 4.0.

	IgE titers ($\log_2$ 1/T)	
η_{OVA}/η_{PE}	PVP(R_2, R_{16})-OVA* $\beta = 5$ mol%	PVP(R_2, R_{16})-OVA $\beta = 7$ mol%
2.0	6.6	7.8
3.0	5.6	5.5
4.0	4.0	5.1
5.0	3.4	4.0
6.0	4.0	3.5
8.0	3.0	4.8

The binding of PEC to the SC surface was estimated by three independent methods [230]. At first isolated SC (10^7 cells/ml) were incubated with radiolabelled OVA in a serum-free medium 199 in the presence of fluorescent isothioeyanate (OV A-F) or its complexes with

PVP(R_2,R_{I6}) (PEC-1) and CP(AA-MVP) (PEC-2). The percent content of the fluorescent cells and the mean values of the fluorescent intensity were measured with the help of a flow cytofluorimeter. In the second case SC (10^7 cells) were incubated with aqueous solutions of OVA or OVA-PEC for 10 min, after which SC were sedimented by centrifugation. Matrix solutions obtained after incubation with SC were analyzed by spectrophotometric methods. In the third series SC were incubated with ^{125}I-0VA or its complexes with PEC (100 μg/ml of OVA) in the medium 199. The radioactivity of the cell samples after their incubation and subsequent washing was measured on a specially designed counter. The degree of clusterization (aggregation) of SC in the presence of OVA or PVA-PEC was assessed by light microscopy and expressed as the number of cell aggregates (three or more cells) per 100 single and aggregated SC.

The results of these experiments are listed in Table 21. It can be seen from these data that in the case of OVA-PE the concentration of the fluorescent cells reached the maximum (> 80%) already during the first 5-12 minutes of incubation of the PEC-SC mixtures and remained at this level throughout the observation period. At the same time, the percent adsorption of the pure protein and its complex with the polyampholyte (CP) was markedly decreased, being about 10-15%. Similar results were obtained during the analysis of mixtures by radioisotopic and spectrophotometric techniques (Table 22).

Table 21. The dependence of the per cent content of fluorescent spleen cells on the time of their incubation with OVA-PE complexes

mixture	incubation time, min 5	15	60	120
SC + [PVP(R_2, R_{16})-OVA]	68	85	90	70
SC + [CP(AA-MVP)-OVA]	5	10	22	20
SC + OVA	4	5	7	8

Thus, the adsorptive capacity of OVA-PEC on the SC surface markedly exceeds that of OVA complexes with the linear polyampholyte. Stipulating that the presence on the cell surface of OVA-specific antibodies or receptors can be neglected, it may be assumed that the observed difference in the PEC binding is due to their physico-chemical peculiarities. The high adsorptive capacity of PEC-1 can be explained by the presence in its composition of positively charged free sites (loop-like structures, free ends) of PE containing fragments of hydrophobic radicals able to interact in a nonpolar fashion with cell membranes. The complex formation between OVA and polyampholytes is due to the nonpolar interactions (very probably, "forced" ion-to-ion contacts) of OVA molecules with the hydrocarbon fragments (methylvinylpyridinium links, MVP bonds) of the CP chain, eventually resulting in partial "fractionation" of the functional groups depending on the composition of PE.

Table 22. The adsorptive capacity of pure OVA and OVA-PEC measured by different methods. *- OVA concentration, 100μg/ml. C_{PEC} – concentration of adsorbed OVA within the composition of PEC; C_{OVA} – concentration of adsorbed OVA

immunogen	spectrophotometry (% of maximum)	radiolabeling (C_{PEC}/C_{OVA})
OVA*	55	—
PVP(R_2, R_{16})-OVA	100	27.92
CP(AA-MVP)-OVA	20.33	1.08

Analysis of SC + PEC mixtures by light microscopy revealed that PEC-1 added to SC suspensions markedly increased the intercellular aggregation which at 0.1 μg/ml OVA was 2%, reaching 40% at 100 μg/ml OVA. Pre-incubation of SC with 10% embryonic calf serum decreased the SC aggregation down to 10%. No cell aggregation was observed in the presence of OVA or its complexes with polyampholytes. Stipulating that protein molecules can be aggregated by short chain polycations whose length is smaller than the circumference of the protein globules (Kabanov and Mustafaev, 1977, 1981), it may be supposed that in our case, when the length of the PEC chains was smaller than the cell size, we dealt with a very similar phenomenon. Cell clusterization occurred as a result of SC interactions with PEC-1 and the dusters formed thereby were stabilize d by relatively short chain macromolecules of PEC whose chain length was much smaller than the cell circumference but sufficient to induce their adherence on the surface of two adjacent cells via the multipoint cooperative interactions between them. After incubation of SC with PEC in the presence of embryonic calf serum PEC-1 added to the mixture appeared to be bound to serum proteins as a result of which the number of free sites of the polycation diminished, eventually resulting in the decreased ability of the complex to interact with SC and to induce their aggregation. In each duster the cells seemed to interact with one another by binding to definite sites on the PEC molecule, such as hydrophobic cetyl radicals of the free sites of PE bound to the membrane lipid bilayer, or fragments containing no cetyl groups but capable of electrostatic interactions with the negatively charged groups situated on the cell membrane surface. As a result of such interactions the negative charge of the SC surface became "neutralized" and it no longer impeded the cell-to-cell contacts. In the case of partial adsorption of PEG-2 the situation was quite different. Partial binding of negatively charged molecules of PEC-2 to cell membranes occurring against electrostatic repulsion might increase the effective negative charge of the cell surface at the expense of polyions "adhering" to it. Quite naturally, this process was unaccompanied by SC aggregation.

One may expect from these data that in living organisms PEC-1 and PEC-2 will behave in a similar way. As a matter of consequence, the ability of artificial polymer-subunit antigens in multipoint cooperative interactions with chemically complementary molecules (blood proteins, intrinsic membrane proteins of immunocompetent cells, etc.) plays a role in their immunogenic activity. This circumstance obviates the need for the development of highly immunogenic artificial antigens.

5.5. Immunogenicity of multicomponent (chymeric) PEC

Investigators engaged in the construction of multicomponent artificial vaccinating materials are usually faced with the problem of developing complex antigenic systems for vaccination against several infections at a time. Needless to say, the question "Are PEC mixtures more effective than multicomponent complexes?" comes to the foreground.

In our attempts to perform PEC synthesis we used BSA and BGG as model antigens and a copolymer (CP) of 4-vinyl-Nethylpyridinium bromide with 4-vinyl-N-cetylpyridinium bromide [PVP(R_2,R_{I6})] as polycationic carrier.

Individual water-soluble complexes of BSA and BGG with CP were obtained as described previously (Kabanov *et al.,* 1977; Mustafaev, 1981). A hypothetical scheme of synthetic PEC is shown in Figure 138. In aqueous solutions each polycation of PC binds two BSA and one BGG molecule. Some hydrophobic radicals of the polycation appear to be bound to the nonpolar regions of the protein, while others interact with one another, thus stabilizing the overall structure. The positively charged fragments of CP form salt bridges with the negatively charged functional groups situated on the surface of the protein globules. Their hydrophobic interactions promote the stability of PEC under physiological conditions. These finding leaves hope that such PEC will not dissociate under conditions of the whole organism.

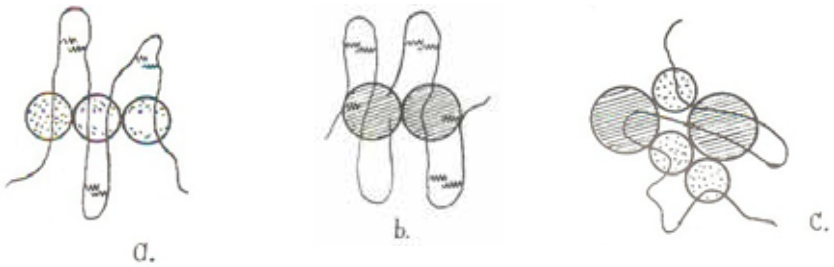


Figure 138. A hypothetical structural scheme of the individual complexes PEC-1 (a) and PEC-2 (b), and of a chymeric (PEC-1 – PEC-2) complex (c).

These complexes were further used in immunological studies. To this end we prepared a mechanical mixture of two PEC, (BSA-CP) + (BGG-CP) at a 1:1 ratio. Specially designed experiments revealed that the complexes retained their stability upon mixing which was unaccompanied by their dissociation or by any other structural changes.

The two-component (chymeric) CP-BSA and CP-BGG complexes were obtained as described previously *(*Kabanov *et al.,* 1980). In these complexes one macromolecule of PE was bound to three molecules of BSA and to one molecule of BGG.

To obtain chymeric PEC, equal volumes of aqueous solutions of CP and BSA + BGG were mixed at room temperature (pH 7.5) at the 0.07 g/dl CP + (0.1 g/dl BSA + 0.1 g/dl BGG) ratio. Under these conditions the reaction equilibrium was fully shifted to the left (Figure 140).

In experiments designed to investigate the immunogenic activity of PEC and their mixtures, solutions of pure antigens, individual PEC-1 (CP-BSA), PEC-2 (CP-BGG), their mechanical mixtures and chymeric PEC were intraperitoneally (i/p) injected to mice at a constant (0.5 mg/animal) concentration of the proteins. The AFC levels in the spleen cells of mice immunized with the individual complexes, PEC-1 and PEC-2 and their mixture are shown in Figure 138. It can be seen from these data that immunization of mice with PEC-1 and PEC-2 elicited strong immune responses to both proteins. Interestingly that the immunogenic activity of PE exceeded (almost 10-fold) that observed after immunization of mice with the pure antigens. Immunization of animals with the mechanical PEC-1 + PEC-2 mixture was unaccompanied by any significant changes in the immunogenic activity of these complexes: the AFC levels were high enough, being commensurate in strength with the immune responses to the individual PEC [178].

These results testify to the fact that the immunogenicity of each individual PEC in the composition of mechanical mixtures does not change, i.e., PEC as artificial immunogens function independently, without any antigenic competition.

The situation was quite different when mice were immunized with the two-component chymeric CP + (BSA + BGG) complex. It can be seen from the Figure 139 that in this case the immunogenic activity of BSA and BGG was markedly decreased in comparison with animals immunized with the mechanical mixture of individual PEC. Similar results were obtained during the analysis of AFC titers in the blood sera of mice immunized with the chymeric complexes or individual PEC.

These data suggest that the immunogenicity of PE formed by various protein antigens is higher in mechanical mixtures of individual PEC than in uniform multicomponent complexes. The physicochemical mechanism of this effect consists, apparently, in the ability of free sites of PE within the PEC structure in the cooperative adsorption on the surface of charged cellular membranes, which, in turn, facilitates the antigen interaction with B-lymphocytes. The efficiency of these interactions may increase due to the high epitope density of the antigen within the PE molecule and the more regular distribution of the antigenic determinants, which creates sterically

favourable conditions for the multipoint interactions of the antigen with cell receptors.

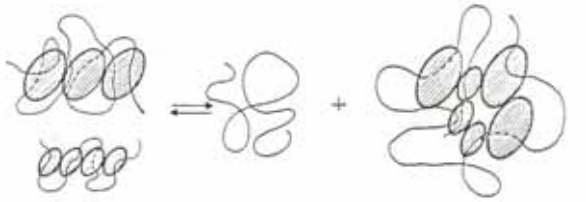


Figure.139. The redistribution of BSA and BGG globules between the polycations [PVP(R_2,R_{16})]

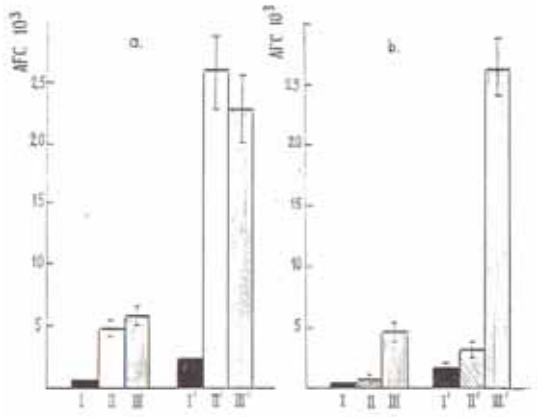


Figure 140. AFC accumulation in the spleen cells of mice immunized with BSA (I), BGG (I'), PEC-1 (II), PEC-2 (II') and PEC-1 + PEC-2 (III, III'). (b) AFC accumulation in the spleen cells of mice immunized with (BSA + BGG) (I, I'), PEC-1 – PEC-2 (II, II') and PEC-1 + PEC-2 (III, III').

These results are of paramount importance for they provide a basis for the construction of artificial multicomponent polymer-subunit materials allowing a simultaneous vaccination against several infections and shed lighter on their mechanisms of action.

The working model of immunogenic PEC

In our studies we set ourselves a task to construct PEC possessing an immunogenic activity. A model of such immunologically active (or passive) PEC is shown in Figure 141, structure I. The physicochemical criteria that were taken into consideration during the construction of such PEC are as follows.

1. The antigen and the polymeric molecule are linked together by tight bonds that are resistant to physiological conditions and exogenous influences (e.g., multipoint electrostatic and hydrophobic interactions, ion-coordinate and covalent bonds, etc.).

2. PEC have a nonstoichiometric composition. The high epitope density of their antigenic determinants is due to the binding of several antigenic molecules to one macromolecule of PE.

3. PEC have a loop-like organization due to the presence in their composition of flexible free polymeric sites. Excessive ionized groups of PE, both adhering to the protein globule surface and included into the loops, form an extensive hydrophilic area on the PEC surface, which promotes the solubility of the PEC particles.

4. PEC have a rigid rod-like carcass structure which is made up of antigenic molecules stabilized by the polymeric carrier Such a structure promotes the regular distribution of antigenic determinants and allows one to increase the interacting surface.

5. PEC are capable of multipoint cooperative interactions with individual components of

biological systems, such as proteins, immunocompetent cells, etc. This ability is conferred on PEC by free, noninteracting with the antigenic molecules functional groups of PE situated in the loops. The reactivity of PEC within the composition of complex multicomponent system s depends on the surface charge, length and hydrophobic-hydrophilic balance of the se sites. One characteristic feature of highly immunogenic PEC is their ability to form complexes with different components of biosystems.

6. The regular distribution of antigenic determinants creates sterically favourable conditions for multipoint interactions of the antigen with cell receptors. Therefore mixtures of individual PEC are far more effective tools in the construction of polyvalent vaccines than multicomponent (chymeric) complexes.

The structural model of low immunogenic PEC differs from that described above (Figure 141, structure II) and meets the following physico-chemical criteria: tight binding of the antigen to the polymeric carrier, stoichiometric composition of PEC, screening of antigenic molecules from the deleterious effects of environmental factors by the "nonreactive" polymeric envelope surrounding the protein globule, etc.

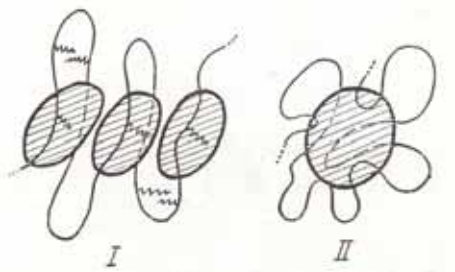


Figure 141. The working model of highly (I) and low immunogenic (II) antigen-PE complexes

The physico-chemical mechanism underlying the immunological activity of protein (hapten) complexes with PE consists, in all probability, in the high cooperative adsorptive capacity of free sites of PE that are not involved in the interaction with the protein globules situated on the heterofunctional surface of negatively charged membranes of immunocompetent cells. This process resembles in many features the PE adsorption on the oppositely charged surfaces of colloid particles (so-called flocculation of colloid systems). In this case the polycationic carrier "sticks together" the antigenic substance and the B-lymphocytes triggering immune responses and thus provokes their interaction. The efficiency of such "forced" interactions is high enough for the immune response to be triggered on even in the absence of T - helper lymphocytes or under conditions of their strong deficiency. The T-independence of PEC antigens described earlier in this chapter provides an illustrative example of this phenomenon. Figure 142 is a schematic presentation of a complex antigen attached to the B-cell membrane surface by "sticky" PE sites, which promote its interaction with cell receptors. One may conclude from these data that the sites of PE that are not involved in the binding to the protein antigen will interact via multipoint cooperative complex-forming contacts with the membrane proteins of immunocompetent cells, eventually resulting in the redistribution of these proteins within the lipid bilayer (so-called clusterization of lipids).

This process resembles the events taking place in the cellular membrane in the presence of the original PE. The ability of the polymeric molecule to induce the aggregation of intrinsic proteins of cellular membranes was reported by a group of Japanese investigators who studied the interaction of human red blood cells with polycations [poly(N-vinyl-3-alkylimidazole bromides)] by measuring the fluidity of membrane vesicles in model systems (Tsuchida, 1979) [231]. Similar results were obtained by a Mustafaev and co-workers [230] who studied SRBC interactions with quaternized PVP by using scanning electron microscopy (Figure 143).

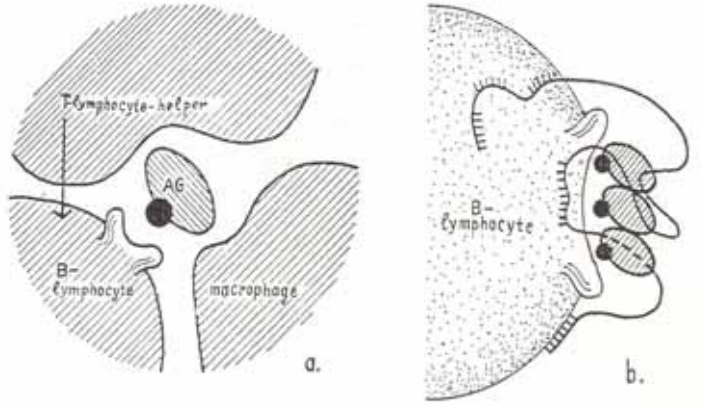


Figure 142. The hypothetical schemes of antigen-cellular complexes able to induce T-independent immune responses

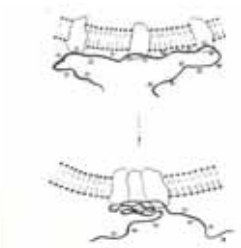


Figure 143. Clusterization of cell membrane proteins induced by positively charged PE in the presence of SAS (lipid bilayer).

It seems likely that the mechanism of the PE-induced clusterization of membrane-bound proteins in the presence of lipid molecules resembles, in some features, the mechanism of self-assembly of PEC formed from linear PE and globular proteins in diluted aqueous media (Kabanov and Mustafaev, 1977, 1981). This mechanism was confirmed by the results of model studies carried out in the past decade.

Our studies showed that the binding of linear polycations to globular proteins is practically insensitive to the addition of negatively charged molecules of surfactants, e.g., SDS. We succeeded in demonstrating that within a rather broad range of [SAS]/[BSA] molar ratios in the PVP(R_2) - SAS-BSA system the mode of binding is always the same. The reaction occurs via the "all or nothing" mechanism and consists in the self-assembly of asymmetrical aggregates from ternary PEC particles. As for SAS, they exclusively affect the composition and stability of the complexes being formed [9,9a].

Specially designed experiments employing the use of electron microscopic and luminescent methods were aimed at the elucidation of the ability of intrinsic membrane proteins of major immunocompetent cells to form clusters with PE purified from protein antigens as well as at the clarification of their role in the immunomodulating activity of synthetic PE. However, all these studies were conducted with cells separated from serum proteins, i.e., in serum-free media; therefore the conclusions made the re in should not be generalized. Valuable information can be derived from physico-chemical analyses of cell-PEC mixtures. Serum proteins added to such mixtures induced multipoint interactions between the cell membrane surface and PEC molecules resulting in the reciprocal aggregation of antigen-cell particles.

5.6. Immunogenic Cu(II)-induced polycomplexes

The increasing interest of investigators in polyelectrolyte-metal complexes (PMC) is due, primarily, to the crucial role of metal ions in biological processes [232-234] as well as to the unique capabilities of the PMC proper whose physico-chemical characteristics differ drastically from those of original components, polymer and metal. It is known, for instance, that PMC formed from imidazole-containing PMC are convenient models of hemoproteins, hemochromes, etc.

The use of such models allows one to get a deeper insight into the mechanisms of action of many naturally occurring polymers and to mimic their behavior in the presence of transient metal ions. PMC were used as a basis for the construction of a vast variety of biomedical preparations and drugs. Transient metal ions as well as other biphylic low molecular weight compounds (e.g., SAS) possess the ability to bind to neutral or weakly charged water –soluble polymers, and they confer on them adhesive properties and the capacity to form complexes with complementary surfaces (proteins, cell surface, etc.).

Recent studies demonstrated the important role of some metals (Cu, Zn, Fe) in the functional activity of immunocompotent cells [235-238]. Thus, iron deficiency leads to the inhibition of hemopoiesis and lymphopoiesis, to morphological changes in the thymus and to cell depletion in the T- and B-dependent zones of the spleen. Lithium ions markedly enhance the mitogenic effect of lymphocytes on lipopolysaccherides. Zinc salts injected to mice increase the immune response to SRBC, stimulate the migration and proliferation of stem cells and change their differentiation in the direction of erythropoiesis. $FeCl_3$ markedly increases the cooperation of T- and B-lymphocytes. All these studies were conducted, as a rule, with relatively high concentrations of metals. Chemical synthesis of polymeric carriers for antigens and analysis of their effects on individual steps of immunogenesis are impracticable, however, without systemic studies of physico-chemical regularities of PMC formation under conditions when the metal content in PMC is relatively low, whereas the toxicity of the original PE is rather high. The large body of experimental evidence makes it possible to follow the relationships between the chemical structure and composition of PMC and the mechanisms of their immunomodulating effects.

Some publications in the current literature deal with description of an original method for obtaining drugs on the bases of natural polyelectrolytes and metal ions [108,112]. These authors succeeded in demonstrating that polysaccharide-protein mixtures supplemented with metal ions are effective means of prophylaxis and treatment of some microbial infections in animal and man. By illustration, the antigenic preparation with represents a ternary complex of an *N.meningitis* (serogroup B)-specific envelope polysaccharide and protein and a pharmacologically suitable metal, is now being widely used in clinical practice.

Polyanions induce a variety of biological activities and as such have received considerable attention in the immunological literature (Ottenbrite et al., 1978; Petrov et al., 1992) [9,11,228]. Synthetic polyanions, such as polyacrylic acid (PAA), dextran sulfate and pyran copolymer exhibit immunoadjuvant activity in several model systems and, given prior to inoculation, confer protection against viruses (Ottenbrite et al., 1978; Petrov et al., 1992). They have also been shown to enhance the primary antibody response to sheep erythrocytes (SRBC) (Petrov et al., 1992).

Compared to other related polyanions such as dextran sulfate, polystyrenesulfonate and polyvinyl sulfate, PAA appears to have significantly higher antiviral activity and to be less cytotoxic. PAA has also been shown to induce interferon release [2]. Analysis of size fractionated PAA reveals that the adjuvant activity is manifested by moieties having certain 'critical' values of molecular mass (Kabanov and Mustafaev, 1984).

The use of PAA as a carrier for model protein antigens such as bovine serum albumin (BSA) or ovalbumin (OA), with the components linked by covalent bonds, has made it possible to stimulate the production of protein-specific antibodies (Abramenico et al., 1983; Mustafaev et al.,

1990 a, b). These immunogens are apparently both thymus and Ir-gene independent. The conjugates of PAA with *Mycobacterium tuberculosis* antigens were shown to be strongly protective (Petrov et al., 1992). However, the conjugation of polyelectrolyte (PE) with antigens may lead to partial changes in the chemical structure of both polymers and of antigenic determinants as a result of their involvement in the formation of covalent bonds. Moreover, this approach is not technically feasible, since the free components formed in the reaction system during the cross-linking process have to be separated from the main product and this involves additional labor and expenditure.

Another ternary complex which is formed in the presence of aluminum and ruthenium and which contains the capsule polysaccharide group and external membrane protein of *N. meningitidis* is also in use for the prophy1axis and treatment of meningitis. However, details concerning the composition and structure of such ternary po1ycomplexes involving polysaccharides have not yet been made available.

We have described in Section II the formation of water solub1e and insoluble ternary complexes of different proteins with synthetic polyelectrolytes (PAA, Poly(N-vinylimidazole), Poly-4-vinylpyridine, copolymers of acrylic acid with different anionic and cationic monomers (CPs) in the presence of transient metal ions. These investigations showed that when protein and polyelectrolytes were incapable of binding to one another, the metal ions promoted the formation of a stable ternary complex. However, the CPs in question was characterized by a greater heterogeneity and a wide distribution of molecular weights. Hence, there is little information concerning the relationship between immunogenicity and the physico-chemica1 properties of ternary CP-metal-antigen complexes.

In this chapter the immunogenic properties of different Cu(II)–induced ternary complexes of proteins with PE are described and the relationship between immunogenicity and complex formation in solutions is analyzed. This report describes a new method for obtaining highly immunogenic complexes of protein antigens. This involves the use of law concentrations of Cu^{2+}, which promote the binding of polymer to the antigen without causing any appreciable change in chemical structure or biological activity.

PAA-Cu(II)-BSA, CP(AA-VPD)-Cu(II)-BSA, CP(AA-MVP)–Cu(II)-BSA, CP(VPD-MA)-Cu(II)-BSA, PVI-Cu(II)-BSA, and CP(AA-MVP)-Cu(II)-OVA were used as immunogens[18,28,29,118,119,125,127,170,171,180,239-243,317].

Eight-week-old male BALB/c mice were immunized intravenously with each of the complexes (subcutaneous as well as intramuscular injections of the polycomplexes were also effective and resulted in similar immune responses). Eight weeks later the mice were boosted using an intravenous injection of the same amount of BSA without PE. The titers of protein-specific antibodies were determined in the blood sera; those of protein-specific AFC (antibody forming cells)–in the spleens of immunized animals. ELISA assessed the magnitude of the antibody response to the antigen.

The dynamics of antibody formation induced by ternary mixtures of PAA-Cu^{2+}-BSA at two different Cu^{2+} concentrations (n_{Cu}/n_{AA} = 0.1 and 0.2) are presented in Figure. 2. A single immunization of mice with BSA elicited the production of very few antibodies. The immunization of mice with solutions of BSA-Cu^{2+}-PAA mixtures using both Cu^{2+} concentrations resulted in the development of a pronounced primary immune response to BSA. The presence of higher concentrations of Cu^{2+} (n_{Cu}/n_{AA} = 0.2) induced an immune response to BSA at doses, which were not otherwise immunogenic.

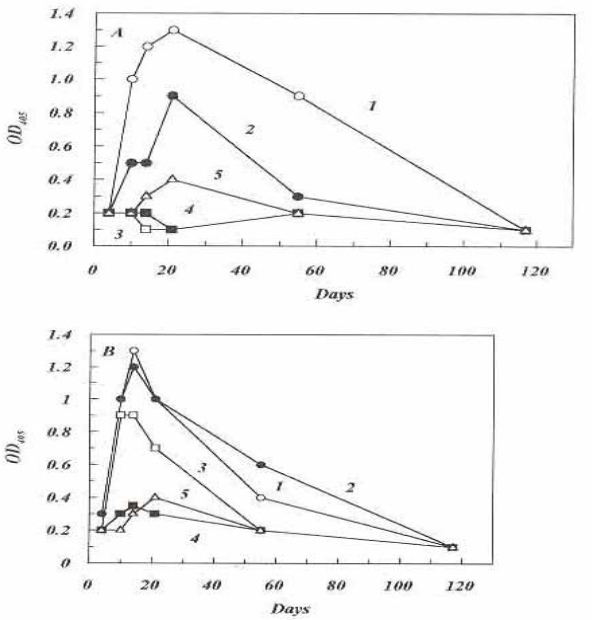


Figure 144. Dynamics of antibody formation as revealed by ELISA. Effect of immunization with complexes of PAA-Cu-BSA of differing protein content at two different Cu^{2-} concentrations. A: n_{Cu}/n_{AA}=0,1; B: n_{Cu}/n_{AA}=0,2; C: n_{BSA}/n_{PAA}=1,0. BSA dose (mg) was: 1, 0,5; 2, 0,25; 3, 0,1; 4, 0,05; 5, control, free BSA. 0,5mg. Serum was assayed at 1/50 dilution.

Figure 145 shows the kinetics of the immune response elicited using different ratios of the components *(n_{PAA}/n_{BSA})* of the ternary mixture PAA-Cu^{2+}-BSA with constant n_{BSA} and differing n_{PAA} values. The ratios n_{Cu}/n_{AA} were adjusted to 0.1 (Figure 144A) and 0.2 (Figure144B). Consistent with the data in Figure 144C, at n_{Cu}/n_{AA} = 0.2 the strong immune response was nearly fivefold higher than the control and remained high for about 20 days before subsequently dec1ining irrespective of the n_{AA}/n_{BSA} values used. The secondary immune responses observed following booster injections with these solutions were also identical and each was characterized by a rapid rise and an extended duration. At n_{Cu}/n_{AA} = 0.1 the immune response was weaker. Antigen solutions with prepared *(n_{AA}/n_{BSA}* of 0.17 and 1.0 gave rise to two and a half- and fourfold increases, respectively, in the primary immune response compared to the control. The response was limited in duration, and after peaking promptly dec1ined. Thus, antibody titers depended on the n_{PAA}/n_{BSA} ratio and increased in proportion to the concentration of PAA in the ternary mixture. The secondary response was negligible at n_{Cu}/n_{AA} = 1 and failed to reach the intensity of the primary response even after 30 days.

$((OD_{405})_{exp}/(OD_{405}\backslash_{cont})$ corresponding to the peaks in the primary immune response (on the tenth day) are plotted relative to n_{PAA}/n_{BSA} and n_{Cu}/n_{AA} in Figure. 4. Antibody titers increased with PAA concentration to attain a maximum at n_{PAA}/n_{BSA} ratios of about 1.0 and 0.5 corresponding to n_{Cu}/n_{AA} ratios of 0.2 and 0.1, respectively. Moreover, as shown in curve 3, the immunogenicity depended on the concentration of Cu^{2+} in the PAA-Cu^{2+}-BSA mixture and increased above a critical n_{Cu}/n_{AA} ratio of 0.05 %.

Hence, under these conditions metal ions appeared to promote stable complex formation between negatively charged antigen molecules and polyanions.

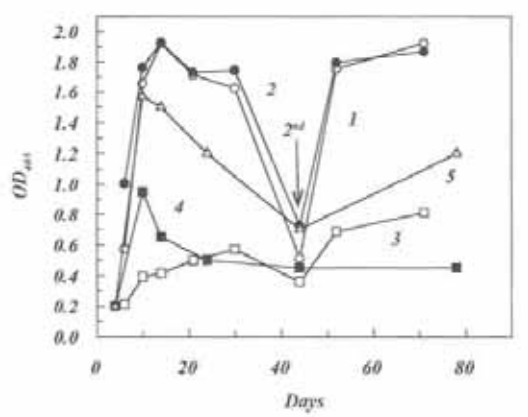


Figure 145. Kinetic of formation of BSA-specific antibodies in the sera of mice immunized with free BSA versus ternary complexes of BSA-Cu^{2+}- PAA at different ratios of the components (n_{PAA}/n_{BSA}). The experimental procedure used was as described in section 2. BSA-specific antibodies were determined by ELISA. 1 and 2. n_{PAA}/n_{BSA} of 0,17 and 1,0 at n_{Cu}/n_{AA}=0,2; 3. Control, free BSA. 0,5 mg; 4 and 5. n_{PAA}/n_{BSA} of 0,17 and 1,0 at n_{Cu}/n_{AA}=0,1

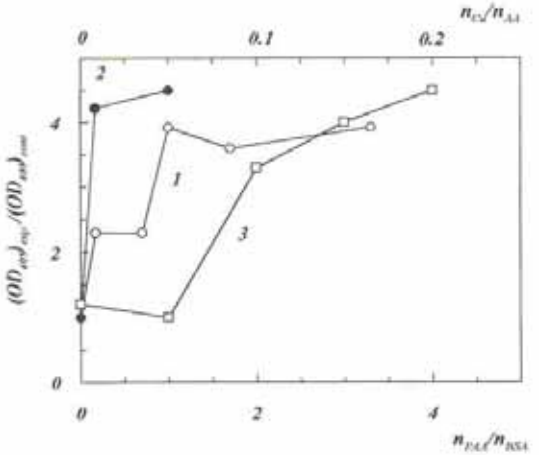


Figure 146. Dependence of relative values of BSA-specific antibodies (OD_{405}exp. /OD_{405}cont.) on n_{PAA}/n_{BSA} and n_{Cu}/n_{AA}. ELISA values determined on the tenth day post immunization were taken as the peak values of the immune response. Data from Figure 145. Plus a series of additional experiments were compiled and evaluated. Dependence on n_{PAA}/n_{BSA} at the ratios n_{Cu}/n_{AA}=0,1 (1), 0,2 (2), and on n_{Cu}/n_{AA} at n_{PAA}/n_{BSA}=2 (3) are plotted.

An analysis of the physico-chemical properties of BSA-Cu^{2+}-PAA mixtures has revealed that the ratio of the components (n_{BSA}/n_{PMC}) and the copper concentrations in the mixture strongly affects interactions in solution and, correspondingly, the stability and composition of the ternary polycomplexes thereby formed.

It was found that at n_{BSA}/n_{PAA} ratios < 1.0 the addition of Cu^{2+} to solutions of binary mixtures (n_{Cu}/n_{AA} < 0.15) caused the BSA-PAA components to form stable ternary complexes with a lower negative charge than free BSA. These systems exhibited a considerably higher immunogenic activity.

A further increase in the BSA content of the mixture ($n_{BSA}/n_{PAA} > 1$) resulted in a breakdown of the polycomplex. The level of BSA-specific antibody production was very law in this case. On the basis of these results, one may conclude that stable ternary BSA-Cu^{2+}-PAA complexes possess the highest immunogenic activity. However, in contrast to solutions of PAA-Cu^{2+}-BSA prepared at $n_{Cu}/n_{AA} = 0.10$, the ternary complexes obtained at $n_{Cu}/n_{AA} = 0.20$ were relatively stable and did not break down when the n_{BSA}/n_{PAA} ratio was increased. These complexes exhibited a much higher immunogenic activity regardless of the n_{BSA}/n_{PAA} ratio. These data agree with the results obtained at the $n_{Cu}/n_{AA} = 0.10$ and suggest that: (1) the highest immunogenic activity is exhibited by stable ternary complexes; (2) immunoactive, polyelectrolyte complexes must have a non-stoichiometric composition, with a high epitope density of antigenic determinants being achieved by the binding of several protein molecules to one particle of polycomplex.

Analogous results were obtained for all studied Cu-induced ternary complexes of proteins with copolymers. The results of the immunological tests for the ternary CP(AA-VPD)-Cu(II)-BSA and CP(VPD-MA)-Cu(II)-BSA complexes are presented in Table 23.

Table 23. The secondary anti-BSA immune response in mice immunized with BSA, BSA-Cu2+ -CP, and BSA-PVP. Results given as the mean number of antibody producing cells (APC) in the spleens of treated mice 7 days after immunisation

Immunogen	Mice	APC (M ± SD, $p = 0.05$)	
		IgM	IgG
BSA	18	23.5 ± 2.1	5.5 ± 2.3
BSA	11	20 ± 4.2	6 ± 2.1
BSA + CP-1	10	20 ± 4.0	5 ± 2.0
BSA – Cu^{2+} – CP-1	15	22400 ± 1570	14720 ± 1200
BSA + CP-2	10	20 ± 4.0	6 ± 2.0
BSA – Cu^{2+} – CP-2	15	24000 ± 1670	10500 ± 750
BSA – Cu^{2+}	10	20 ± 4.0	5.5 ± 2.3
BSA – PVP	14	5920 ± 724	7446 ± 678

Double immunization of mice with pure BSA barely induces the production of antibodies. Solutions of BSA–CP and BSA-Cu(II) mixtures also proved to be immunologically inactive. In contrast to this, immunization of mice with solutions of the ternary complexes led to the development of pronounced primary and secondary immune responses to BSA. For both CP-1 (CP(VPD-AA)) and CP-2 (CP(VPD-MA)), addition of Cu(II) solutions to these with BSA gave rise to immunological activity much higher than that observed with immunization of mice with solutions of pure BSA and control mixtures. Bearing in mind that the immunogenicity of BSA with either CP or Cu(II) was barely changed, one may conclude that the increased immunological activity seen when all three substances were present was due to the formation of water-soluble triple polymer-metal complexes with the protein antigen. It is notable that the immunogenicity of the triple complexes and the nature of the complex molecules were both independent of the nature and distribution of the monomer units (random for CP-1 and regular for CP-2) in the copolymer, and that preliminary immunization of the animals with these complexes induced increased formation of memory cells at very low concentrations of added copper ions. As it follows from Table 23 B-mice cannot practically develop the immune response to SE, what can be accounted for by T-helper deficiency. However, the introduction of the antigen in the composition of ternary complexes causes a considerable immune response, i.e. ternary Cu-induced polycomplexes manifest the properties of T-cell-independent artificial immunogens.

The physico-chemical mechanism(s) underlying the immunogenic activity of protein complexes with polyelectrolytes may be related to an adjuvant effect of polymeric macromolecules. Free sites on PMC may have the capacity, via copper ions, to interact strongly with the negatively charged membranes of immunocompetent cells. This may facilitate and stabilize the interaction of polymer bound antigen (PE-Cu^{2+}- BSA) with specific cell receptors and hence enhance the immune response.

In summary, in this chapter we have described our discovery of water-soluble complexes of various protein antigens with homopolyanions and nontoxic copolymers, which in the presence of very small amounts of divalent copper ions induce, pronounced immunogenicity and immunological protection (see below) and manifest the properties of T-cell-independent artificial vaccines. These polycomplexes can be obtained by technologically simple procedures-in a single step by mixing solutions of the selected components. The macromolecular structure of the complexes formed does not depend significantly on the in homogenecity of the composition of the polymer chain, the molecular mass of the PE (and polycomplex particles), or the nature of the protein antigen. The results of our investigations open the way for the creation of universal polymeric carriers, which could be used to bind a wide range of polymeric substances to various antigens by means of different metal ions, thus enabling various artificial vaccines to be created.

5.7. "Intelligent" Immunogens

5.7.1. New Amphiphilic Immunogens by Poly(N-Isopropylacrylamide)-Modified Bovine Serum Albumin

Introduction of synthetic polymers to biomolecules has been studied for the application in the fields of medicine, pharmacy and engineering. Polyethylene glycol (PEG) has been widely studied for protein modification, reducing the immunoreactivity and/or immunogenecity of originally antigenic proteins and improving their *in vivo* stability with prolonged clearance times. Food and Drug Administration have already authorized a few of them for clinical use.

On the other hand, nonimmunogenic synthetic polyelectrolytes (PE) reveal immunoadjuvant activity in several systems, and their complexes/conjugates with antigens given prior to inoculation confer protection against viruses. [9]. A novel method based on Cu^{2+}-meditated soluble and insoluble complex formation of PE adjuvants with proteins for enhanced protein-specific antibody responses was proposed recently [125]. However, some problems remain to be solved before the promising synthetic PE can be practically introduced into vaccines for medical and veterinary applications. In this context, it is important that PE is non-toxic, biodegradable and/or has a low molecular weight.

To endow new functions to proteins, functional polymers such as poly(N-isopropylacrylamide) (PIPAAm) have been introduced [244-248]. PIPAAm is a well-known water-soluble polymer showing reversible hydration-dehydration changes in response to small temperature changes [249,250]. An aqueous solution of PIPAAm demonstrates phase separation and polymer precipitates at a certain temperature, the so-called lower critical solution temperature (LCST). IPAAm gels have various functional applications such as artificial muscle [6], drug-release systems [7,141], and recovery of cultured cells [147,148].

In this study, the IPAAm-bovine serum albumin (BSA) conjugate was prepared and its immunogenic properties were investigated and discussed in terms of a novel immunogenic system [251]. This report demonstrates a new approach developed for highly immunogenic conjugates of protein antigens. We are currently investigating alternate adjuvants, which have a low molecular weight (7000D), and effectively enhanced immunogenecity of protein antigens, and also seeking a new route of immunization, which may circumvent the adjuvant problem.

Co-oligomerization procedure. Semitelechelic N-Isopropylacrylamide co-oligomer (IDc) was prepared by radical oligomerization of IPAAm with N,N-Dimethylacrylamide (DMAAm) in the

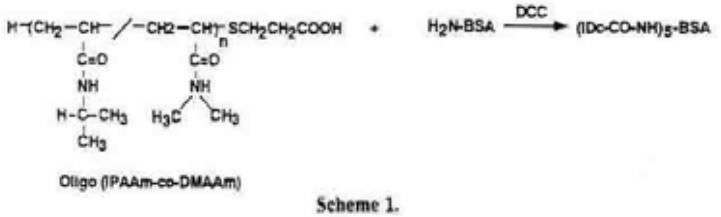
presence of 3-Mercaptopropionie acid as a chain transfer agent in N, N-dimethylformamide as described in the literature [148]. Mole fraction of DMAAm was 11 mol % analyzed by proton NMR spectra and its LCST was 36.5°C. Molecular weight was estimated to be 7000, determined by gel permeation chromatography. BSA was modified by the same method described previously [148]. The degree of modification was determined by fluorescamine assay.

IDc-BSA was injected into 8-week-old Balb/c mice intravenously, intradermally and intramuscularly, at varying concentrations, such as: 0.2 mg, 0.1 mg, 0.05 mg and 0.01 mg/mouse (intravenously). A second immunization was carried out with 0.1 mg injected intravenously. As control groups, 0.1 mg BSA/mouse (intravenously and intradermally) and 0.1 mg-0.05 mg BSA-IFA/mouse (intradermally) were injected.

For another set of experiments, IDc-BSA + IFA, 0.1 mg/mouse, was injected intradermally. Serum titer was determined by bleeding a tail vein by ELISA.

Data shown are the means of 5-8 separate immunizations. Statistical evaluation of the experimental data was done by use of a Grafit computer program based on Bevington. Standard errors in the antibody levels have been estimated for each set of experiment *(M + m,* where $m <$ 0.02).

IDc-BSA conjugation. BSA was modified with IDc via a condensation reaction between carboxyl group of IDc and amino groups of BSA, a conventional method as shown in Scheme 1.



Scheme 1.

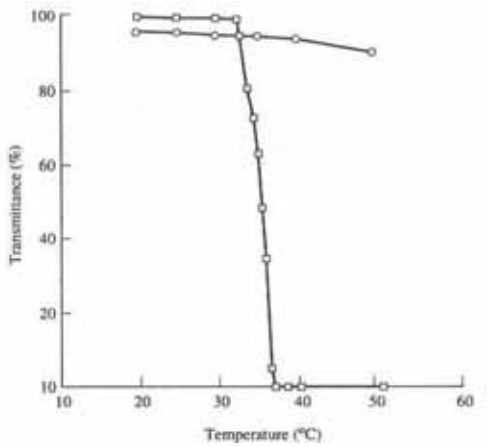


Figure 147. Temperature dependence of transmittance for aqueous solutions of IDc-conjugated. O: native BSA; Í: IDc-BSA.

Number of grafted IDc molecules per BSA was determined by fluorescamine assay, indicating that 6.8 % of amino groups on the surface of BSA molecules had reacted with IDc. As each BSA has 58 amino groups originating from lysine residues, an average of 5-7 amino groups per BSA molecule are modified with IDc. The conjugate was soluble in water at room temperature and in organic solvents such as ethanol and chloroform. Transmittance change for the

aqueous solutions of IDc-BSA conjugate at 500 nm is shown in Figure 147. Native BSA solution was transparent over all temperatures examined, while IDc-BSA conjugate exhibited phase transition at 36.5°C, which corresponds to the LCST of IDc. Dehydrated, precipitated IDc-BSA conjugate resolubilized upon cooling, demonstrating their reversible properties.

Immunogenecity of IDc-BSA conjugates. Mice were injected intravenously, intradermally or intramuscularly with solutions of BSA and IDc-BSA. Antibody levels in blood of mice were determined by ELISA [252]. Both native BSA and IDc-BSA solutions were normalized as protein concentration by the HPLC method. The dynamics of anybody formation, (OD_{405}) induced by BSA (as control) and IDc-BSA conjugates are presented in Figures 147 and 148. Intravenous administration of BSA to mice barely induced the production of antibodies. The immunization of mice with solutions of IDc-BSA conjugates led, in turn, to the development of pronounced primary immune response to BSA. Immune response could be detected in the blood sera on the l0th day post-immunization, the peak of the immune response being observed on the 14th day with the onset of decline on the 20th day. A single immunization of mice with IDc coupling BSA without adjuvants evoked increased specific immune responses to BSA.

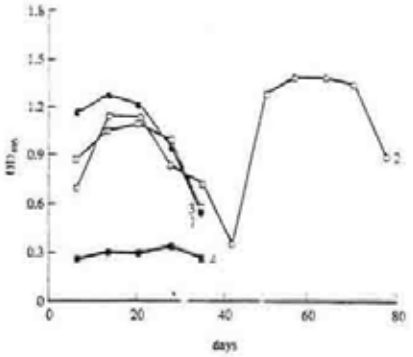


Figure 148. The kinetics of formation of primary and secondary BSA-specific antibodies (OD_{405}) in the blood sera of mice immunized intravenously with IDc-BSA conjugates at different protein dose (mg): 0.2 (1), 0.1 (2), 0.05 (3), 0.01 (4).

Moreover, the increase in the protein dose caused a proportional increase in values of antibody titers in the blood sera. The development of the high immune response was observed already with 0.05 mg and the antibody titers in sera remained practically at a maximum level in the range of 0.05-0.2 mg BSA. The mice, which were boosted six weeks later intravenously with the same concentration of free BSA and traced for the secondary immune response revealed no further increase in the antibody titers. In contrast, a second immunization of mice with IDc-BSA conjugates evoked increased immune responses to BSA.

The dynamics of antibody formation induced by IDc-BSA conjugates with different routes of immunization are compared in Figure 148. Intravenous, intradermal and intramuscular administration of BSA barely induced the production of antibodies. At the same time, the intravenous route of immunization of mice with IDc-BSA conjugates displayed an essentially higher BSA-specific immunogenic activity.

When the intramuscular and intradermal routes carried out immunization, however, IDc-BSA conjugates did not elicit higher antibody production. It is noteworthy that intradermal administration of BSA and IDc-BSA together with Freund's adjuvants (BSA + IFA, IDc-BSA + IFA) both gave rise to high immunological activity. The weak immunogenecity of free IDc- BSA by intramuscular and intradermal routes injection may be due to diffusion dependent dilution of the conjugates in the organism before they can precipitate.

It is known that covalent attachment of PEG to BSA and bovine liver catalase reduces

the immunoreactivity [24]. Abuchouwski *et al* [19] suggested that a protein molecule might be surrounded by a flexible hydrophilic shell composed of PEG and its bound water. Such a shell would cover antigenic determinants and render the protein inert to immune processes. We report that the immunogenecity of polyelectrolyte-protein complexes (multi point attachment) depends on the composition and that the structural model of low immunogenic polycomplexes inc1udes the following physico-chemical criteria: screening antigenic molecules from the interaction with the immunocompetent system through the "nonreactive" polymeric envelope surrounding the protein globule [9]. The physicochemical mechanism underlying the high immunogenecity of antigen (protein, polysaccharide) complexes with polyelectrolytes consists, in all probability, of the high cooperative adsorptive capacity of polycomplex particles situated on the heterofunctional surface of negatively charged membranes of immunocompetent cells [9].

We use IDc-BSA in which only about five IDc chains were attached to the BSA molecule. The chromatographic properties are consistent with the picture that attachment of these amounts of IDc did not produce substantial changes in the physical and chemical properties of the albumin. In the aqueous solutions, IDc transfer from a hydrophilic to a hydrophobic state at 36.5°C (body temperature). The hydrophobic aggregation site on the surface of IDc-BSA in contrast to hydrophilic (nonreactive) shell on PE-protein complexes/conjugates would increase the absorptive capacity of BSA (antigenic determinants) on the immunocompetent cells. The efficiency of such "forced" interactions of conjugate aggregates (high epitope density) are high enough for the immune response. As changing the mole fraction of DMAAm in the IDc oligomer the LCST of the IDc-BSA conjugate can be regulated, this "forced" interaction can also be controlled by temperature, which will lead to the control of antibody production.

A temperature-responsive bioconjugate was prepared via condensation reaction between carboxyl group of IDc oligomer and amino group on the surface of BSA. The conjugate demonstrated reversible hydration-dehydration changes in response to small temperature changes and had a LCST at 36.5°C, which revealed phase separation at body temperature. A single immunization with these conjugates increased specific immune responses to BSA, whereas the intramuscular and intradermal did not elicit higher antibody production. In the IDc-BSA conjugates the hydrophobic interaction due to the IPAAm at body temperature in the blood of mice seemed to play an important role to absorb to the immunocompetent cells, which increased the immunological activity of IDc-BSA antigens.

5.7.2. New Amphiphilic Immunogens by Cu(II)–Mediated Complexes of Poly(N-isopropylacrylamide) and Bovine Serum Albumin

As it was mentioned above, polymer-protein conjugates of BSA with poly(N-isopropylacrylamide) oligomers showed high immunogenicity at the intravenous route of single immunization without classical adjuvants. However, these covalent conjugates have a high primary and secondary immunogenicity for a short time [251]. This chapter describes new high immunogenic protein antigen polycomplexes with specific antibody production with relatively prolonged times which are very important for immunization and vaccine production. This involves the use of low concentrations of Cu(II) ions which promote the binding of water-born polyN-isopropylacrylamide-co-acrylic acid) copolymers to BSA. The polymer carriers are practically non-toxic and the CP-Cu(II)-BSA complexes showed reversible hydration-dehydration changes in response to temperature which produced phase separation at body temperature (see chapter) [127,241]. We are investigating alternate adjuvants, which effectively enhance immunogenecity of protein antigens as well as searching for new routes of immunization, which may circumvent the adjuvant problem.

Immunogenicity. The dynamics of antibody formation, induced by ternary mixtures of CP1-Cu^{2+}-BSA (CP-1, copolymer of acrylic acid and N-isopropylacrylamide with composition AA/NIPAAm =1:1) prepared in water and in physiological salt solutions with different Cu^{2+}

concentrations are presented in Figure 149. A single intravenous immunization of mice with BSA and double mixtures of CP1-BSA independent of the nature of solvent elicited the production of very few antibodies.

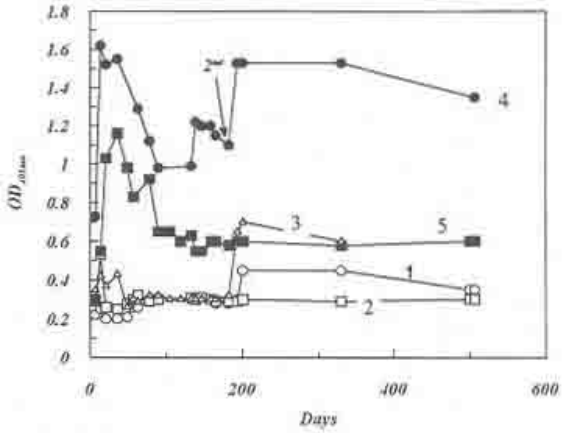


Figure 149. The kinetics of primary and secondary BSA-specific antibody (OD_{405}) formation in mice immunized intravenously with BSA (1), CP1-BSA (2) and (CP1-Cu^{2+}-BSA) mixtures in water (3) and in 0.154 M NaCl (4,5). n_{Cu}/n_{AA}=0.2; pH 7.0; n_{BSA}/n_{CP}=1.0 (4), 2.0 (5). Free BSA and BSA in polycomplex dose 100μg

When the mice were immunized with soluble ternary mixtures of CP1-Cu^{2+}-BSA prepared in water, immune response was also weak. In contrast the immunization of mice with the CP1-Cu^{2+}-BSA mixtures prepared in physiological salt solutions resulted in a pronounced primary immune response to BSA.

An immune response was detected in the blood serum on the 10th day postimmunization, and peaked on the 20th day with the onset of partial decline on the 50th day. A high level of immunogenic activity lasted more than 18 months. Thus a single immunization of mice with ternary CP1-Cu^{2+}-BSA complexes in physiological salt solutions without adjuvants evoked increased specific immune responses to BSA.

The mice, which were boosted 78 days after intravenous immunization with the same concentration of free BSA, revealed a weak increase in the antibody titers. In contrast, a second immunization of the mice with CP1-Cu^{2+}-BSA + NaCl complexes caused a sustained increase in secondary immune responses to BSA.

The immunogenicity of ternary CP-Cu^{2+}-BSA was dependent on the composition of CP and ratios of components: n_{Cu}/n_{AA} and C_{BSA}/C_{CP} . A weak immunogenicity was observed when animals were immunized with a ternary CP2-Cu^{2+}-BSA mixture (CP-2, copolymer with AA/NIPAAm =1:3). In this case more insoluble and less stable soluble polycomplexes are formed, as the number of carboxyl groups is fewer in CP2 than in CP1. Moreover, as shown in Figure 146, the immunogenicity, which is dependent on the ratio C_{BSA}/C_{CP}, decreased with increasing CP concentrations at constant n_{Cu}/n_{AA} and BSA concentrations in the mixture. Insoluble polycomplexes prepared in the presence of high concentrations of Cu^{2+} in the ternary mixtures exhibited a weaker immunogenic activity. This may be due to administration of insoluble polycomplexes products.

The immune response kinetics required using physiological salt solutions of CP1-Cu^{2+}-BSA which were prepared by dissolving the precipitate obtained after heating (37°C) the ternary mixtures are shown in Figure 150. The highest immunogenic activity is exhibited by the soluble polycomplexes. Moreover, the increase in the protein caused a proportional increase in antibody

titers in the blood. The development of the high immune response was observed with 10 μg and the antibody titers in the serum remained practically at a maximum level in the range of l0-100μ*g* BSA.

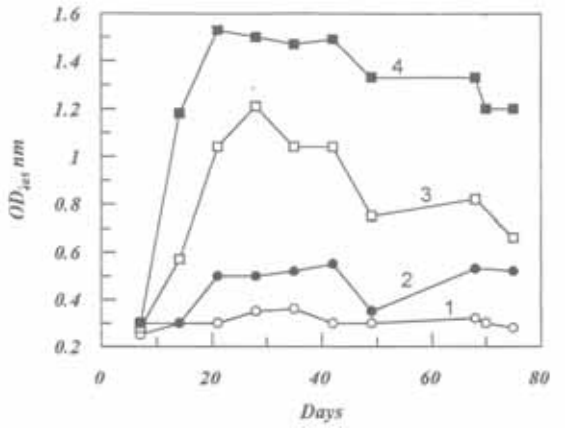


Figure 150. The kinetics of primary BSA-specific antibodies (OD_{405}) formation in mice immunized intravenously with CP1-Cu^{2+}-BSA complexes prepared in 0.154M NaCl. Solution was prepared by dissolving in 0.154M NaCl solvent of the precipitate obtained after heating (37˚C) of soluble ternary polycomplexes at different protein dose g/(μg): 10 (2), 50 (3), 100 (4), free BSA 100 μg (1). N_{Cu}/n_{AA}=0.2; n_{BSA}/n_{CP}=1.0; pH=7.0

A structural model of law immunogenic antigen containing polyelectrolyte complexes meets the following physicochemical criteria: tight binding of the antigen to the polymeric carrier, stoichiometric composition of polycomplexes and screening of antigenic molecules from the deleterious effects of environmental factors by the "nonreactive" polymeric envelope surrounding the antigen molecule [9]. The electrostatic and hydrophobic complexes of BSA, ovalbumin (OA), *Clostridium perfringens a-anatoxin* and bovine gamma globulin (BGG) with copolymers of acrylic acid and 2-methyl-5-vinylpridine [9], Cu^{2+}-induced equimolar complexes of poly(N-vinylimidazole) with BSA and BGG [9] and covalent conjugates of poly(ethylene glycol) with BSA and liver catalase [19,24] were characterized by an analogous structural model and possess lower immunoreactivity. One can propose that the lower immunogenicity of the ternary EP-Cu^{2+}-BSA complexes prepared in water is a result of similar physicochemical behavior. The physicochemical mechanism underlying the high immunogenicity of the antigen complexes with PE is probably due to the high cooperative adsorptive capacity of the polycomplex particles situated on the heterofunctional surface of negatively charged membranes of immunocompetent cells.

In physiological salt (0.154 M NaCl) solutions at pH-7, the CP1-Cu^{2+}-BSA complexes transform from a hydrophilic to a hydrophobic state at >30°C and at body temperature are practically insoluble. The efficiency of such "forced" interactions of polycomplex aggregates (high epitope density) are apparently high enough for the immune response.

The insoluble polycomplex in the body seems to effect antigen targeting to immunocompetent cells. It should be noted that the copolymers used are practically nontoxic (LD_{50} > 2 g/kg) and the highest level of immunogenic activity lasts more than 18 months. This result implies that amphiphilic; temperature-sensitive polycomplexes have a long immunological time, which is necessary for the creation of effective artificial vaccines.

It seems that the binding of antigen containing polycomplexes to a membrane is also an essential step for penetration into the cell. This approach was successfully used to introduce proteins, oligonucleotides and DNA into intact mammalian cells [253].

5.7.3. New Amphiphilic Immunogens by Covalent Conjugates of Anionic Poly(N-isopropylacrylamide-co-acrylic acid) with Bovine Serum Albumin

In previous investigation we use IDc-BSA in which about five IDc chains (molecular weight about 7000 D) were attached to one BSA molecule, i.e. the protein globule was the carrier for the IDc chains [251]. This conjugate has a short-time primary and secondary immunogenicity. The objective of the present study is to examine the immunogenic properties of the "intelligent" bioconjugates obtained by covalent conjugation of BSA with the NIPAAm and acrylic acid copolymers characterizing by higher values of inherent viscosity (and molecular weight) [153]. The covalent binding mechanism of poly(N-isopropylacrylamide-co-acrylic acid) copolymers (CP) with BSA molecules and the structure of forming conjugates are described in chapter.

To clarify the effects of thermal transition of polymer–protein conjugate chains on the immunogenicity of protein antigens, the immunological activity of modified BSA was examined in comparison with that of the free protein. For the immunization of mice, different concentrations of CP–BSA conjugate solutions were used: dilute solution (0.01–0.10 g/dl), that does not precipitate thermally and more concentrated solution (0.448–2.286 g/dl, thermally precipitate). These solutions containing the same dose of BSA were intravenously injected to mice.

The dynamics of antibody formation, (OD_{405}) induced by BSA (as control) and CP-BSA conjugates are presented in Figure 151.

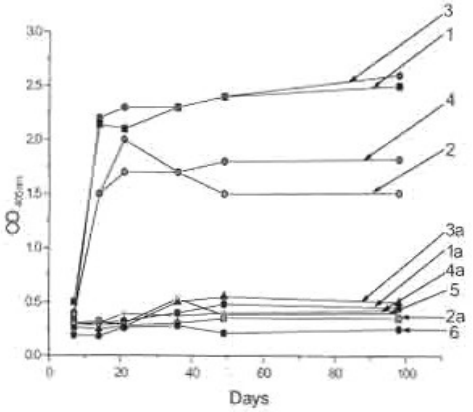


Figure 151. The dynamics of formation of BSA-specific antibodies (OD_{405}) in the blood sera of mice immunized with CP-BSA conjugate at dilute solutions. [0.1 (1a), 0.05 (2a), 0.03 (3a); 0.01 (4a) g/dl] and thermally precipitated concentration [2.286 (1); 1.346 (2); 0.673 (3); 0.448 (4) g/dl] preparing at different C_{BSA}/C_{CP}: 0.28 (1,1a); 0.59 (2,2a); 1.14 (3,3a); 1.70 (4,4a) pure BSA (5), serum of mice without immunization (6); protein dose: 100μg; phosphate buffer (pH 7.2)

Intravenous administration of BSA to mice barely induced the production of antibodies. A single immunization of mice with dilute solutions of polymer–protein conjugates, independent of initial C_{BSA}/C_{CP} ratio of components, which were synthesized samples, elicited the production of very low number of antibodies (practically at the same level as free BSA). At the same time, the immunization of mice with more concentrated solutions (same dose of BSA) of CP–BSA conjugates with different C_{BSA}/C_{CP} ratios of conjugation led, in turn to the development of a pronounced (more than 10 times) primary immune response to BSA. Immune response for all conjugates could be detected in the blood sera on the seventh day post-immunization and the highest level of immunogenic activity lasted more than 50 days. Therefore, a single immunization of mice with CP–BSA conjugates at the thermally precipitated concentration without adjuvant

evoked increased specific immune response to BSA. The immunogenicity of conjugates does not depend on the initial conjugation ratio of components (C_{BSA}/C_{CP}) and is observed practically at the same levels.

In section I the schematic representation of the carbodiimide-induced conjugation of BSA with polyanions and hypothetical structures of the CP-BSA conjugates was given. The character of the binding depends on the [BSA]/[polymer] ratio and two types of bioconjugate particles are formed: at $r<1$, the protein molecules in the structure of conjugate particles are densely covered as a shell by polymer chains and practically "fenced off" from water environment; at $r>1.0$ forming conjugate particles possess more friable structures in which protein molecules are practically exposed to the solution.

It is reasonable to conclude from these observations that the negatively charged carboxylic groups remaining in composition of poly(NIPAAm-AA)–BSA conjugates could eventually prevent phase separation if the conjugate concentration is very low. At the same time, when the temperature is raised above the LCST of poly(NIPAAm), the precipitating poly(NIPAAm) segments of the conjugates will flocculate together if the concentration is high enough, similar to free poly(NIPAAm-AA). In this study, we see the thermally induced precipitation and flocculation of conjugates in all studied C_{BSA}/C_{CP} ratios since we used more concentrated solutions. A single immunization with these conjugates increases specific immune responses to BSA, whereas, the level of the antibody titers does not depend on the composition of soluble conjugate molecules. In the aqueous solutions, poly(NIPAAm-AA) transforms from hydrophilic to a hydrophobic state at 36.5°C (body temperature). The precipitation of conjugate molecules leads to "leveling off" of the effect of conjugate composition on their immunogenicity and the hydrophobic aggregation site on the surface of poly(NIPAAm-AA)-BSA molecules would increase the absorptive capacity of BSA (antigenic determinants) on the immunocompetent cells. The efficiency of such "forced" interactions of conjugate aggregates (high epitope density) is high enough for the immune response.

5.7.4. Some Practical Applications of PEC

In the foregoing chapters we have demonstrated the practical utility of PE as helpful tools in theoretical immunological studies. Analysis of macromolecular substitution reactions in PEC both *in vitro* and *in vivo* has made it possible to calculate the minimal time needed for the induction of immune responses by artificial thymus-independent antigens. This finding is very important for the implementation of directed control over immune reactions occurring in living organisms. In the present chapter we will consider some of the most important results obtained through the use of PEC, such as identification of antigenic determinants whose immunogenic activity is not suspected but is manifested in the isolated state, interactions between the neuroendocrine and immune systems, relationships between cell-type and humoral immunity, allergenicity and immunogenicity as well as possible applications of PEC as progenies of future vaccines.

PEC in allergology. Studies designed to investigate the effects of synthetic PEC on, the allergenic activity of the antigenic component of PEC acquire special importance during the transition from artificial antigens to synthetic vaccines.

The results of experiments aimed at the analysis of immunogenic and allergenic activities of several PEC: PVP(R_2,R_{16})-OVA (PEC-1), CP(AA-MVP)-OVA (PEC-2) and CP(AA-MVP)-Cu^{2+} -OVA (PEC-3), obtained with the help of EIA are listed in Table 23 [29,254].

The allergenicity of PEC *in vivo* was estimated by the ability of these complexes to induce histamine release from the mast cells of presensitized mice as well as in the passive skin anaphylaxis test (PSAT) (Popov, 1990). The results of these studies are listed in Table 24. It can be seen from these data that the intensity of PSAT in rats immunized with both OVA and PEC was nearly the same, the differences in the immune responses being statistically insignificant. PEC used at 0.1,1.0 and 10μg/ml of OVA and the pure protein were both able to induce the *in*

vivo release of histamine from the mast cells of presensitized mice (Popov, 1989). This finding suggests that the antigenic and allergenic activities of OV A did not change after the protein binding to PEC. These data are in good agreement with the results of physico-chemical studies aimed at the preservation of the native structure of the protein component of PEC (Mustafaev, 1981). Stipulating that OVA is a convenient model in many allergological studies, it seemed important to examine the changes in the ability of this protein to induce antibody production after its binding to PE (Popov *et al.,* 1989, 1990). In this study mice were immunized with low doses of the antigen known to elicit immune responses of the IgE type [255].

The dynamics of changes in the titers of IgG- and IgE-homocytotropic (HAT) antibodies to OVA after a three-fold immunization of mice with pure OVA or OVA-PEC (0.5, μg/animal) is shown in Table 25. It can be seen from these data that the sub maximal level of the IgE response was reached only after immunization of mice with OVA or OVA complexes with the polyampholyte. The positively charged PEC were unable to elicit IgE-specific immune responses.

Table 24. the ability of OVA and its polyelectrolyte complexes to induce passive skin anaphylaxis in rats. * - β=4.6 mol%. **- optical density of formalin-extracted Evans' blue at λ=620nm

immunogen	content of Evans' blue in the extravasate** (M ± m)
OVA	0.57 ± 0.12
PVP(R_2, R_{I6})*-OVA	0.58 ± 0.06
CP-OVA	0.71 ± 0.12
CP-Cu^{2+}-OVA	0.52 ± 0.12

Table 26 shows the dependence of the titers of IgE antibodies to OVA measured on the 10th day after a single immunization of mice with pure OVA or OVA-PEC on the antigen dose. In this case the immunogenic activity of the pure protein increased drastically after its binding to PE, whereas PEC-2 and PEC-3 displayed no immunogenic activity at all doses used. This was unobserved in the case of the IgG response. As can be seen from the data depicted in Table 27, OVA complexes with both polycations and polyampholytes were able to induce the synthesis of IgG antibodies. High doses of PVP(R_2,R_{I6})-OVA evoked an early drastic increase in the levels of protein-specific antibodies of both types.

Table 25. The dynamics of the immune response to OVA on the 1st and 3rd weeks after the third immunization of mice with pure OVA, OVA-PEC and OVA-$Al(OH)_3$ mixtures. OVA dose – 0.5μg/animal. * - w/w ratio of OVA/$Al(OH)_3$ on OVA-$Al(OH)_3$ or PEC-$Al(OH)_3$ was 1:5000. IgG – optical densities of the immunoenzymatic reaction product in the total pools mouse blood sera used in a single dilution. IgG – log IgE of titers of homocytotropic antibodies in the total pools of blood sera of inbred rats as determined by PSAT. β=4.6 mol%

	1st week		3rd week	
immunogen	IgG*	IgE*	IgG*	IgE*
OVA	0.430	6.6	0.385	5.6
PVP(R_2, R_{I6})-OVA	0.305	0	0.355	0
CP-OVA	0.355	5.0	0.370	1.0
CP-Cu^{2+}-OVA	0.340	0	0.295	0
	OVA + $Al(OH)_3$			
OVA	0.385	10.0	0.375	9.75
PVP(R_2, R_{I6}-OVA)	0.275	9.8	0.310	9.5
CP-OVA	0.300	9.0	0.490	9.0
CP-Cu^{2+}-OVA	0.345	10.0	0.320	9.75

Table 26. The dose dependence of the IgE response to OVA on the 10th day after immunization of mice with pure OVA or OVA-PEC. *β=4.6 mol%

dose, μg/animal	antibody titers (log_2 1/T) OVA	PVP(R_2, R_{16})*-OVA	CP-OVA	CP-Cu^{2+}-OVA
0.5	0	0	0	0
5.0	0	09	0	0
50.0	4.3	3.5	0	0
500.0	3.0	7.7	0	0

Table 27. The titers of HAT-specific IgE and OVA-specific IgG antibodies determination on the 10th and 18th days afters a single immunization of mice with pure OVA and OVA-PEC, OVA dose – 1 mg/animal. * β=4.6 mol%

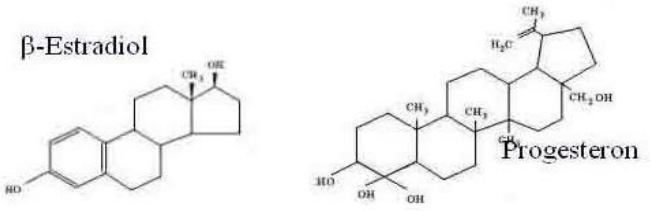
immunogen	IgE HAT (log_2 1/T), M ± m 10th day	18th day	IgG (M ± m) 10th day	18th day
OVA	0	0	7.0 ± 0.3	6.2 ± 0.6
PVP(R_2, R_{16})*-OVA	7.25 ± 0.14	4.5 ± 0.3	7.8 ± 0.5	10.5 ± 0.5
CP-OVA	0	0	7.3 ± 0.2	7.4 ± 0.2
CP-Cu^{2+}-OVA	0	0	7.5 ± 0.4	7.1 ± 0.3

Thus, the dependence of the immunological activity of PEC on the physico-chemical peculiarities of their composition and chemical structure was also demonstrated in experiments, in which the model allergen was used as protein antigen. Evidence in favour of the crucial role of the PEC structure in the immunogenic activity of these complexes can be derived from the results of immunological experiments, in which mice were immunized with PEC-adjuvant (aluminum hydroxide) mixtures (Table 25). These studies revealed that in mice immunized with such PEC-adjuvant mixtures there were no differences between the IgE and IgG immune responses. This finding can be explained as being due to the leveling-off of the physicochemical differences in PEC within the PEC-$Al(OH)_3$ complexes.

5.8. Novel Hapten Containing Polyelectrolyte Complexes

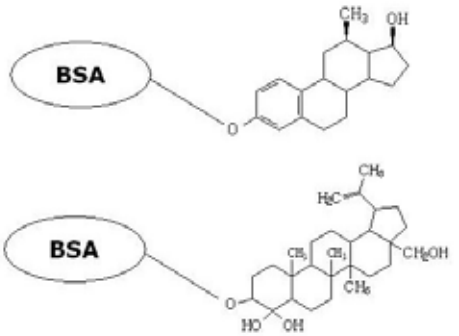
5.8.1. Steroid Hormones

Steroid hormones (estradiol, progesterone and testosterone) are widely used as contraceptive, anti-inflammatory, and anticancer drugs and, in general, have extremely low immunogenicity [256]. Estrogen acts as a regulator of various physiological processes in the body, progesterone is important in preparing the uterus for the implantation of the blastocytes and in maintaining pregnancy.



Therapeutic application of progesterone is the treatment of certain types of endocrine dysfunction such as amenorhea and dysfunctional uterine bleedings. In order to elicit an immune response to such antigens, small hapten molecules must be coupled to carrier structures, most

often proteins, and administered into the body with classical adjuvants-$Al(OH)_3$, Freund's mineral oil adjuvant, liposomes, or immunostimulating complexes (ISCOM). However, all of these common adjuvants suffer from serious disadvantages. Beta-estradiol contains no functional groups that are themselves amine reactive; therefore, it is necessary to use estradiol derivatives having an amino reactive group to couple the derivative to carrier proteins. A series of estradiol derivatives was prepared [257-262].



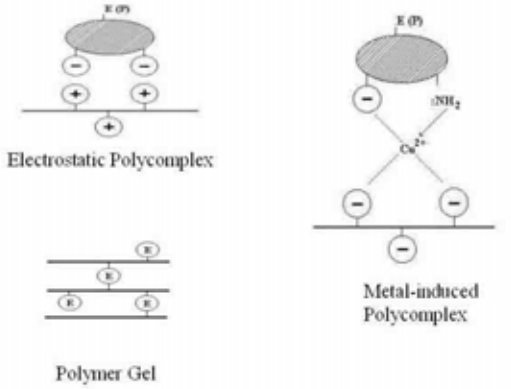
Using azocoupling techniques, Erlanger et al [263] have prepared steroid-bovine serum albumin (BSA) conjugates that elicited production of antisteroid antibodies. Later, amino phenyl derivatives were used for conjugation of steroids. Niswender et al. pointed out that the ratios of carrier/protein were critical [264]. However, each derivative required extensive complex synthetic procedures for its preparation, which diminished their convenience and utility in the preparation of immunogens. To enhance the immunogenicity of the steroid hormones keyhole limpet hemocyanin (KLH), bovine gamma globulin (BGG) and ovalbumin (OVA) were also used as carriers for coupling of steroid hormones [265]. However, the effective immune response to steroid hormones, constructed in this way, was developed in the presence of classical adjuvants (Freund's complete adjuvant), which sets limits to practical applications.

Reports on the production of monoclonal antibodies (MAbs) against steroids have shown that the yields of high affinity antibodies are low. On the other hand, the high affinity and high specificity have so far been attained only after long periods of immunization, for example, four to six administrations. Thus, using different immunization regimens and the antigens 6-hydroxyprogesterone hemicucinate conjugated to BSA, Fantly et al. [265-268] have produced 35 monoclonal antibodies against progesterone with a wide range of specifities and affinities (Ka $=8x10^7$-$3x10^{10}$ M^{-1}). Simultaneous production of monoclonal antibodies to mixtures of different steroid antigens linked to BSA was investigated in [269,270]. Only six antibodies were developed against progesterone in the first fusion experiment despite the relatively high binding to this steroid shown by the mouse serum. However, in the second fusion experiment, relatively few monoclonal antibodies developed against these antigens. In the two fusion experiments, these were shown to be of the IgG1s subclass. However, in all cases antibodies with both high affinity and high specificity have so far been produced only after long periods of immunization [271-273]. A very promising alternative to classical adjuvants is the use of nonimmunogenic synthetic polyelectrolytes (PE) (negatively or positively charged polymers) as a carrier for the antigens [9] and chemical modification of some steroid hormones with polymers for the controlled release of these hormones has been reported [274-285,286-300]. Polymers used include micro particles of poly (lactic acid-co-glycolic acid), poly(lactide-co-epsilon-caprolactone), polyethylglycollactide copolymers containing levonergesterel and estradiol and water-soluble and insoluble polymer-hormone conjugates. However, it must be noted that polymers used in these systems are not water-soluble, and the immunological properties of these Biopolymer Systems have never been published in the current literature.

Recently some results on the physico-chemistry of new amphiphilic polyelectrolyte

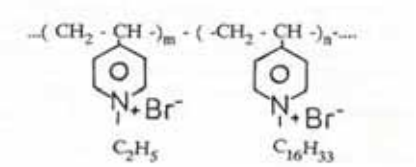
complexes containing steroid hormones (or anticancer betulin hapten) and protein antigens, monoclonal antibodies produced against steroid hormones in regard to association constants, specificity and immunoglobulin subclass obtained by author's colleagues and collaborators are summarized in this chapter. This chapter reports also on the synthesis of novel polymer conjugates and gels comprising 17-estradiol and betulin directly cross-linked to nonimmunogenic anionic polyelectrolytes with high hormone-specific immunogenecity and the production of estradiol-specific MAbs after single administration [186,239,240,242,243,302,303].

The proposed strategy consists in the covalent bonding of a haptens (steroide hormone and betulin) to BSA globules and incorporating the BSAxHormone conjugate into polyelectrolyte complexes by electrostatic (and/or electrostatic-hydrophobic), and Cu(II)-induced complexing, i.e. the water-soluble polycation-protein complexes and Cu(II)-induced polyanion-protein complexes were used as a carriers for steroid hormones and betulin hapten.



The use of water-soluble complexes of PE and proteins as a carrier for model protein haptens (PAA-BSAxtrinitrophenol) has made it possible to increase the hapten-specific immune responsiveness of the organism by several orders of magnitude.

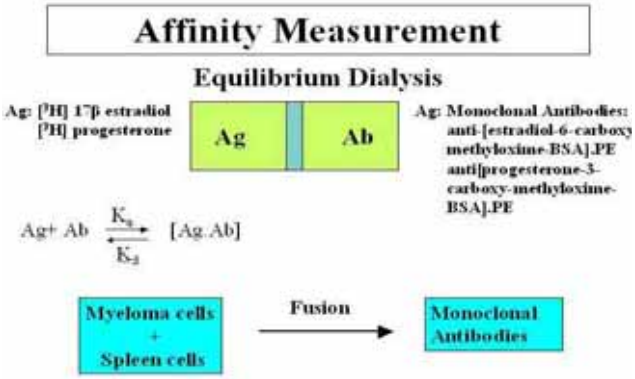
Estradiol containing PEC. The polyelectrolyte complexes chosen was the copolymer of 4-vinyl-N-ethylpyridine bromides PVP(R_2, R_{16}), where *(n/m* + n)xl00 = 10 mol%. PE was obtained by quaternization of narrow fractions of poly-4-vinylpyridine (PVP) *(Pw* = 10^3) with ethyl (R_2) and cetylbromides (R_{16}):



The molecular weight of PE was around 200,000. For inclusion of estradiol (E) molecules into PEC we used the covalent conjugates of BSA with (-estradiol 6-(o-carboxymethyl) oxime (BSAxE) (32 mol steroid per mol BSA) .To prepare a polymer-protein complex, various concentrations of the protein solution were added to PE, dissolved in phosphate buffer (PBS), pH=7.2. In practice, 1,2, and 5 mg/ml BSA-estradiol (BSAxE) solutions were mixed with 1 mg/ml PE solution. The supernatant was taken and diluted to 4 ml in PBS and investigated by HPLC, UV spectrophotometric and electrophoretic methods. The concentrations of free polyelectrolytes were obtained from the calibration curve of $OD_{254} = Kx$ C (C is the concentration of PE).
The protein/polymer (n_{BSA}/n_{PE}) ratio was calculated using the equation $n = CxN_A!M$, where n is the number of the molecules in one ml, M is the molecular weight of components, N_A is Avagadro's number, and C is the concentration in g/l00 ml.

Immunization. PE-(BSA.E) complexes were used as the immunogen (BSA.E) and (BSA.E) administered together with incomplete Freund's adjuvant were used as controls. Eight-week-old BALB/c mice were immunized with each of the antigen samples by intravenous and intraperitoneal injections. For serum titer detenninations, the mice were bled through the tail vein. The blood was collected in a microfuge tube in sodium citrate and centrifuged at 6000 rpm to remove red blood cells. Serial dilliistions of serum were made in PBS (dilutions 1/200, 1/400, 1/1000). The serum samples were tested with ELISA. Fusion was done by using classical fusion protocols. Monoclonal antibodies were purified from the hybridoma supernatant by $(NH_4)_2SO_4$ precipitation between 30 and 50% saturation. The precipitate dissolved and dialyzed against PBS and the antibodies were purified by gel filtration chromatography using a Bio Sil Sec 250 column on HPLC. Immunoglobulin typing or monoclonal antibodies was done with a hybridoma subisotyping kit (Boehringer Mannheim).

Affinity measurements of monoclonal antibodies were performed by equilibrium dialysis. [3H] 17-estradiol (NEN, specific activity 87 Ci/mmol) was used as radiolabelled antigen. Antigen and antibodies were incubated in equilibrium dialysis cells for 20 h at room temperature under slow shaking. Radioactivity measurements were done in an LKB (Wallac) 1212 Rackbeta liquid scintillation counter. Slope was estimated by using the Grafit program.



Preparation of PE-BSA complex. The complex formation between BSA and PE was investigated as described previously. BSA molecules were found to interact with polycations and to form soluble as well as insoluble protein-PE complexes. Preparations of complexes of BSA.E with PE were carried out by the methodology as elaborated in this system.

Starting with very low BSA.E/PE ratios, that is $n_{BSA}/n_{PE} = 0.1$, a phase separation took place in this system [PE-(BSA.E)] at pH 7. Analysis of the matrix solution of insoluble mixtures PE-(BSA.E) was carried out with spectrophotometric, electrophotometric, and chromatographic (HPLC) methods that showed only the presence of one substance corresponding to free PE with absorption at 254 nm in the matrix solution of mixtures PE-(BSA.E). An absorption at 280 nm corresponding to free BSA.E or soluble PE-protein complex was absent in the matrix solution of mixtures PE-(BSA.E) as indicated by electrophoretic methods. Thus, binding of added BSA.E to PE resulted in the formation of an insoluble PE-(BSA.E) complex. It can be seen that at the $n_{BSA}/n_{PE} = 1$, free fractions of PE remained in the matrix solution. The existence of free PE under these conditions indicates a nonrandom distribution of the conjugate molecules between the coils of polycations (self-assembly of polycomplexes).

Such a type of distribution was previously found upon complexation of globular proteins with oppositely charged polycations in aqueous solutions. The demonstrated disturbance of the randomness of the distribution in PE-protein solutions appears to be due to a positive interaction between the proteins globules absorbed by one chain. In our case, with the hydrophobicity of BSA.E being higher than BSA, the proposed mechanism is very probable.

Dependence of absorption at 254 nm (OD_{254}) on the ratio of components on free PE is shown in Figure 149a Taking into account the above indicated fact of the quantitative binding of BSA.E to PE, one may consider that $\lim(n_{BSA}/n_{PE}) = Ni$, when $OD_{254} = 0$. This limit equals the number (Ni) of the protein molecules bound by a single chain of PE of a given degree of polymerization under given conditions (Ni = 2).

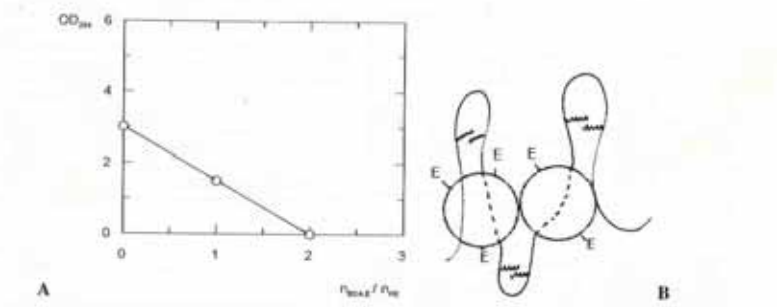


Figure 152. (a) Dependence of values of optical density (OD_{254}) of matrix solution of mixture PVP ($R_2.R_{16}$)-BSA.E obtained by UV spectrophotometric analysis at 254 nm on n_{BSA}/n_{PE}- (b) A schematic presentation of the structure of BSA.E complexes with PVP ($R_2.R_{16}$). For explanations see text

From the result, a hypothetical scheme of the structure of a particle of the PE-BSA.E complex was constructed (Figure. 152b). Electrostatic and hydrophobic interactions of BSA.E with PE lead to the formation of interpolymer-protein complexes with the realization of self-assembly of the nonstoichiometric particles of polycomplexes. Protein globules in each particle of the complexes apparently contact each other and are wrapped by the polycation-carrier. Hydrophobic portions of PE contact each other, stabilizing the structure as a whole. The polycation fragments not containing cetyl groups are probably, in part, in the form of free loops and form salt bonds with the negatively charged groups on the surface of the protein globules.

Immunogenecity of PE-BSA.E. The polycomplex PE-BSA.E with composition $n_{BSA}/n_{PE} = 2$,i.e., two molecules of BSA.E bound by one chain of polycation, was used for the immunization. The dynamics of antibody formation, induced by free BSA.E and polycomplexes PE-BSA.E at different protein doses (corresponding to different polycomplex concentrations), are presented in Figure 153.

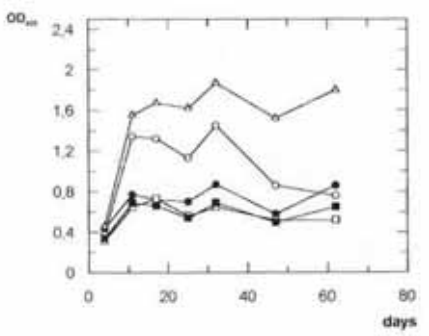


Figure 153. The dynamics of estradiol-specific (BSA.E-specific) antibody formation [as assayed by ELISA (OD405)], induced by free BSA.E and by polycomplexes of PVP (R2.R16)-BSA.E at different protein doses: 100 µg (○), 50 µg (●), 10 µg(□)50 µg free BSA.E (■), prepared by dilution of polycomplex solution, (Δ) Mixture of BSA.E with IFA with protein dose of 50 µg

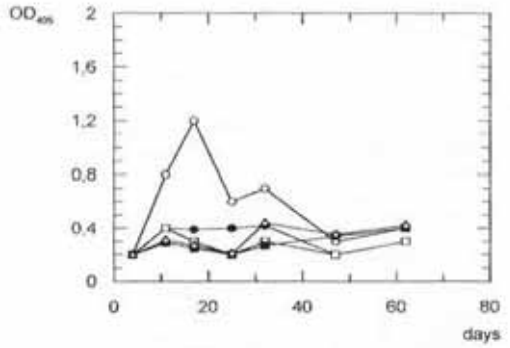


Figure 154. The dynamics of formation of BSA-specific antibodies (OD405) induced by free BSA.E polycomplexes of PVP ($R_2.R_{16}$)-BSA.E and mixture of BSA.E + IFA at different protein doses. For PVP ($R_2.R_{16}$)-BSA 100 μg (○), 50 μg (●), 10 μg (□); for BSA.E 50 μg (■); and for BSA.E + IFA mixture, 50 μg (Δ)

It can be seen from the data that a single immunization of mice with BSA.E barely induced production of antibodies. The immunization of mice with 100 g PE-BSA.E complexes led, in turn, to the development of a pronounced primary immune response. The peak of the immune response was observed on day 10-30 postimmunization with subsequent decline by day 50. The mice immunized with this dose of (PE-BSA.E) (100 g) showed the second highest antisteroid serum activity, following those immunized with 50 g (BSA.E + lFA). In mice immunized with (BSA.E + lFA), the antibody activity increased in about 10 days and then kept the same level up to 65 days. In mice immunized with (PE-BSA.E) an enhanced antibody activity could be detected up to 35 days with subsequent decline. The specificity of antibodies formed was determined on plates coated in parallel with BSA.E conjugate and free BSA. Only mice immunized with 100g (PE-BSA.E) gave activity against BSA up to 20 days with subsequent decline (Figure 154). These data show a pronounced primary immune response to BSA.E conjugate and BSA simultaneously.

Fusions following immunizations with PE-BSA.E. **Table 28 gives comparisons of antibody-producing hybrids obtained following immunization with PE-BSA.E and immunizations with BSA.E + lFA.**

Table 28. Comparison of total and antibody producing hybrids obtained after fusions using immunization with PE-BSA.E versus BSA.E+IFA

Immunization/ fusion	*Total hybrid number/ total well*	*Estradiol reactive hybrid number*	*Estradiol specific hybrid number*
PE–BSA.E			
1	493	21	5
2	481	33	3
3	466	26	2
IFA–BSA.E			
1	420	31	3
2	392	26	3
3	150	9	0
BSA.E	—	—	—

The two different immunization procedures appeared to yield comparable results in regard to total as well as to estradiol-reactive and -specific hybrid numbers. Sixteen estradiol-specific monoclonal antibodies obtained after these fusions were found to be of IgM class. They revealed, as tested in ELISA, moderate to negligible reactivity with progesterone, testosterone,

aldosterone as well as with BSA (the ratios of relativities with nonestradiol antigens to reactivity with estradiol A_{405}(nonestradiol)/A_{405}(estradiol) = 0.1-0.4

The relativities of some of the antibodies with corticosterone were, however, more pronounced: A_{405}(corticosterone/A_{405}(estradiol) = 0.2-0.7. The affinities of 11 of these antibodies for estradiol were subsequently determined by equilibrium dialysis. As shown in Table 3, the antibodies obtained by the two different immunization methods did not show any considerable differences in terms of the determined apparent dissociation constants *(Kd).*

We have shown that inclusion of steroid hormones (estradiol) in water-soluble polyelectrolyte complexes (polycation/protein) can efficiently enhance their immunogenecity.

The formation of PVP (R_2,R_{16})-BSA.E complexes is promoted by cooperative electrical (salt) attraction of oppositely charged polycation and protein molecules and by hydrophobic interactions.

A comparative study of immunogenic activity of BSA-steroid polycomplexes and BSA-steroid + lFA mixtures revealed that at the same level of immunogenicity, they differed in regard to the specificity of antibody produced. The PVP (R_2,R_{16})-BSA.E complexes were able to generate both estradiol-specific and BSA-specific antibodies (Figures. 153 and 154). However, BSA.E + lFA mixtures generated mainly estradiol-specific antibodies. Polycomplexes employed for immunization produced antibodies reactive with the native BSA. Such a response is determined possibly by an increase(s) in the immunogenicity of weak antigenic polypeptide or conformational determinants present on the surface of protein globules and/or by the representation of "dormant" determinants existing in the inner side. A decrease in fluorescence of BSA molecules in the complex formation with the polycations loaded by hydrophobic groups was observed recently (unpublished results). The conformational transitions as implicated by these results may expose dormant determinants and increase the immunogenicity of weak determinants (i.e., through clusterization of surface antigenic determinants on the polymer matrix by the formation of interpolymer complexes).

Table 29. Apparent dissociation constants (K_d) of monoclonal antibodies obtained using immunizations with PE-BSA.E versus BSA.E+IFA

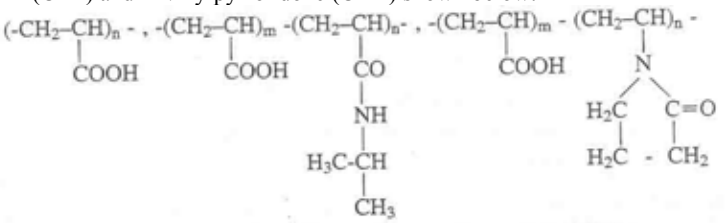
Monoclonal antibody	K_d *(M)*
PE–BSA.E	
2F3	6.5×10^{-7}
4F1	1.2×10^{-7}
8F9	8×10^{-6}
6B8	3.8×10^{-8}
6B11	7×10^{-8}
IFA–BSA.E	
8H7	3.3×10^{-8}
8H9	2×10^{-8}
10F4	1.6×10^{-7}
15D4	5.5×10^{-8}
14D2	3.8×10^{-6}
16G4	1.2×10^{-7}

In conclusion, a method is described for increasing the immune response to steroid hormones of immunological and practica1 interest. Selective use of the degreased polyelectrolytes and of other polyelectrolytes as well as of coupling methods may lead to more efferent use of weak antigens like steroid hormones and to a better understanding of the influence of the structure and orientation of polydeterminant antigens on the immunogenicity in polycomplexes and/or conjugates. Chemical modification of proteins and other bioactive molecules with PE can be used to "tailor" molecular properties to particular applications, eliminating disadvantageous properties or conferring new molecular functions. Finally as demonstrated in this study, the use of PE-BSA.E in immunizations can aid in the development of hybridomas and in the production of

monoclonal antibodies comparable in yield and affinity to those obtained through conventional immunizations using FIA.

Steroid hormone containing Cu(II)-mediated PEC. In the present study, the mechanism of the including of covalent conjugates of bovine serum albumin (BSA) with Estradiol (E) (BSAxE) and Progesterone (P) (BSAxP) hormones into Cu(II)-induced complexes of Polyacrylic acid (PAA) and nontoxic copolymers (CP) of acrylic acid with N-isopropylacrylamide and N-vinylpyrrolidone at the relatively low concentrations of metal ions have been investigated. The immunogenicity of steroid hormones and protein antigen in polyelectrolyte complexes, after a single immunization without traditional adjuvants was analyzed.

The polyelectrolyte components of this invention are the PAA and copolymers of AA with NIPAAm (CP-I) and *N*-vinylpyrrolidone (CP-2) shown below:



Monomer composition (m/n) VP/AA = 1:1;Molecular weights were 100kDa (PAA), 30kDa(CP-1) and 40kDa(CP-2).

To produce the polymer-metal complexes (PMC) (PAA-Cu(II) and CP-Cu(II)) the $CuSO_4x5H_2O$ (pH:4) solution was added to PE, dissolved in phosphate-buffered saline (PBS) . The pH values were adjusted with 1 M NaOH to desired pH. The ternary PE-Cu(II)-(proteinxhormone conjugate) complexes were, in turn, prepared by adding conjugate solution to the PE-Cu(II) solution.

The fraction composition of polymer-protein mixture was estimated by gel-filtration chromatography using Bio Sil Sec.250 column (7,8 X 30 cm). For spectrophotometric measurement, the UV- visible measurement (200-1000 nm) was carried out using a Shimadazu UV-I60 A spectrophotometer equipped with a temperature controlled attachment Proteins and their mixtures with PE were analyzed by polyacrylamide gel electrophoresis.

The complex formation between BSAxHormone and PE was investigated as described previously. At pH 7, both polyelectrolytes (PAA, copolymers of CPl and CP2) and BSAxHormone conjugates have negative electrical charges and are incapable of binding to one another; the divalent Cu(II) -ions acı as "fasteners" promoting the formation of fairly stable soluble Cu(II) -BSA and colloidal PE-Cu(II) -BSAxHormone ternary complexes. Starting with very low conjugate/PE ratios, that is, for example, C_{BSAxE}/C_{PE} 0.1 (C_{BSAxE} and C_{PE} -the concentration of protein-hormone conjugates and polymers in g/100 mL) and [Cu(II)]/[COOH] 0.1; (one Cu(II) mole per 10 mol-COOH groups of polymers), weakly water soluble (colloidal) polycomplexes was formed upon addition of BSA.E conjugate to PMC solution. After centrifugation, the fraction composition of mixtures was analyzed with UV spectrophotometry, electrophoresis, HPLC, and a Zeeman Atomic Adsorbtion spectrophotometer. Analysis of the matrix solution and of the sediment mixtures showed that Cu(II) mediated complex formation in all investigated systems in a similar manner. At low concentrations of the proteinxhapten covalent conjugates, free BSAxhapten fraction was absent, as part of the PE-Cu(II) complexes remained in matrix solution. When the ratio of components was similar, (C_{BSAxE}/C_{PE} =1.5-2.0 for the CPl and CP2 copolymers and 2.5-3.0 for the PAA homopolymer), free BSA.E as well as PMC were absent in the matrix solution of these mixtures. All components were obtained in the sediment of the mixtures. Therefore, under these conditions, all components were involved in the composition of polycomplex particles. Taking into consideration that the BSA.E and polymers (PAA, CPl and

CP2) at pH 7.0 do not form stable polycomplexes in the absence of Cu(II), we can ass time that the copper ions led to the binding of similarly (negative) charged protein hapten conjugates with polyanions by the formation of "cheIate" units in which the copper ion was central (Figure. 155).

The hydrophobic hapten containing BSA globules in each molecule of the polycomplex were apparently in contact with one another and cross-linked with a linear polyion via copper ions

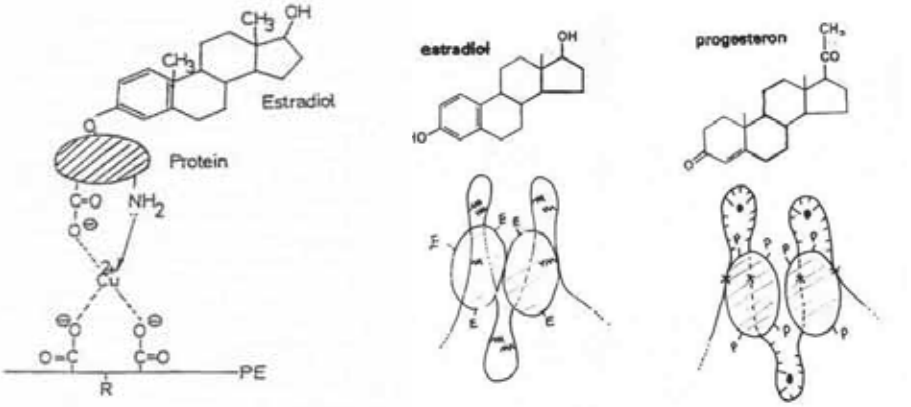


Figure 155. Hypothetical scheme of the structure of BSA.E conjugates with Polymer-metal complex. For more explanation see text

and CP2-Cu(II)-BSAxE complexes prepared at the ratios of components, $n_{BSA\ Hapten}/n_{PMC}$ =2.0 and $n_{Cu(II)}/n_{AA}$=0.2, were used for the immunization of mice. The dynamics of antibody formation, induced by free proteinxhapten conjugates and different Cu(II) -induced polycomplexes of these conjugates with anionic PE at different polycomplex doses are presented in Figures 158, 159 and 160. It can be seen from the data that a single immunization of mice with free BSAxHapten conjugates barely induced a primary immune response (production of antibodies). The immunization of mice with polycomplexes without traditional adjuvants even at the 3-4 time low antigen doses than free BSAxHapten led, in turn, to the development of a pronounced primary immune response to BSAxE and BSAxP correspondingly. The relative values of titers of antibodies $(OD_{405})_{exp}/(OD_{405})_{control}$ increased 2-to 5-fold and a broad peak of immune response was observed in the 10-70 day post immunization period with subsequent gradual decline.

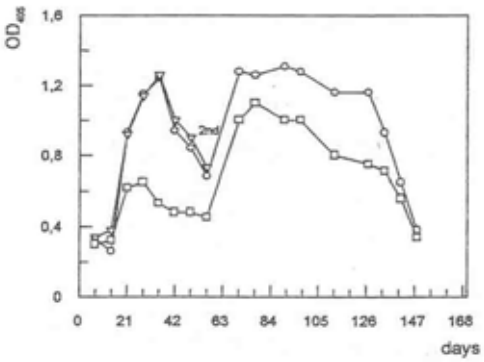


Figure 156. Estradiol-specific antibody response to PAA-Cu^{2+}-BSA.E complex and BSA.E conjugates. Mice were immunized with 100μg PAA-Cu^{2+}-BSA.E (0); 50μg PAA-Cu^{2+}-BSA.E (Δ); 100μg BSA.E (ف). Second immunizations were performed with BSA.E on day 56. Anti-estradiol antibody activity was measured with ELISA at weekly intervals

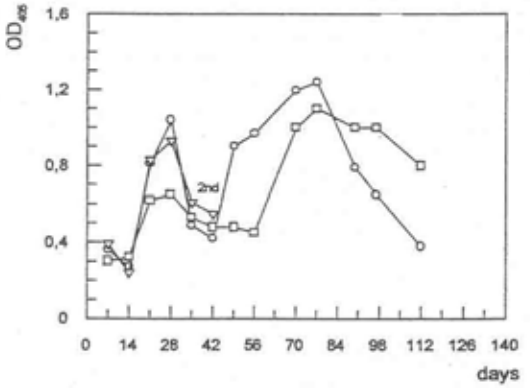


Figure 157. Estradiol-specific antibody response in mice immunized with CP1-Cu^{2+}-BSA.E complexes at different doses: 100μg CP1-Cu^{2+}-BSA.E (0); 50μg CP1-Cu^{2+}-BSA.E (Δ); and 100μg BSA.E alone (ڤ). Second immunizations were performed with BSA.E on day 42 Anti-estradiol antibody activity was measured with ELISA at weekly intervals.

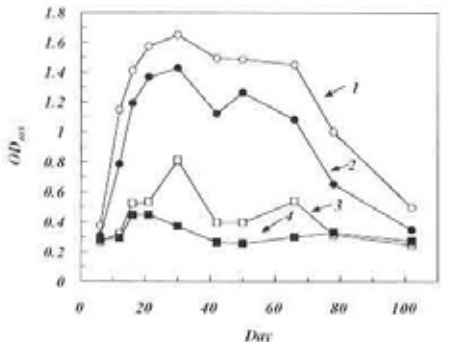


Figure 158. The dynamics of formation of P-specific (BSA.P-specific) antibodies (OD_{405}) in the blood sera of mice immunized with free BSA.P and PAA-Cu^{2+}-BSA.P complexes at different protein doses prepared by dilution of polycomplex solutions: 100μg (1); 50μg (2); 10μg (3) BSA-P in PAA-Cu^{2+}-BSA.P complexes and 50μg (4)-free BSA.P n_{BSA}/n_{PAA}=2.0 n_{Cu}/n_{AA}=0.20

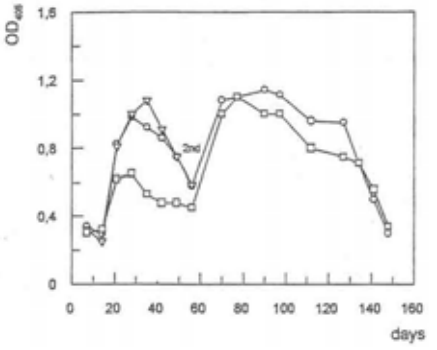


Figure 159. Estradiol-specific antibody response to CP2-Cu^{2+}-BSA.E complex and BSA.E conjugates. The groups of mice were given with 100μg CP2-Cu^{2+}-BSA.E (0); 50μg CP2-Cu^{2+}-BSA.E (Δ); and 100μg BSA.E (ڤ). Second immunizations were performed with BSA.E on day 56 Anti-estradiol antibody activity was measured with ELISA at weekly intervals

Moreover, antibody titers depending on the concentration of polycomplexes and the increase in the antigen dose caused a proportional increase in values of titers in the blood sera. The secondary immune responses observed following booster injections with the solutions of free BSA.E were characterized by a rapid rise and an extended duration, although the level of antibody production was not strongly different from the control experiments.

In a second experiment, the antibody response induced by BSAxP in the ternary polycomplexes was compared to the antibody response elicited by BSAxP in incomplete Freund's adjuvant. Antibody titers in both experimental groups increased sharply reaching a plateau after 3 weeks and subsequent decline. The levels of antibody titers were practically the same in both cases.

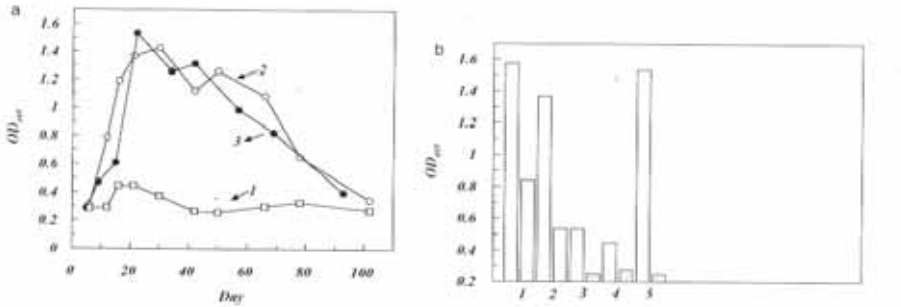


Figure 160. (a) Comparison of the dynamics of formation of P-specific antibodies (OD405) in the blood sera of mice, immunized with free BSA.P (1), PAA-Cu2 + BSA.P (2) and BSA.P +I.F.A. (3) mixtures $n_{BSA.P}$ n_{PAA}= 2.0. n_{Cu}/n_{AA}=0.20; protein dose is 50 μg, (b) Comparison of P- and BSA-specific antibody production induced by PAA-Cu^2 –BSA.P (1-3), by free BSA.P (4) and BSA.P + I.F.A (5) at day 21. Different doses of BSA.P (μg) used; 100 (1); 50 (2); 10 (3); (4) only BSA.P (50 μg); (5) BSA.P + I.F.A; (μg), Empty columns, BSA-P-reactive antibody activity: shaded columns, BSA-reactive antibody activity

Biopolymer systems were also able to generate both BSA- and BSAxHapten-specific antibodies simultaneously.

Table 30. Results of the fusions following immunizations based on use of BSA-progesterone-Cu^2 -PAA

	Fusion number				
	1	2	3	4	5
Number of spleen cells	1×10^8	1×10^8	12×10^8	2×10^8	18×10^7
Number of myeloma cells	1×10^7	1×10^7	12×10^7	2×10^7	18×10^6
Total number of wells at fusion	384	384	576	576	576
Number of hybrid cell clones	36	40	197	425	333
Number of hybrid cell clones having antibody activity	9	8	5	20	18
Number of hybrid cells producing specific antibody for progesterone	0	0	1	0	6

Table 31. Apparent dissociation constants, (Kd) and immunglobulin class of monoclonal antibodies obtained using immunizations with PAA- Cu^{2-} -BSA.P versus BSA.P+IFA

Monoclonal antibody	K_d (M)	Immunoglobulin class
PAA-Cu^{2+}-BSA.P		
MAM-6G10	1.3×10^{-7}	IgG2a
MAM-5F12	2.1×10^{-7}	IgG2a
MAM-3H8	9×10^{-8}	IgG2a
MAM-4H5	1.8×10^{-7}	IgG1
MAM-2B7	2.4×10^{-7}	IgG1
MAM-5G10	1.0×10^{-8}	IgG2a
MAM-4A4	7×10^{-7}	IgG2a
IFA-BSA.P		
MAM-2A9	1.0×10^{-8}	IgG1

Table 32. The rations of reactivities with estradiol antigens to reactivity with BSA $[(OD_{405})_E/(OD_{405})_{BSA}]$ in sera of mice immunized with BSA.E and different biopolymer systems

Biopolymer systems	$(OD_{405})_E/(OD_{405})_{BSA}$
BSA.E	1.1/0.3 = 3.7
BSA.E-Cu^{2+}-CP1	1.2/0.6 = 2
BSA.E-Cu^{2+}-CP2	1.25/0.58 = 2.1
BSA.E-Cu^{2+}-PAA	1.38/1.11 = 1.25

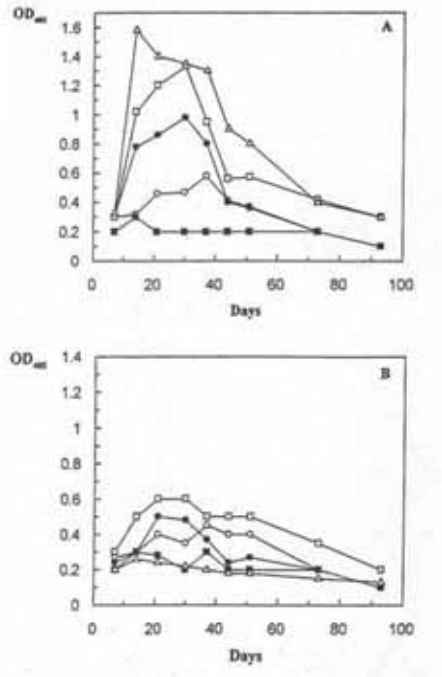


Figure 161. The dynamics of pcogestercee-specific (BSA.P-specific) and BSA-specific setibody formation [as assayed by ELISA (OD_{405})], induced by free BSA.P, BSA.P-IFA, and by polycomplexes of BSA.P-Cu_2*-CPI at different protein doses; 10 μg (antigen dose 3 μg) (○), 50 μg (antigen dose 15 μg) (●), 100 μg (antigen 30 μg) (□), 100 μg free BSA.P (■), prepared by dilation of polycomplexes solution, (Δ) Mixture of BSA.P with IFA with protein dose of 100 μg (A) Plate was couted with BSA.P and (B) Plate was couted with BSA

Table 33. Apparent dissociation constants (K_d), and immunoglobulin class of mabs obtained after using immunizations with BSA.P-Cu^{2+}-CP1 polycomplexes and BSA.P+ IFA system

Monoclonal antibody	K_d	*Immunoglobulin class*
BSA.P-Cu^{2+} CP1		
MAM 6G11	1.7×10^7	IgG_1
MAM 4G8	8×10^7	IgG_1
MAM 4B7	5×10^7	IgG_1
BSA.P+ IFA		
MAM2 A9	$1. \times 10^7$	IgG_1
MAM4 H10	1.6×10^7	IgG_{2a}

The specificity of antibodies formed was determined on plates coated in parallel with BSAxHapten conjugates and free BSA. The relative titers of estradiol- and BSA-specific antibodies E_{OD}/BSA_{OD} for the BSA.E conjugate and different polycomplexes obtained after the second immunization (90 days after primary immunization) are summarized in Table. The antibodies generated by BSA.E reacted with the estradiol antigen about four-fold more intensively than with BSA. However, reaction of the antibodies generated in the biopolymer systems with BSA alone was more intensive, amounting to about 50% (CP1 and CP2 polymers) and more than

80% (PAA polymers) of the reaction observed with BSA.E as antigen. These data show that polycomplexes were able to simultaneously generate both estradiol- as well as BSA-specific antibodies. Such a creator immune response to BSA in PAA-Cu(II)-BSA Biopolymer system may be conditioned by the stronger interaction of PAA-Cu(II) with BSAxE molecules. The conformational transitions, as implicated by strong interactions between components, may expose dormant determinants and increase the immunogenicity of weak determinants (i.e., through clusterization of surface antigenic determinants on the polymer matrix by the formation of ternary polymer-metal-protein complexes). For the mixture PAA-Cu(II)-BSAxP the antibody generated by this complexes reacted with the same antigen about twice more intensively than with BSA. However, the reaction of the antibodies generated in the adjuvant-mediated system with BSA alone was less intensive, amounting to about 15% of the reaction observed with BSAxP as antigen.

Table 34. Comparison of total and antibody-producing hybrids obtained after fusions using immunization with BSA.P-Cu^{2+}-Cp1 versus BSA.P+IFA

Fusion	*Total hybrid number/total well*	*Progesterone reactive hybrid number*	*Progesterone-specific hybrid number*
BSA.P-Cu^{2+} CP1			
1	67/576	8	2
2	100/576	7	1
BSA.P+ IFA			
1	36/384	9	1
2	40/384	8	1

Cell fusions were performed using the spleen from a BALB/c donor which had received a booster injection 2 weeks after the primary intravenous injection by PAA-Cu(II) – BSAxP and,CP1-Cu(II)-BSAxP ternary complexes and BSAxP-IFA mixture.. The results of these fusions are summarized in Table. Of 60 progesterone-reactive clones obtained in five fusions, 7 proved to be progesterone-specific for PAA-Cu(II)-BSAxP complex and 3 progesterone-specific Mabs obtained for CP1-Cu(II)-BSAxP complex. They revealed, as tested in ELISA, moderate to negligible reactivity with estradiol, aldosterone, corticosterone, and testosterone as well as with BSA (the ratios of relativities with non-progesterone antigens to reactivity with progesterone, A_{405} (non-progesterone)/ A_{405} (progesterone) = 0.1-0.2 and 0.06-0.2 for PAA and CP1 complexes correspondingly).

From the analysis of supernatant samples, 10 progesterone-specific monoclonal antibodies obtained after these fusions were found to be of IgG class, six of which were IgG2a and four IgG_1 type. All of these antibodies contained kappa light chains. The affinities of these ten antibodies for progesterone were subsequently determined by equilibrium dialysis. Affinity constants *(Kd,* M) measured for the purified monoclonal antibody are shown in Table 31 and 33. Tables show comparisons of antibody-producing hybrids obtained following immunization with polycomplexes and BSAxP +IFA system. The affinity constant was determined also for the antibodies obtained immunization of mice with BSA.P + IFA followed by two booster injections with BSA.P + lFA. Hence, the antibodies obtained by the two different immunization methods did not appear to have considerable differences in terms of the determined affinities.

We have shown that the inclusion in Cu(II) -induced polyanion-protein complexes can efficiently enhance the immunogenicity of estradiol and progesterone steroid hormones. In solutions, complex particles are formed with low concentrations of transient metal ions playing the role of a cross-linking agent between the appropriate functional groups of negatively charged complex components. The physico-chemical mechanism underlying the immunological activity of protein (hapten) complexes with polyelectrolytes lies, in all probability, in the high cooperative absorptive capacity of free sites (loops) of PE. These sites, which are not involved in the

interaction with the protein molecules, may be situated on the heterofunctional surface of negatively charged membranes of immunocompetent cells. In our case, the PE-Cu(II) carrier is implicated to facilitate and stabilize the interactions between the antigenic substance and B-lymphocytes via positively charged fragments of polycomplexes, so triggering the immune response. Cu(II) ions play, probably, the main role in the stabilization of the antigen bound on the surface.

A comparative study of immunogenic properties of BSAxsteroid polycomplexes and of BSAxsteroid + lFA mixtures revealed that at the same level of immunogenicity, they differed in regards to the specificity of the antibody produced. The ternary polycomplexes were able to generate both E-, P-specific and BSA-specific antibodies, where as BSAxsteroid + lFA mixtures generated mainly E- and P-specific antibodies. Polycomplexes employed for immunizations thus appear to induce antibodies reactive with the native BSA. A decrease in the fluorescence of BSA molecules during Cu(II) -induced complex formation with the polyanions was observed recently (unpublished results). The conformational transitions as implicated by these results may expose dormant determinants and/or increase the immunogenicity of weak determinants, i.e. through clustering of surface antigenic determinants on the polymer matrix by the formation of interpolymer complexes.

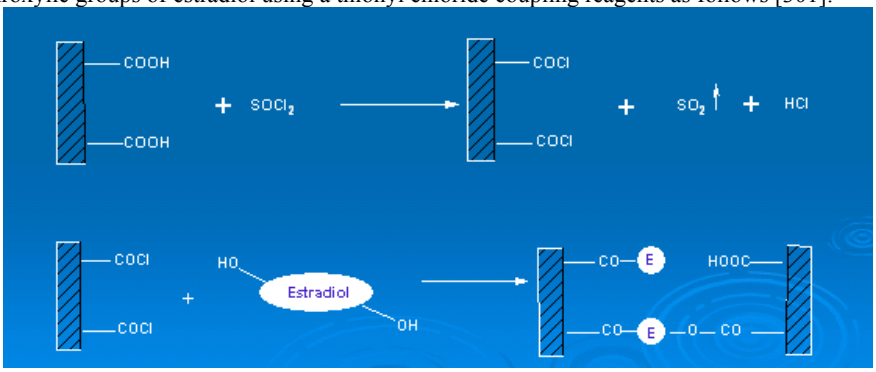
It should be noted that these polycomplexes stimulate the production of polyclonal antibodies. At the same time, the technique of raising monoclonal antibodies provided the possibility to determine the specificity and affinity of the antibodies to estradiol and progesterone produced by using synthetic polyelectrolytes as carrier (adjuvant activity and structure formed) compared to the conventional Freund's adjuvants. The ten antibodies raised against the 3-(O-carboxymethyl)-oxime antigen after a short immunization procedure showed similar results in terms of specificity and affinity to those raised against 6- and 11- hydroxy-progesterone hemisuccinate conjugates.

In conclusion, a method is described for increasing the immune response to steroid hormones and production of monoclonal antibodies of immunological and practical interest. As demonstrated in this study, the use polycomplexes in immunizations can aid in the development of hybridomas and in the production of Mabs comparable in yield and affinity to those obtained through conventional immunizations using Incomplete Freund’s adjuvant. Selective use of the described and of other polyelectrolytes as well as of coupling methods may lead to a more efficient use of weak antigens and to a better understanding of the influence of the structure and orientation of polydeterminant antigens on the immunogenicity in polycomplexes as well as to the production of monoclonal antibodies to other haptens. The surplus of Cu(II) ions induced crosslinking self-assembly of polycomplex particles, which led to a “leveling off” the effect of polymer chain molecular mass on structure formation and to an effective increase in the stability of such Biopolymer Systems. Therefore, the macromolecular structure of the polycomplex particles and the immunogenicity of the Biopolymer systems formed did not depend on the compositional heterogenicity and the molecular mass of the polymeric chain. This Cu(II)-induced crosslinking procedure is universal and opens new approaches for the creation of universal polymeric carriers , which could be used to bind a wide range of polymeric substances to various antigens, thus enabling various immunogenic Biopolymer systems to be created. These polycomplexes can be obtained by technologically simple procedures, e.g., in a single step by mixing solutions of the polyelectrolyte, protein, and Cu(II) ions (absence of free components in mixture and no additional reagents or purification procedures are required). Chemical modifications of proteins and other bioactive molecules with PE can be used to 'tailor' molecular properties to particular applications, eliminating disadvantageous properties or conferring new molecular functions.

In recent years, much research has focused on the development of combined vaccines, which reduce the number of vaccine administrations and thereby, the cost of vaccination. Our data obtained with different Biopolymer systems shown here reveal the generation of an antibody

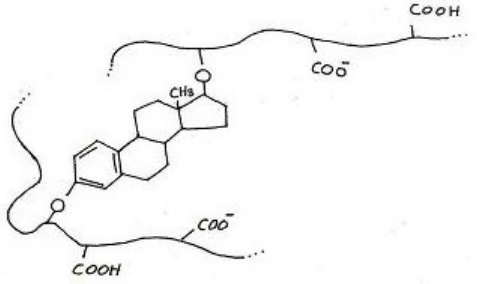
response to both protein and hapten when the antigens (BSAxHapten) are administered with polymeric systems. These results may suggest that polymeric carriers may be promising adjuvants in approaches to combined vaccines. On the contrary, injection of estradiol and progesterone with protein carrier or with Freund's mineral oil (FIA), produced only steroid hormone-specific antibodies Keny et al. [286] and Gonzales et al. [287] showed that preparation of antigen in FIA led to the production of antibodies reacting better with epitops present in the unfolded protein and suggested that the emulsification process itself led to unfolding of the antigens, resulting in antibodies that prefentially recognize linear epitops. However, in our experiments, the production of only estradiol-specific antibodies with FIA indicates that the antigen (BSAxE) was present in its native form.

Estradiol containing polymer gels. A new estradiol comprising negatively charged network was synthesized by the covalently cross-linking of carboxylic groups of PAA and copolymers of acrylic acid with N-isopropylacrylamide (CP-1) and N-vinylpyrrolidone (CP-2), to two hydroxylic groups of estradiol using a thionyl chloride coupling reagents as follows [301]:



The formation of polychloranhydryde after the mixing of polymers and thyonil chloride was accompanied by the educe of gaseous SO_2.This allows to synthesize the polymer-estradiol conjugates by technologically simple procedures-in a single step by mixing the solutions of the selected components.

The products were analyzed by FT-IR, thermo gravimetric and element analysis techniques. Swelling behaviour of PE-estradiol conjugates was investigated in neutral water. Conjugates were dried *in vacuo* and the net weight, Wo, determined. The dried gels were swollen in neutral water at room temperature and then weighted to determine the equilibrium weight, W. The weight-swelling ratio *(WIWo)* is equal to 2.5 for PAA-estradiol conjugate.



It was suggested that the coupling reaction between PE and estradiol should be an intra- and intermacromolecular exchange reaction (the formation of ester bonds) with the participation of both hydroxylic groups of estradiol sequentially and simultaneously with the formation of cross-linked negatively charged network. In aqueous media the fragments of polyanionic chains that are not directly involved in covalent bond exists in the form of free "loops", which dissolve

in water and turn the network into polymer gel. The resulting hydrogels are composed of hydrophobic estradiol core surrounded by the hydrophilic polyanions as corona.

The immunizations were carried out in male BALB/c mice and four mice were used for each group. Because of insolubility in water, PE-estradiol gels were implanted into mice subcutaneously at a dose of 1 mg/mouse after washing the conjugate material in 70% alcohol before implantation. Other groups were immunized subcutaneously with *100g* of BSA.E, IFA + *E,* and BSA.E + IFA. All groups were followed up for development of antibody activity for estradiol for a period of 50-70 days after primary immunization.

The indirect enzyme-linked immunoadsorbent assay (ELISA) was used to detect antibody activity for estradiol. Two mice were selected from the group immunized with subcutaneously implanted PAA-estradiol gels for fusion and the standard fusion protocol was followed. The affinities of MAbs were measured by equilibrium dialysis by using [^{3}H] 17-estradiol (NEN, specific activity 87 *Ci/mmol).* Purified MAbs and radiolabelled 17-estradiol were incubated in equilibrium dialysis cells for 20 h at room temperature under slow shaking. An LKB Wallac 1212 Rackbeta liquid scintillation counter was used for radioactivity measurements, and slopes from the data were determined by using a computer graphics program. Statistical analysis of the experimental data was performed by using a computer graphics program.

The dynamics of formation of estradiol-specific antibodies *(OD_{405})* in blood sera of mice immunized with PAA-estradiol gels, BSA-estradiol conjugates, estradiol + lFA, and BSA-estradiol + lFA mixtures are presented in Figure. It can be seen from these data, determined by ELlSA, that a single immunization of mice with E + lFA mixtures barely induced production of antibodies.

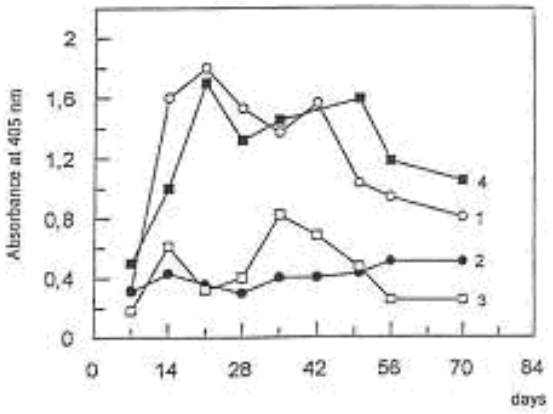


Figure 162. The dynamics of formation of 17β-estradiol specific antibodies (OD_{405}) in the blood sera of mice immunized with subcutaneously implanted PAA-estradiol gels (I), BSA.E (2), E + IFA mixtures (3), and BSA.E + IFA (4). Hapten dose is 100 μg

A subcutaneous administration of, 17β-estradiol trapped in polymer gels without traditional adjuvants led in turn to the development of a pronounced primary estradiol-specific immune response. The peak of the immune response was observed on days 14-40 postimmunization with subsequent decline by Day 50.

Cell fusions were performed using the spleen from *BALB/c* donors after the primary subcutaneous implantation of PAA-estradiol gels. Total number of hybrid cells obtained in two fusions was 1258 and of 60 reactive clones, 10 proved to be estradiol specific. They revealed, as tested by ELISA, moderate to negligible reactivity with progesterone, corticosterone, aldosterone, testosterone as well as with BSA (the ratios of reactivities with nonestradiol antigens [haptens] to reactivity with estradiol, A_{405}(non-estradiol/A_{405}(estradiol) = 0.1-0.4 (Table) The reactivities of

some of the antibodies with corticosterone were, however, more pronounced: A_{405}(corticosterone)/ A_{405}(estradiol) = 0.15-0.9.

From the analysis of supernatant samples, of the 10 estradiol-specific MAbs obtained after these fusions, 7 were found to be of IgM and 3 of IgG classes. The affinities of the estradiol-specific antibodies were subsequently determined by equilibrium dialysis. Dissociation constants K_d ranged between 1.2 x 10^{-7}-8x10^{-8} M (Table 35).

Table 35. Apparent dissociation constants (K_d) of monoclonal antibodies obtained using subcutaneous implantation of 17β-estradiol comprising polymer gels I

Monoclonal antibody	K_d *(M)*
1F10	$2.9 \pm 0.4 \times 10^{-7}$
4G5	$1.6 \pm 0.2 \times 10^{-7}$
2H4	$7 \pm 0.06 \times 10^{-8}$
16D7	$1.2 \pm 0.2 \times 10^{-7}$
1A6	$1.4 \pm 0.1 \times 10^{-7}$
7C4	$8 \pm 0.05 \times 10^{-8}$

We have shown that, the inclusion in polymer gels can efficiently enhance the immunogenicity of 17-estradiol. A single immunization without traditional adjuvants with subsequent fusions gave rise to the development of estradiol-specific MAbs.

The formation of PAA-estradiol gels is promoted by the covalent cross-linking of carboxyl groups of polyanionic PE and two hydroxyl groups of 17-estradiol using a thionyl chloride as coupling agent. In aqueous media fragments of polyanion not directly involved in covalent bond exists in the form of free "loops," which dissolve in water and turn the network into polymer gels, the resulting hydrogels are composed of hydrophobic estradiol core surrounded by the hydrophilic polyanions as corona.

The physicochemical mechanism(s) underlying the immunogenic activity of hormone comprising polymer gels may be related to an adjuvant effect of polymeric macromolecules. Polymeric gels, as a soft and wet biocompatible delivery system, slowly release antigens into the tissue of vaccinated individuals (prolonging effect). Moreover, free sites on the gels may have the capacity, via charged fragments, to interact with the membranes of immunocompotent cells (adjuvant effect). This may facilitate and stabilize the interaction of polymer-bound antigen (high epitope density) with specific cell receptors and enhance the immune response. An increase in the immunogenicity of estradiol trapped in polymer gels was also obtained by direct cross-linking of 17-estradiol with other polyelectrolytes (CP-1 and CP-2) although the immunological activity and swelling effect were slightly lower than in PAA-gels (data not shown). Coupling of estradiol-like haptens to polymers that are modified in their chemical composition might become a general method, extending the use of hapten comprising polymer gels in immunology.

5.8.2.Novel Betulin Conjugates

Betulin and its derivates have been evaluated as cancer and AIDS reagents and have been found to selectively kill human melanoma cells as well as inhibit HIV replication in lymphocyte cells [304]. In addition, betulinic acid has antibacterial and antimalarial properties, low toxicity, and is relatively inexpensive. It is abundantly available from the bark of white birch trees in the form of betulin [305] (Figure 163). The compound is presently undergoing preclinical development. Synthesis and structure-activity relationships of betulin derivatives, as redox cycling agents, were with low molecular weight organic compounds as anti HIV agents and hepatoprotective activity have been studied [304,306-308]. A number of betulin esters were shown to exhibit a pronounced

hepatoprotective effect [307] and protective effects against the cytotoxicity of cadmium in hepatite cells [305].

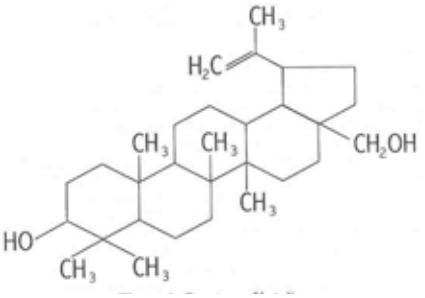
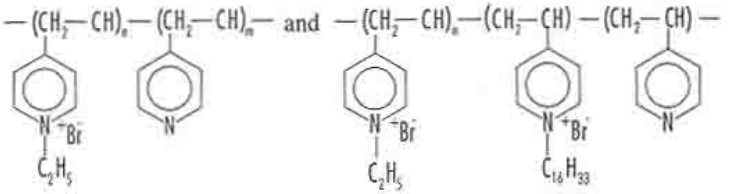


Figure 163. Structure of betulin

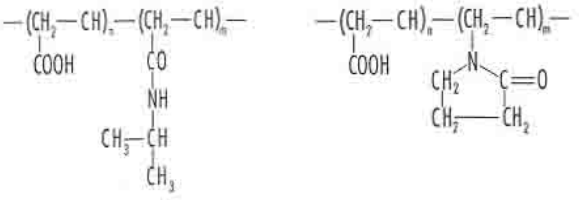
Nitroaromatic betulin derivates as redox cycling agents were single-electron reduced by ferrodoxin: NADP + reductase and flavocytochrome b2 at rates comparable with their simple structured analogs [309]. Poly(vinylpyrrolidone) and Proxanol polymers solubilize betulinic acid from betulin with the same efficiency as liposome's [310]. However, until now there have been no reports of modifications to allow construction of immunogenic polymer-betulin conjugates.

This paper reports the synthesis of novel betulin containing polyelectrolyte conjugates (PEC) by two methods [311]: (1) betulin covalently crosslinked directly to non-immunogenic and non-toxic anionic PE-copolymers of acrylic acid with N-isopropylacrylamide and N-vinylpyrrolidone; (2) betulin covalently crosslinked to bovine serum albumin (BSA*Betulin) forming a water-soluble bioconjugate which was complexed with the synthetic polycationpoly (N-alkyl-4-vinylpyridinium bromide). The immunogenicity of BSA*B and betulin in PEC was found in terms of antibody titer in the serum of the immunized animal.

The conjugates of anionic PE with betulin (B) (PE-B) were synthesized by covalently crosslinking the carboxyl groups of PE to the hydroxyl groups of betulin using thionyl chloride as the coupling agent. The formation of polychloranhydryde in the mixtures of PE with betulin and thionyl chloride was accompanied by the evolution of gaseous SO_2. The synthesis of the chloroanhydride of CP1 and CP2 copolymers and their coupling to betulin was carried out in one step. The total quaternization degree for the PEVP and PECVP used was about 90% [34,35].



The anionic PE were copolymers of acrylic acid (AA) with N-isopropylacrylamide (NIPAAm) (CP1) and N-vinylpyrrolidone (VP) (CP2).



The conjugate products were analyzed by FT-IR (Figure 164). The IR spectrum of betulin was characterized by the absorption at 3900 cm^{-1} (hydroxyl group), 2900 cm^{-1} (alkanes), 1795, 1475 cm^{-1} (aromatic C=C ring) and the distinctly split band at 1000 cm^{-1} ($C=CH_2$ stretching). The IR spectrum of CP1 was characterized by relatively broad hydrogen bonded –OH peak at 3410-3440 cm^{-1} (OH-stretching). The assignments for other major IR bands were 1718, 1700, 1695 cm^{-1} (carboxylic acid, C=O stretching), 1680, 1670-1640 cm^{-1} (O=C-NH-stretching) and 1450-1200 cm^{-1} (C-O stretching coupled with O-H in-plane bending.).

Comparing the IR spectra of betulin, CP1, the physical mixture CP1+betulin and the reaction products showed that for the CP1-B conjugate system the band at 1580 cm^{-1} disappeared, the intensity of 1770 cm^{-1} bands strongly decreased and shifted to 1730 cm^{-1} (C=O stretching), the intensity of the bands 1220, 1226 and 1100 cm^{-1} (C-O-C stretching) increased and changed their character, these were assigned to CP1-coupled betulin (Figureure3). The reaction products spectra obtained for CP2-B mixtures have absorbances at 1724 cm^{-1} (C=O stretch in esters), 1525 cm^{-1} (C-N stretch), 1464 cm^{-1} (C-O-C stretch), and 1300-1200 cm^{-1} (-C-N or –C-O-C asymmetric stretch). These results suggest that the covalent conjugation reaction between chloroanhydride of CP2 and betulin took place.

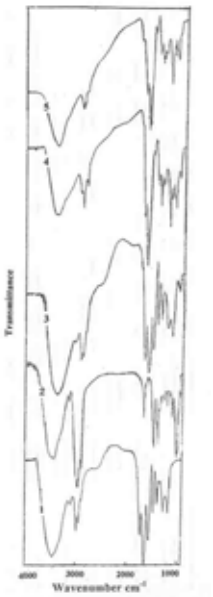


Figure 164. FT-IR spectra of CP1 (1), betulin (2), CP1+betulin physical mixture (3), and the products obtained from the CP1+betulin+$SOCl_2$ mixture at different contents of $SOCl_2$ 1 mL (4) and 0.183 mL (5)

The CP2-B conjugate was poorly water-soluble (colloidal) in neutral water (pH 7), apparently because of the hydrophilic pyrrolidone rings (and/or remains of acrylic acid) in composition of CP2-B conjugate. At the higher initial ratio of betulin to AA in the conjugates were practically insoluble in different polar and nonpolar solvents similar to the conjugate of CP1-B. The N-isopropylacrylamide monomer units increased the hydrophobicity of CP1-B conjugates and additionally decreased their solubility. The poly(n-isopropylacrylamide-co-acrylic acid) which was used as a carrier for the production of CP1-B conjugates, possesses dehydration behavior with increasing solution temperature from N-isopropyl groups. It seems that these conjugates also acquire a negative temperature solubility coefficient.

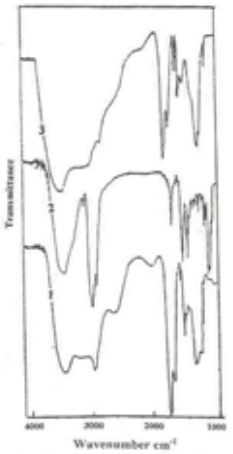


Figure 165. FT-IR spectra of CP2 (1), betulin (2), and the products obtained from the CP1+betulin+$SOCl_2$ mixture (3)

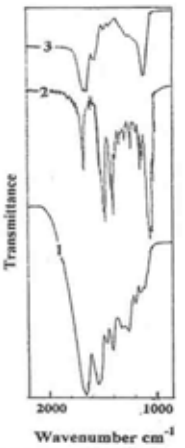


Figure 166. FT-IR spectra of BSA (1), betulin (2), and the products obtained from the BSA + betulin + carbodiimide mixture (3).

In contrast to the PE-B conjugates, the products from the reaction of BSA with betulin by carbodiimide coupling were mainly water-soluble. The FT-IR of the soluble products of the BSA* B conjugate is shown in Figure 166. The BSA spectrum has absorbances at 1580 and 1650 cm^{-1}. In the BSA*B spectrum the band at 1580 cm^{-1} disappeared and the band 1000 cm^{-1} ($C=CH_2$ strongly stretched) appeared, which was assigned to BSA coupled betulin. As seen in Figure 167, the UV spectrum of BSA*B conjugate shows one peak at 280nm corresponding to free BSA in solution, but the character of the absorption spectrum versus wavelength indicate the formation of aggregates.

The HPLC results for the solution of free BSA and BSA*B conjugate prepared at $n_B/n_{BSA}=20$ are shown in Figure 168. The BSA*B was characterized in the chromatograms by two peaks. The retention time (RT) corresponding to peak I in chromatogram 2 is essentially different from the values of RT of free BSA. The RT value of the fraction II (curve 2) in chromatogram 2 has a closer RT to that of free BSA but shows a wider molecular weight distribution. In Figure 168, the fraction, corresponding to peak I, moved more slowly than the free BSA molecules. One may assume that peak I corresponds to the reaction products of BSA*B conjugates, and peak II free BSA molecules (and/or BSA containing a few betulin molecules).

These data indicate that the molecular weight of the molecules forming the conjugate is essentially higher than that of free BSA molecules and that the betulin molecules were unevenly distributed between the protein molecules. Some protein globules may bind the maximum quantity of betulin molecules possible under given conditions, while the others remain practically unpopulated. This type of distribution was found previously in different complex forming systems [35]. Such cooperative populations were obtained at the interactions of BSA with Cu^{+2} in neutral water (Cu^{+2} ions were unevenly distributed between the protein molecules) and covalent binding of BSA with linear synthetic polyelectrolytes by carbodiimide [153,312]. In that last case, the role of the matrix was to place the linear chains of PE macromolecules and protein globules nonrandomly between polymer chains. The reason for the demonstrated disturbance in the randomness of the distribution of the BSA*B-carbodiimide systems is probably due to a hydrophobic interaction of the hydrophobic betulin molecules bound by one protein globule. These hydrophobic fragments on the surface of BSA can act more effectively as a cross-linking agent between two (or more) protein globules. This intermolecular interaction leads to the formation of a soluble conjugate aggregate with a complicated structure.

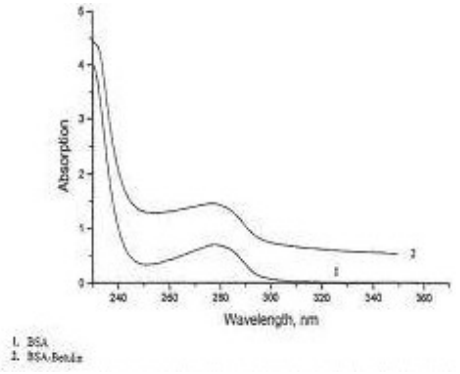


Figure 167. The adsorbance spectra of solution of BSA (1) and the product obtained frost the BSA+ betulin + carbodiimide mixture (2) at pH 7. BSA concentration in the both cases 1mg/1ml

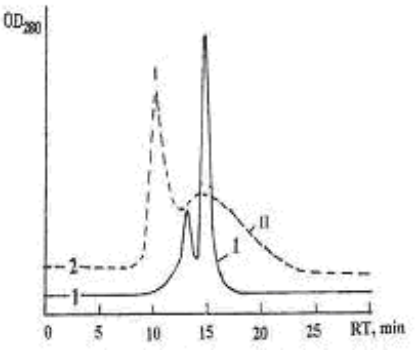


Figure 168. HPLC analysis of the solutions of pure BSA (1) and the product obtained from the BSA+betulin+carbodiimide mixture (2) prepared in phosphate buffer (pH 7); Uv – 280nm; 0.7mg/mL; 40gLc 1mL/min

Shown in Figure 169 are fluorescence spectra of free BSA and BSA*B conjugates. It is well known that tryptophan (Trp) fluorescence of protein varies with their conformational changes resulting in changes in the fluorescence parameters, such as the emission maximum (λ_{max}), quantum yield and lifetime [137].

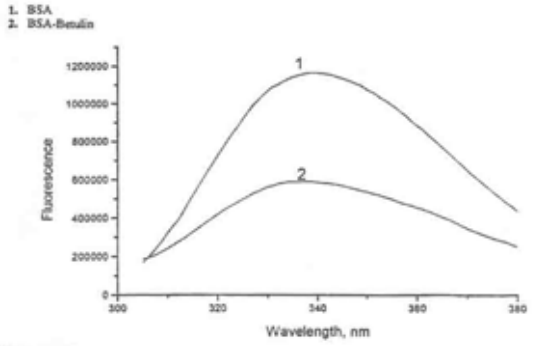


Figure 169. Fluorescence spectra of pure BSA (1) and the product obtained from the BSA+betulin+carbodiimide mixture (2), prepared in phosphate buffer (pH 7), BSA concentration in both solution were 0.71 mg/ml

As seen in Figure 169, the fluorescence intensity (I_{max}) of BSA at pH 7 decreases (quenching) and shows a marked blue shift of the λ_{max} for free BSA and BSA*B conjugate from 340 and 330 mn, correspondingly, BSA contains two Trp [92]. One Trp that has a λ_{max}=340-342 nm and half width 53-55 nm [310] is located on the bottom of the BSA hydrophobic cleft. The second Trp (λ_{max}=350-352 nm, half width $\Delta\lambda$=59-61 nm) has a low quantum yield (1/5 of the total BSA fluorescence) is located exteriorly and completely accessible to aqueous solvent. The results in Figure 169 indicate that in the conjugate formed, BSA*B tryptophanyls were completely isolated from water by the covalently bound betulin, which seems to cover all of the BSA surface. The results obtained by the analysis of the overall physico-chemical measurements are confirmed by the fluorescence analysis.

The complex formation between BSA*B conjugates and PE was first investigated by Mustafaev et al. [92,311]. BSA*B conjugate molecules were found to interact with polycations–

alkylated poly-(4-vinyl pyridines)- to form poor water-soluble (colloidal) protein-PE complexes. Starting with low BSA*B/PE ratios ($n_{BSA.B}/n_{PE}$=0,1), a phase separation took place in both systems, (PEVP-BSA*B and PECVP-BSA*B), at pH 7. Analysis of these mixtures was carried out with spectrophotometric, electrophotometric, and chromatographic (HPLC) methods. Only the presence of one substance corresponding to free polycations produced absorption at 254 nm in the mixtures of PE-(BSA*B); absorption at 280 nm corresponding to free BSA*B or a soluble PE-protein complex was absent. When the ration of components $n_{BSA.B}/n_{PE}$ was 2.0, free BSA*B conjugates as well as PEVP and PECVP were absent in the matrix solution of these mixtures. All components were in the sediment of the mixtures. Thus, the binding of added BSA*B to PE resulted in the formation of a poorly soluble PE-BSA*B complex. When $n_{BSA.B}/n_{PE}$=1, the free fractions of PE under these conditions indicate a nonrandom distribution of the BSA*B molecules between the coils of polycations (self-assembly of polycomplex particles).

This type of distribution was previously found upon complexation of globular proteins with oppositely charged polycations in aqueous solutions [34]. The demonstrated disturbance of the randomness of the distribution in PE-Protein solutions appears to be due to a positive interaction between the proteins globules absorbed by one chain. In our cage, the hydrophobicity of BSA*B is higher than that of BSA; therefore, the proposed mechanism is possible.

The formation dynamics of betulin-specific antibodies (OD 405 nm) in blood serum of mice immunized with CP1-B and CP2-B conjugates and free betulin are presented in Figure 170. These data, determined by ELISA, show that a single immunization of mice with free betulin barely induced production of antibodies.

A subcutaneous administration of betulin trapped in a polymer precipitant without traditional adjuvants led in turn to the development of a pronounced primary betulin-specific immune response. The peaks due to the immune response were observed on days 7-14 for CP1-B and 7-30 for CP2-B post immunization, respectively with subsequent decline by day 35. The best results were obtained with CP2-B conjugates which were more hydrophilic due to the N-vinyl pyrrolidone monomer units in the composition while the CP1-B conjugates contained more hydrophobic N-isopropylacrylamide monomer units.

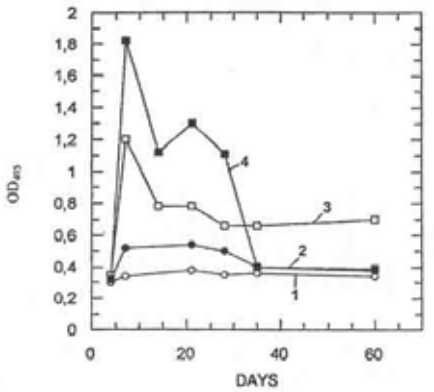


Figure 170. The kinetics of betulin-specific antibody formation (OD_{405}) in blood of sera of mice immunized subcutaneously with betulin (1), CP1-B (2) and CP2-B conjugates (3); 1-control, nonimmunized mice; Dose 1 mg conjugate/mouse

The dynamics of the antibody formation induced by intravenous administration of a colloidal sample of CP2-B conjugates and water-soluble BSA*B conjugates are compared in Figure 171. The intravenous route of immunization of mice with CP2-B conjugates as well as intradermal routes displayed an essentially higher betulin-specific immunogenic activity. As betulin is not water-soluble, we could not immunize mice with betulin intravenously. It is

noteworthy that the administration of BSA*B conjugates also gave rise to high immunological activity with maximal level of immunogenicity duration of 60 days which is longer than that of the polymer-betulin conjugates (40 days).

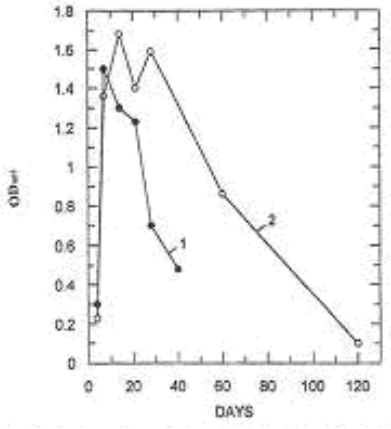


Figure 171. The kinetics of betolin-specific antibody formation (OD_{405}) in blood of mice immunized intravenously with CP2-Betulin (1) and BSA-Betulin (2) conjugates. The results by immunization with pure betulin (Figure 7) (2); Dose 1 mg conjugates

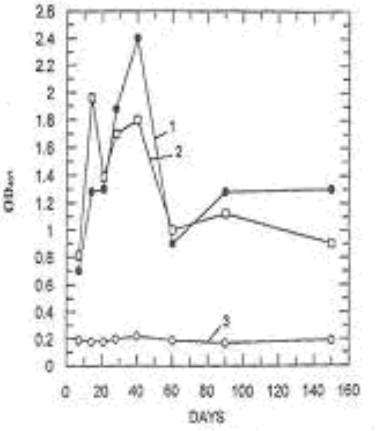


Figure 172. The kinetics of betolin-specific antibody formation (OD_{405}) in mice serum immunized intravenously with PEVP-BSA B (1) and PECVP-BSA B (2) diseases. 3 – nonimmunized mice; dose 700 μg for coch mice

The polycomplexes PE-BSA*B ($n_{BSA}./n_{PE}$ = 2), with two molecules of BSA*B bound by one polycation chain, were used for the immunization in the next series of experiments. The dynamics of the antibody formation, induced by polycomplexes PEVP-BSA*B and PECVP-BSA*B after intravenous administration of samples are presented in Figure 172. It can be seen from the data that the level of immunogenicity of the BSA*B conjugates was not essentially changed after complexing with polycations. In mice, immunized with PE-BSA *B, the antibody activity increased for 10 days and then kept that same level for up to 40 days. A comparative study of the immunogenic activity of BSA*B conjugates and their polycomplexes with polycations revealed that they had similar levels of immunogenicity at the time of administration; they differed with regard to the duration of immunogenicity: The immunogenicity of the BSA*B conjugates strongly decreased with time while the PE-BSA*B complexes were able to generate betulin-specific antibodies for 160 days.

5.9. From artificial immunogens to vaccinating macromolecules

If individual antigens or antigen determinants of pathogens acquire a capability to induce a sufficient immune protection as a result of binding with a polymer stimulant, then, we are facing a discovery of a way for designing of artificial vaccines, may be against still unconquered infections.

Salmonella typhimurium. One group of investigations was carried out using moose typhus as a model infection, which is caused by *Salmonella typhimurium,* and is typical of mice [313] Mice were primary immunized with the conjugates of PAA or CP(VPD-AA) with H-antigen and polysaccharide of 0-antigen of *Salmonella typhimurium* in dosage varying from 1 to 625 μg. After two weeks, all animals were infected with a certain virulent strain of these microbes in doses of 1-5x10^6 of microbial cells, which represent a 20-100 LD_{50}, that is an absolutely mortal

dosage. After introduction of such doses of microbes to intact (control) animals 100% mortality was observed within the first 5-7 days. The antigens conjugated with PEs protected the animals from death practically in all of used doses. The pure antigens particularly the *Salmonella* polysaccharide protected the animals only upon the introduction of rather high doses.

Innfluenza. In other investigations [9,9a,28,180,171,314-318] Cu(II)-induced complexes and covalent conjugates of the individual antigens: hemaglutinin (HA) or mixture of HA of the influenza virus, which imitate subunit influenza vaccine and protein fraction of BCG(TPF) ,with copolymers of MVP with AA (CP-1), VPD with AA (CP-2) and VPD with MA (CP-3) as well as with the homopolymer-PVI were prepared. Immunization of mice with such complexes and conjugates leads to 50-100 times increase in the number of specific AFC as compared with the analogous characteristics in the case of immunization with an individual influenza antigens.

And in these cases, the conjugates and Cu(II)-induced complexes appeared to possess distingly expressed protective properties.

Table 36. The proliferative activity of the pathogenic virus P94 in the lungs of mice immunized with a ternary PMC (a mixture of isolated surface antigens of the influenza virus)

group of animals	immunogen HA + NA	immunogen CP-1 + Cu^{2+} + E	proliferative activity Ig EID_{50}
Nonimmunized	–	–	8.2 ± 1.6
Immunized			
A	+	–	6.8 ± 2.4
B	–	+	2.4 ± 0.6

Table 37. The protective effects of a ternary polymer-metal complex containing the protein fraction of the BCG *Mycobacteria* cell envelope on immunized and control B-mice

group of animals	immunogen TPF	immunogen TPF – Cu^{2+} – CP – 1	survival, %
Nonimmunized	–	–	(0/12) 0
Immunized			
A	+	–	(1/15) 6.6
B	–	+	(23/27) 85

The protective activity of ternary complexes containing BCG-TNP was studied in thymectomized B-mice. Prior to experiment the animals were lethally irradiated and i/p immunized with a pure protein fraction or PEC. After one month the mice were i/v immunized with a laboratorial strain of BCG (2 mg) with a subsequent follow-up during 2 months.

Two months after immunization of mice with a live BCG vaccine both control (nonimmunized B-mice) and their TPF-vaccinated counterparts died from the disseminated infection. At the same time, 85% of animals immunized with the TPF-Cu^{2+}-CP complex survived. In the control group (sham operated mice) the survival was 100%. It should be noted that in of mice immunized with water-in-oil mixtures of TPF neither CFA nor lFA could afford effective immune protection from the infecting pathogen. It appears, therefore, that ternary highly immunogenic PMC made up of PPD, an AA copolymer with vinylpyrrolidone (CP) and Cu^{2+} provide effective immune protection in thymectomized animals as well as in T-deficient mice restituted by bone marrow infusions from lethal dissemination of live attenuated BCG bacilli.

These results clearly demonstrate that antigen-PE complexes based on nonimmunogenic T-dependent proteinaceous microbial pathogens display the activity of T-independent immunogens and afford effective immune protection, especially in T-deficient organisms. The

construction of vaccinating materials on the basis of such complexes not only prevents the deleterious effects of routinely used corpuscular vaccines but also affords effective immune protection against infection.

The model of tuberculosis infection. Complexes and covalent conjugates of PPD and the total protein fraction (TPF) of tuberculosis infection with poly-4-viyl-N-ethylpyridinium bromide (PE-1), poly-4-vinyl-N-ethyl(cetyl)pyridinium bromide (PE-2), PAA (PE-3) and an AA copolymer with N-vinylpyrrolidone (PE-4) were prepared. It was examined the immunogenic activity of PEC and conjugates whose physico-chemical properties are documented in [28,180,183,317]. Humoral immune responses were assessed by the previously described procedure (Romanova *et al.,* 1988). The levels of circulating tuberculin-specific antibodies were determined in a PHA test using PPD-Ioaded SE. The titers of tuberculin-specific antibodies were measured two weeks after immunization and 3, 6 and 8 weeks after infection of animals with a virulent culture ($H37R_v$).

The results of experiments in which mice were immunized with PPD complexes with quaternated PVP are depicted in Table 28.

Table 38. The titers of PPD-specific AFC in the spleen cells of mice immunized with pure PPD and its complexes with quaternated poly-4-vinylpyridines

immunogen	AFC titers (M ± m, Ip = 0.05) 6th day	10th day	14th day
PPD	364.4 ± 66.6	708.9 ± 126.0	413.0 ± 152.0
PVP(R_2)-PPD	824.0 ± 130.2	3208.0 ± 130.2	192.0 ± 256.0
PVP(R_2, R_{16}-PPD	392.4 ± 542.2	15558.0 ± 1120.0	6971.0 ± 1632.0
PVP(R_2)	160.0 ± 42.1	132.1 ± 79.8	100.0 ± 44.0
PVP(R_2R_{16})	226.0 ± 50.6	274.2 ± 61.6	160.0 ± 74.0
control	48.0 ± 16.2	100.0 ± 42.1	84.0 ± 12

These data indicate that both types of PEC displayed a much higher immunogenic activity in comparison with mice immunized with the pure antigen. At the same time, the immunogenicity of PEC-2 whose stability under *in vivo* conditions is significantly increased at the expense of nonpolar interactions was much higher than that of PEC-1.

Analysis of humoral immunity in mice immunized with PEC and TPF conjugates with negatively charged polymeric carriers (PEC-3, PEC-4) revealed that the titers of tuberculin-specific antibodies in the blood sera of immunized animals determined two weeks after their immunization were rather low. A several fold increase in the AFC titers in mice of this group was observed after their infection. The immunological parameters of mice immunized with PEC-4 are listed in Table 39. As can be seen from these data, i/p injected PEC-4 elicited specific responses in the limb pads of experiment al animals that were manifested on the 14th post-immunization day. This effect testifies to a strong stimulation of cell-type immune responses by the PPD-containing conjugate. Interestingly that a still more pronounced DTH response to tuberculin was observed on the 3rd week after infection of mice with the pathogenic microbe ($H37R_v$).

The increase in the SI values and the simultaneous enhancement of the DTH response (Table 40) testified to the granulomatous response. At the same time, in mice immunized with TPF alone the DTH and SI values were rather low.

Table 39. The PPD-specific responses in the limb pads of mice immunized with TPF and TPF-PAA conjugate and infected with a virulent culture of *M. tuberculosis* ($H37R_v$)

immunogen	tuberculin test, mm immunization 2nd week	infection 4th week	6th week
TPF	0.1	0.4	0.1
TPF-PAA	0.5	0.8	0.35
control	0	0.35	0

Table 40. The values of the spleen index (SI) in mice immunized with pure TPf and TPF-PAA and infected with a virulewnt culture of *M. tuberculosis* ($H37R_v$)

	SI			
immunogen	immunization 2nd week	3rd week	infection 6th week	8th week
TPF	1.05	1.15	1.0	1.0
TPF-PAA	1.15	2.00	1.2	1.05

These results provide compelling evidence that immunization of mice with PPD-containing PEC leads to the development of strong cell-type and humoral immune responses. It may therefore be expected that in animals infected with virulent cultures such PEC will also afford effective antimicrobial protection.

The mean life span of animals immunized with PEC and infected with different doses of the virulent culture, $H37R_v$ is shown in Table 41. These data suggest that PEC-3 and PEC-4 were able to produce a strong statistically significant protective effect at all doses, the survival being 126-167%. In the experimental group the mean life span exceeded by 10-18 days that in the control group. A comparative study of protective activities of PE-PAA complexes containing TE fractions (PEC-6), TE-1 (PEC-7), TE-2 (PEC-8) and TE-3 (PEC-9) revealed that the PEC-6 and PEC-8 caused a statistically significant increase in survival.

Table 41. The mean survival of mice immunized (s/e) with PPD, PPD complexes and conjugates with PAA and infected with a virulent culture of *M. tuberculosis* ($H37R_v$)

		infecting dose, mg					
		0.1		0.05		0.025	
immunogen	dose, μg	days	%	days	%	days	%
control	—	17.60±0.44	—	23.67±1.3	—	26.50±4.25 42.33±4.12	—
TPF	100	—	—	24.50±2	101	—	—
PAA-TPF(CC)	100	—	—	29.0±0.7	126	39.36±2.53	148
PAA-TPF(PEC)	100	28.67±2.62	163	—	—	44.07±2.7	167
	450	—	—	—	—	—	—
BCG (live)	450	29.80±1.11	169	—	—	—	—
PAA	450	18.70±1.80	106	—	—	—	—

Antifertile PEC. The construction of vaccinating materials for fertility regulation in animals and man and analysis of their mechanisms of action is one of the most burning problems of present-day medicine. In the past decades this problem has acquired special importance in Asian and African countries, especially in India and China. Among the vast variety of antifertile drugs presently available immune contraceptives are considered as the most effective ones, because they promote partial or complete (depending on their immunogenic activity) sterility and even castration. Besides, such vaccines can be use d in the conservative treatment of hormone-dependent diseases and tumors, e.g., prostate carcinoma (Talwar, 1986) [319].

One of the most interesting events of the past decade is the discovery of the luteinizing hormone releasing hormone (LHRH), a hypothalamic decapitate able to stimulate the release of LH. The β-OLH hormone is a basic protein, which in neutral aqueous solutions bears an excess positive charge. Therefore we used as polymeric carriers for β-OLH the negatively charged copolymers of VPD with AA (CP-1) and MA (CP-2).

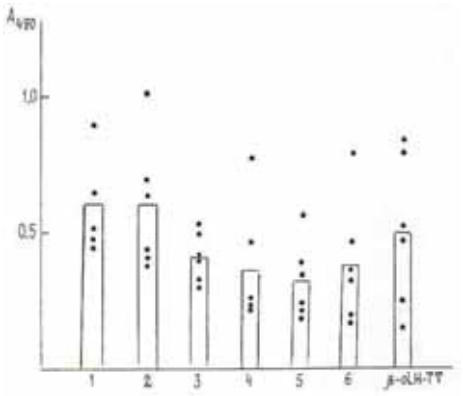


Figure 173. The primary immune response of beritary PMC of β-OLH-CP-I + Cu^{2+} +β.OLH: 1 – (CβC =2;

Immunological studies were conducted at the National Institute of Immunology (New Delhi, India) by Drs. Talwar and Arunan with the participation of Mustafaev (Moscow, USSR) [9].

Albino rats were i/v immunized with 0.5 mg and the antibody titers in the blood samples were determined in a radioimmunoassay (ELISA) using a ^{125}I labelled antigen. It may be inferred from these data that all ternary PMC were able to induce strong immune responses to the protein hormone included into PMC. Interestingly the strength of the immune response was either commensurate with or, in some cases, exceeded that of the control nontechnological conjugate. It follows from these data that the strength of the immune response does not depend on the distribution pattern of the carboxyl groups within the composition of CP on the protein/polymer ratio as well as on the transient metal ion concentration in experimental mixtures. Stipulating that ß-OLH possesses no immunogenic activity of its own and that the CP used in this study were nontoxic, technological and had low M_r (10 and 50 kDa), it may be concluded that the proposed method designed to increase the immunogenic activity of protein hormones opens new ways to the chemical synthesis of antifertile vaccines for birth control in animals and man.

5.9.1. Synthetic peptide vaccines.

The need for development of new vaccines and the improvement of currently used ones is defined as one of the prime goals of the World Health Organization. Although vaccine technologies and manufacturing methods have come a considerable distance over the past 50 years, much more development will occur. There will be challenges for biotechnology to arrive at safer, more effective vaccines for an ever-increasing number of antigen targets. Vaccines will remain one of the most cost-effective and logical biomedical technologies of the next century, as diseases are prevented rather than treated. Challenges are also posed in bringing existing vaccines to technologically undeveloped nations, where they are needed most. Vaccines are biologic preparations that elicit immune system responses that protect an animal against pathogenic organisms. The primary component of the vaccines is an antigen, which can be a weakened (attenuated) version of an infectious pathogen or a purified molecule from the pathogen or, more recently, chemically synthesized or recombinantly expressed viral (or bacterial) subunits. However, these antigens are not as immunogenic as live organisms and the vaccines contain as a secondary component of adjuvants to enhance immune response as well as formulation agent to preserve the antigen during storage or upon administration, to provide proper delivery of antigens,

and to minimize side reactions. Therefore, development of new vaccines is an interdisciplinary field that has a broad impact on biotechnology, synthetic chemistry, and immunological developments.

The current vaccines are killed virus vaccines, and they can be hazardous due to contamination by live viruses in the manufacturing process. Taking into account the safety and contamination problems involved in the production of vaccines for living virus and the relatively short-term storage possibility in addition to the narrow protection range, the development of synthetic peptide vaccines is a challenge. Synthetic peptide vaccines are expected to be stable and inexpensive to produce. They also provide two possible methods for dealing with antigenic variation. First, since linear synthetic epitopes are fairly easily prepared, mutations, once located, can be readily incorporated into the synthesis. Second and perhaps of greater importance, invariant antigenic sequences of the pathogen can be utilized to promote longer-lasting immunity [320,321].

Investigation of synthetic peptide vaccines has largely centered on antiviral agents, such as foot and mouth disease virus (FMDV), hepatitis B, influenza virus, poliovirus, and human immunodeficiency virus(HIV). It should be noted that antibacterial peptide vaccines are also of current interest, such as for diphtheria and cholera toxins, as well as antiparasitic immunogens for prevention of malaria [320-324].

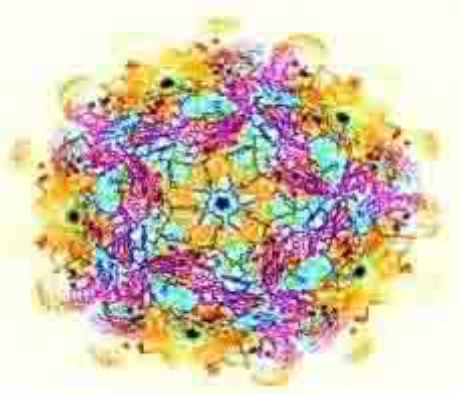


Figure 173. Virus of Foot and Mouth Disease.

Peptides manifest a variety of physiological and therapeutically properties. However after administration into living organism they are very often subjected to biodegradation (e.g. by proteolytic enzymes) or they are of immunogenic nature and they may start the corresponding immune reaction of the organism. Besides, the weak immunogenecity of them prevent immune protection from infection. Thus, they lifetime and the magnitude of the immune response *in vivo* depends on the nature of these bioactive compounds and have to be regulated and optimized in each case. The project proposes to study a novel approach to solve this problem by including of such polypeptide epitops of viruses of different disease to Biopolymer systems.

The use of peptide epitops of viruses particles as vaccines have several potential advantages over whole viral or bacterial preparations [322-325,338]. However, to elicit the maximum immunogenic response from such antigens, they have to be used with traditional adjuvant, which limit their practical applications. Besides, it is generally necessary to bind the peptide molecules also to a carrier protein, which may initiate a corresponding immune (e.g. allergic) reaction of the organism.

The peptides (the 140-160 fragment of VP1 protein of FMDV [326], immunogenic peptide of human hepatitis B virus pres (120-145) [339,340], rabies virus polypeptide antigens (130-141) [341]), prepared by chemical synthesis, and coupled to large carrier proteins like

keyhole limpet hemocyanin (KLH) were elicited neutralizing antibodies and protected guinea pigs in the mixture of incomplete Freund's adjuvant. Cattle that received a high dose of peptide (5 mg) or that had been vaccinated twice with a smaller dose, developed high levels of neutralizing antibodies. However, full protection against challenge was not obtained. Such immunizations, at the same time with disadvantageous of classical adjuvants, suffer from difficulties in producing conjugates of reliable composition, and from unwanted anti-carrier immune responses. Also, the protein carrier can induce hypersensitive, allergenic side reactions in the patient after repeated inoculations. These difficulties can lead to irreproducibility in the immune response [342]. To circumvent these problems, two new protocols in vaccine design are now emerging that is based entirely on synthetic peptides. The multiple antigenic peptide (MAP) concepts were recently introduced by Tam [343]. Such systems were obtained by stepwise solid-phase synthesis of a MAP in which the final multibranching MAP core bound with eight copies of the antigenic peptide. The dense peaking of so many copies of a highly antigenic epitope has been shown to produce a strong immunogenic response.

To elicit full immunogenic activity, another current thinking suggests that a vaccine should consist of B at T cell epitops to be most effective. This theory was first supported by Francis and co-workers [344], who noted that the carrier- free FMDV vaccine derived from residues 141 to 160 of VP1 did not protectively immunize the H-2^d strain of mice. By coupling this B cell antigen to known T cell epitops of ovalbumin and sperm whale myoglobin, high levels of cross-selective antibodies were invoked, which neutralized FMDV in subsequent challenge experiments.

Utilizing the template-assembled synthetic protein engineering techniques of Mutter [345], Kobbs-Conrad and coworkers [342] designed a totally synthetic vaccine consisting of single or multiple copies of B and T cell epitopes built into a β-sheet template peptide. They observed high titers of antibodies in response to this template-assembled vaccine bearing B cell epitopes of LDH-C and T cell antigens of tetanus toxoid. When a chemically synthesized peptide, bearing hepatitis B virus α-determinant specificity, was conjugated to a dipalmityllysine moiety (enhancement by conjugation to a fatty acid carrier), a significant improvement in anti-hepatitis B surface antigen response was obtained, in comparison to the corresponding peptide conjugate [346].

Novel low-molecular-weight synthetic vaccine against FMD containing a patent B-cell and macrophage activator (T-cell epitope) was obtained by conjugation of peptide fragment (135-154) of VP1 protein of FMDV to tripalmitoly-s-glyseryl-cysteinylserylseryl [347], which shows protection in guinea-pigs again FMD viruses. However, the MAP system has not yet been proved to be an effective vaccination vehicle, although it does offer exciting possibilities for the future. Gel filtration experiments suggest that the above-mentioned conjugates form large aggregates, possible micelles, which may play a significant role in the enhancement of the anti-peptide response.

It is known that Lactide-co-glycolide polymer microsphere technology is feasible and holds great promise for improving human vaccines [349]. Peptides carrying an immunodominant T-helper delineated from the rabies virus nucleoprotein either alone or in combination with liner B-cell epitope was incorporated into poly(DL-Lactide-co-glycolide)(PLG) microspheres and stimulated a peptide-specific T-cell line[348]. Such formulations of PLG upon subcutaneous immunization of mice induced the best immune response, in magnitude comparable or even superior to that induced by peptide emulsified in complete Freund's adjuvant. Despite the potential of microencapsulated vaccines, a number of unsolved questions persist. Some of them: residinal solvents and monomers in the microspheres, adverse reactions with slowly released antigen, control of allergic reactions, the size of microspheres, etc.

Biodegradable water-soluble polyelectrolytes developed over the past decade for the activation of the immune system (immunostimulants) have significant potential for the creation of highly immunogenic preparations for human and veterinary medicine. It was shown that the

quaternary polycondine salts, which contain biodegradable N-C bonds in structure, increase the immunogenecity of weakly antigenic biomolecules by several times and can effect the immune system avoiding T-helpers [9]. The Cu^{2+}-complexes of copolymers of piperazin with metilenbisacrylamide possess own sufficiently immunostimulant (adjuvant) activity in models of mice and have broad physico-chemical potential for the preparation of stable polycomplexes with different antigens [185].

Recently, vaccine "Grippol"-trivalent polymer-subunit vaccine containing the sterile conjugate of influenza virus surface proteins, types A and B, bound with copolymer polyoxidonium (polyconidine derivatives) has been developed [318]. The administration of "Grippol" to children of school age (6-18 years) demonstrated low reactogenicity of the vaccine, its safety and sufficient prophylactic effectiveness, and no side effects produced by "Grippol" were registered.

In the light of these findings it was very important to establish the probability of inducing synthesis of peptide containing immunogenic polyelectrolyte conjugates. The synthetic peptide analog HA^{-}_{2} docapeptide of HA^{-}_{2} subunit of hemagglutinin influenza virus was turn into highly immunogenic preparations by the covalent cross-link with synthetic carbochain PE [9]. By the incorporation of synthetic peptide analogous (decapeptide) of utilizing hormone releasing hormone (LHRH) into polymer-metallic (PE-Cu^{2+}) complexes synthesized ternary PE-Cu^{2+} peptide polycomplexes which were able to induce strong peptide-specific immune response in the experiments on rats [9].

Foot-and-Mouth Disease Virus (FMDV) Vaccine. Recently, we report a novel approach to a totally synthetic vaccine, which consists of a Hb_SAg and food-and-mouth disease virus(FMDV) VP1 peptides, prepared by chemical synthesis and nonimmunogenic membrane active carbochain polyelectrolytes[355,356].

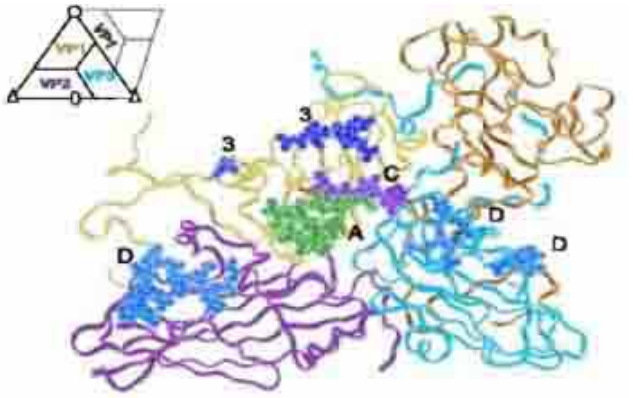


Figure 173. The structure of FMDV VP1 protein

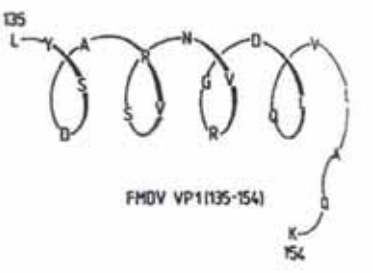


Figure 174. Amphipathic α-helix in VP1 protein of FMDV

FMDV terminally afflicts domestic livestock and has had devastating economic effects on the agricultural industry. FMDV consist of four protein subunits, VP1-4, of which only VP1 (1D) showed immunogenic activity when the individual proteins were used [325,326]. However, the antigenicity of VP1 was noted to be considerably reduced relative to the whole intact virus, which suggests that the other subunits are required for the correct folding of VP1. Strohmaier at al. [327] and Bittle at all [328] indicated that the peptide residues 141 to 160 of VP1 was the most effective immunogen. This segment has been found actually more antigenic than whole protein VP1 and to be cross selective for several stereotypes of FMDV [325]. The peptides that consist of the three regions of VP1, 138-154, 140-160 and 200-213, are able to induce neutralizing antibodies against the homologous virus type and protected guinea-pigs [328,329]. Further studies undertaken to locate antigenic determinants on FMDV suggested the presence of several antigenic regions. Residue at positions 40-60, 130-171, 141-160 of VP1 (1D) of different stereotypes of O, A and C have been shown as antigenic [330,333]. Analysis of crystallographic X-ray diffraction data suggest that type O amino acids 41 to 50 could form a surface loop close to the 141 to 160 region [334]. Also it was suggested that the 41-50 loop may either have insufficient amino acids exposed to the virus surface for recognition by antipeptide antibodies, but those that are alternatively, its proximity to the epitope may indirectly effect the conformation [335]. Recently, 43-49, 135-151, 166-170 and 195-206 amino acid sequences of VP1 protein were also determined as antigenic sites of type C [336,337].

One of the promising alternatives to classical adjuvants is the use of nonimmunogenic synthetic polyelectrolytes (PE) that are negatively or positively charged polymers, as carrier for antigens [9,19,21-24,28,125,240,242]. We, as well as the others, have previously shown that the attachment of weak microbial and viral protein antigens to various charged polymers allows the modulation not only of their immunogenicity, but also protective activity.

In the present study, polypeptide antigens corresponding to amino acid sequences predicted from the nucleotide sequence of Foot-and-Mouth disease virus (FMDV) VP1 protein were synthesized chemically, the polyelectrolyte-polypeptide conjugates were prepared and their immunogenic properties were investigated and discussed in terms of a novel immunogenic system. The polypeptide-comprising Biopolymer Systems were obtained by two methods: 1) inclusion of polypeptides in polyelectrolyte complexes (PEC) of PE with weak protein antigen (bovine serum albumin - BSA) which were stabilized by electrostatic and/or Cu^{2+}-induced interaction of compounds; 2) covalent cross-linking of peptides with PE directly. Immunogenecity of Biopolymer Systems without traditional adjuvants and recognition of antibodies in blood sera were investigated.

The peptides were synthesized by using the solid-phase methods developed by Merrifield, with Millipore's Automated Peptide Synthesizer. The characterization steps include chromatographic, spectroscopic, and fluorometric analyses while the purification step includes gel electrophoresis techniques. After coupling of all desired amino acids in the chain, the product was cleaved from the support with TFA cocktails. Each synthetic peptide was subjected to acid hydrolysis at low pressure (6 M HCl, 110 ^{o}C, 72 h) and its amino acid composition was determined. In this study, 40-60 (P1), 135-160 (P2), and 140-160 (P3) fragments of FMDV VP1 antigens were synthesized.

40-60 residues

TRP -VAL-LYS-ILE-ASN-ASN-THR-
SER-PRO-THR-HIS-VAL-ILE-ASP-LEU
MET-GLN-THR-HIS-GLN-HIS-GLY

135-160 residues

TRP -LYS-TYR-SER-ALA-THR-GLY-
GLU-ARG-THR-ARG-GLY-ASP-LEU-
GLY-ALA-LEU-ALA-ALA-ARG-VAL-
ALA-THR-GLN-LEU-PRO-ALA-CYS

The cationic polyelectrolytes (PE) are the copolymers of 4-vinylpyridine with 4-vinyl-N-ethylpyridine (PEVP) and 4-vinyl-N-cetylpyridine (PECVP). PE was obtained by quaternization of narrow fractions of poly-4-vinylpyridine ($P_n = 10^3$) with ethyl and cetylbromides by the method previously described.

The anionic PEs are polyacrylic acid (PAA), copolymers of acrylic acid (AA) with N-isopropylacrylamide (NIPAAm) (CP1) and N-vinylpyrolidone (VP) (CP2):

To carry out PE-peptide (PE. Pep) and BSA-peptide (BSA. Pep) conjugation reactions, we used carbodiimide activation method [355].

To prepare PE-BSA.Pep electrostatic complexes, various concentrations of the BSA.Pep conjugate solutions were added to PEVP (or PECVP), dissolved in phosphate buffer (PBS), pH 7.2. In practice, 1,2 and 5 mg/ml BSA.Pep conjugate solutions which were mixed with 1 mg/ml PE solution and 200 /μl of this mixed solution were centrifuged at 10000 rpm for 10 min. The supernatant was taken and diluted to 4 ml in PBS and investigated by different methods. The concentrations of free PE were obtained from the calibration curve of $OD_{254} = K.C$ (C is the concentration of PE). The protein / PE ratio (nBSA.Pep/nPE) was calculated using the equation $n = C.N_A/M$, where n is the number of the molecules in 1 ml,N_A and M are the Avogadro's number and molecular weight correspondingly. To produce the PE-Cu^{2+} complex, the $CuSO_4.5H_20$ (pH 4) solution was added to PE, dissolved in PBS. The desired pH values were adjusted with 1 M NaOH. The ternary PE-Cu^{2+}-protein complexes were, in turn, prepared by adding BSA.Pep conjugate solution to the PE-Cu^{2+} solution.

The heterogenicity of PE, proteins, peptides and the fraction compositions of the reaction products were estimated by using gel filtration chromatography (SIL-10Ai HPLC).

PEVP-BSA.Pep, PECVP-BSA-Pep, PAA-Cu^{2+}-BSAPep, CPI-Cu^{2+}-BSA(Pep, CP2-Cu^{2+}-BSA-Pep complexes, PE-Pep and BSA-Pep. covalent conjugates were used as the immunogen. Eight week-old BAIB/c mice were immunized with each of the complexes by intravenous injections. All groups were followed for development of antibody activity for polypeptides (FMDV VP1) for a period of 50-150 days after primary immunization.

The indirect enzyme-linked immunoadsorbent assay (ELISA) was used to detect antibody activity for polypeptides.

PE-peptide conjugates. HPLC analysis of the free components and reaction products in the mixture of PAA-Pep, CP1-Pep and CP2-Pep with water-soluble carbodiimide are provided. HPLC results of the reaction products prepared under this condition are shown in Figure 175. The solution of the reaction products between PE and peptide molecules in all cases was characterized in the chromatograms practically by one peak at the RT region corresponding to RT of peptide solution. The peak, corresponding to free PE was absent in chromatograms around RT = 1O min. Thus, under conditions where PE and polypeptide molecules are incapable of binding to one another, the WSC promoted the formation of water-soluble polymer-peptide covalent conjugates.

The conjugates were studied by ion exchange HPLC method (Figure 176). The solution of free P2 peptide as well as CP2-P2 reaction products is characterized by a bimodal distribution of elution components on ion-exchange chromatograms. On the other hand, conjugation induces an increase in the values of RT as compared with pure EP, a slight decrease of RT and width of the peaks as compared with pure peptide molecules. This indicates that in the CP-P conjugates formed, conjugate particles possess *more* friable structures in which *more* of the reactive groups are open for interaction with column materials.

The yield of conjugation should be directly proportional to the concentration of ε-aminolysil groups of Lys amino acid of polypeptide molecules. The ε-aminolysil contents of the FMDV and polymer-peptide conjugates were studied by fluorescamine, which interacts with the primary amino groups of samples. it was shown that the number of free amino group s of FMDV and FMDV in reaction products significantly decreased ($N_{exp.}/N_o = 0.8$, N_o and $N_{exp.}$ - the bound number of fluorescamine molecules with free polypeptides (N_o) and peptides after conjugation reaction ($N_{exp.}$)) One can assume that the ratio $N_{exp.}/N_0 = 0.8$ (or 80 percent) also characterized

the yield of conjugation reaction between CP1 and FMDV, polypeptide.

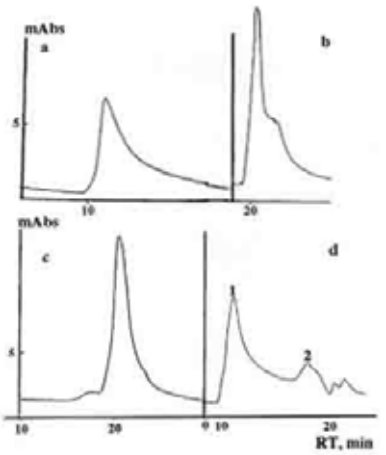


Figure 175. Gel filtration HPLC chromatograms of free CP (a), P2 (b), reaction products of CP2-P2 prepared in HRM systems (c) and mixture CP2-P2 (d). RT-retention times, UV-280 nm C_{P2} = 2mg/ml; 40µl. Concentrations of CP2, conjugate and P2: 3mg/ml; 40µl injection, 1ml/min; 25˚C

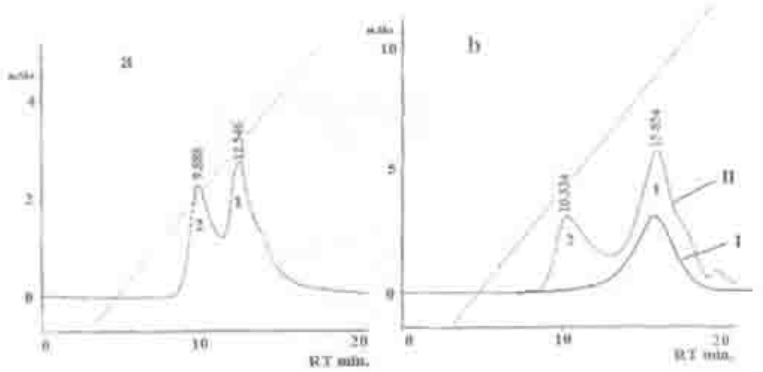


Figure 176. Ion-exchange HPLC results of Poli(VP-AA)-CDI-Peptide mixtures

BSA - peptide conjugates. Figure 177 presents the HPLC results of the covalent conjugation of the reaction products of BSA with FMDV by the activation of dicyclohexylcarbodiimide (DCC). Analysis of reaction products by use of Ultra free- CL high flow filters showed that polypeptide molecules were covalently bonded to BSA, resulting in the formation of bioconjugates with complicated structure. One can speculate that at the same time with monomer and dimer form of BSA.peptide (BSA.Pep) conjugates, the formation of water-soluble bioconjugate aggregates takes place. These aggregates were obtained as a single peak in the free eluent volume (Figure. 3, RT = 10.748 min).

However, in contrast to DCC carbodiimide conjugation reaction of BSA with polypeptides, the activation of water-soluble carbodiimide (WSC) lead to the formation of bioconjugate molecules with more essentially homogenous composition (and structure). A typical HPLC result of BSA-FMDV conjugate prepared by WSC is given in Figure. 4. The conjugate

solution was characterized in chromatograms by a single peak. Moreover, free BSA macromolecules were absent in the solution as indicated by HPLC (see Figure. 177a RT = 16.374 min for free BSA). These findings indicated that, under these conditions water-soluble carbodiimide promoted the covalent cross-linking of the BSA globules with polypeptide molecules with sufficiently increased yield and homogenous composition.

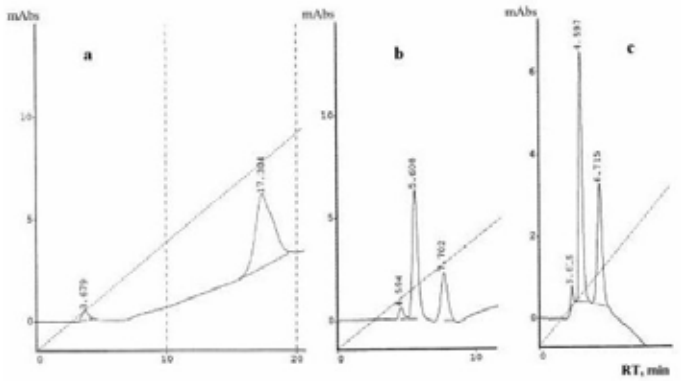


Figure 177. Ion-exchange HPLC results of BSA-CDI-Peptide mixtures

Preparation of PE-BSA.Pep complexes. BSA.Pep molecules were found to interact with polycations and to form soluble or insoluble protein-PE complexes. Starting with very low BSA.Pep/PE ratios, that is $n_{BSA.p}/n_{PE} = 0.1$, a phase separation took place in this system: PEVP-BSA.Pep and PECVP-BSA.Pep. Analysis of the matrix solution of insoluble mixtures shows that at the $n_{BSA.p}/n_{PE} = 1$, free fractions of PE remained in the matrix solution (Figure 178a). The existence of free PE under these conditions indicates a nonrandom distribution of the conjugate (BSA.P) molecules between the coils of polycations. The number (Ni) of the protein molecules bonded by a single chain of PEVP as well as PECVP of a given degree of polymerization under given conditions equal Ni = 2, i.e., two molecules of BSA.Pep. bound by one chain of polycation in composition of water-soluble polycomplexes: PEVP-(BSA Pep) and PECVP-(BSA Pep).

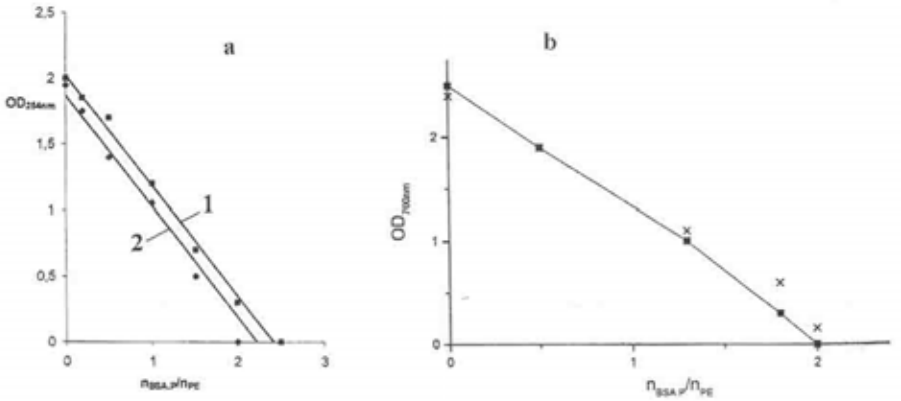


Figure 178. Dependence of optical density (OD_{405} and OD_{700}) of matrix solution of mixture PEVP-BSA*P2 (a,2), PECVP-BSA*P2 and CP2-Cu^{2+}-BSA*P2 (b) obtained by UV spectrophotometric analysis at 254nm and 700nm on the n_{BSA*P2}/n_{PE}

Ternary PE-Cu^{2+}-BSA .Pep complexes. As shown by HPLC analysis, complex cannot be formed between BSA.Pep and anionic PE at pH 7,0 in the absence of copper ions. However, water-soluble and insoluble complexes are formed upon addition of divalent copper ions to the solution of the mixtures PAA – BSA.Pep, CP1 - BSA.Pep and CP2-BSA.Pep. The extent of complex formation was dependent on the amount of Cu^{2+} added and was nearly quantitative at $n_{Cu}/n_{AA} = 0.25$ (Figure 178b).

The CP1-(Trp+(135-160)) conjugate was studied by fluorescence method. The results in Figure 179 indicate that peptide Trp+(135-160) solution shows discrete (structured) emission spectra at $\lambda_{max} \approx 315$ and 325 nm. Such Trp residues were attributed to class S in the hypothesis of discrete states. Therefore, peptide tryptophanyls exist in the hydrophobic environment of polypeptide chain and completely isolated from water solution.

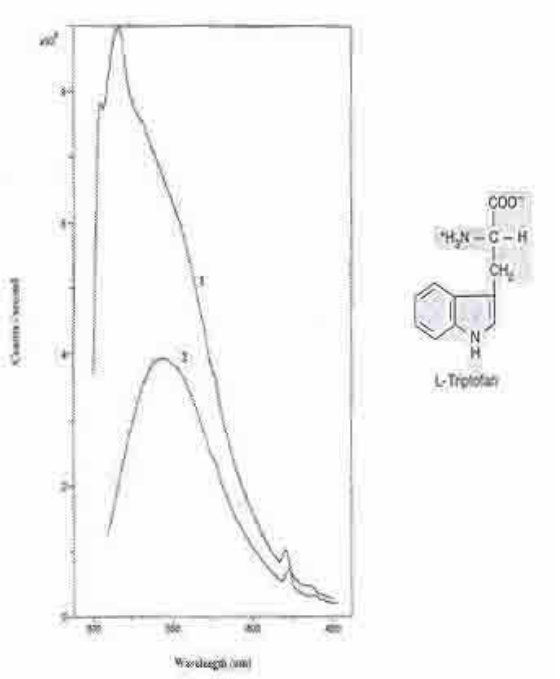


Figure 179. Fluorescence spectra of pure FMDV peptide and P50-peptide conjugate in water solutions. Peptide concentrations 0.07 mg/ml; phosphate buffer (pH 7.0), 25˚C. Quanta Master spectrofluorometer (Photon Technology International, Canada) The excitation wavelength 280nm

Previously, the large hydrophobicity and amphypatic α-helical structure of 135-160 fragments was demonstrated by Pfaff and co-workers [329]. The fluorescence intensity (I_{max}) of peptide after conjugation with CP1 decreases (quenching) which testify conjugate formation. On the other hand, conjugation of peptide with copolymer macromolecules induces a marked red shift of λ_{max}. This indicates that in the CP1-peptide conjugates, peptide Trp as compared with pure peptide molecules are essentially exposed to the solution.

We can assume that the conjugate species can be represented rather as a macromolecule of a segmented (block) copolymer in which the hydrophobic blocks, i.e. the sequences of copolymer and peptide unit pairs which have formed the covalent and salt bonds alternate with hydrophobic ones, i.e. the sequences of the copolymer chain not participating in the formation of double strand blocks. Such mechanism proposed, "frozen" of peptide molecules in the structure of conjugate at the unfolding state, which Trp environment are exposed to the solution.

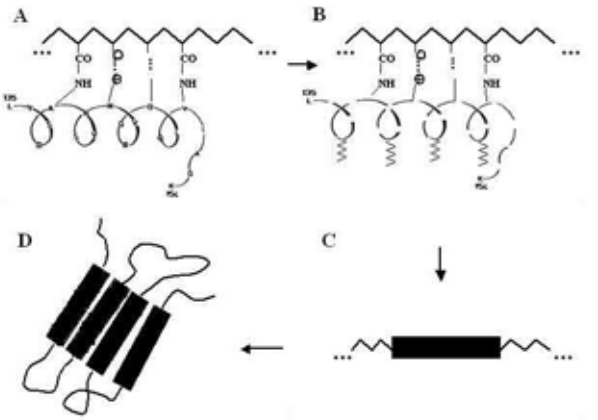


Figure 180. Schematic representation of polyelectrolyte-peptide conjugate species

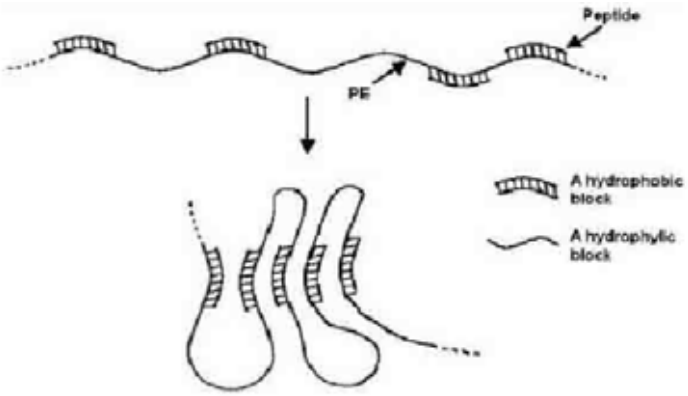


Figure 181. Schematic representation of polyelectrolyte-peptide conjugate species

Recently, we have shown that these peptide containing polymeric conjugates characterize the higher immunogenicity. This "intelligent" immunogens alike with another peptide containing Biopolymer systems were used in vaccinating guinea pigs for estimation of the potency against FMDV and dose dependent high protection was achieved. Such a modulated system is attractive for application as a novel immunogenic system in vaccine technology (see below).

Immunogenicity. For immunological experiments in ternary mixture [BSA.Pep]/[PE] = 2.0 ($C_{BSA\text{-}P}/C_{PE}$ = 2.0) and metal/polymer (n_{Cu}/n_{AA} = 0.25) ratios were used.

The dynamics of antibody formation, induced by covalent bioconjugates of PE-FMDV peptides are presented in Figures 182 and 183.

It can be seen from these data, as determined by ELISA, that a single immunization of mice with FMDV polypeptide antigens solutions barely induced production of antibodies. The immunization of mice with solution of the bioconjugates PE-polypeptide led, in turn, to the development of a pronounced primary peptide-specific immune response. The mice, which were boosted 8 weeks later intravenously with the same concentration of free FMDV polypeptides and traced for the secondary immune response revealed no further increase in the antibody titers. In

contrast, the immunization of mice with conjugates evoked increased immune responses to polypeptides.

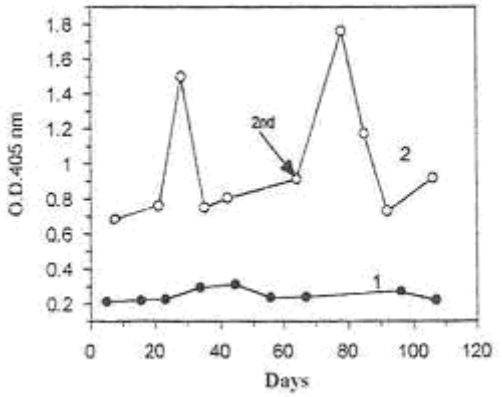


Figure 182. The dynamics of P2-specific (BSA-P2-specific) antibody formation [as assayed by ELISA (OD_{405})], induced by free P2(1) and CP2-P2(2) conjugates. 100 μg conjugate and P2 doses; intravenous injection

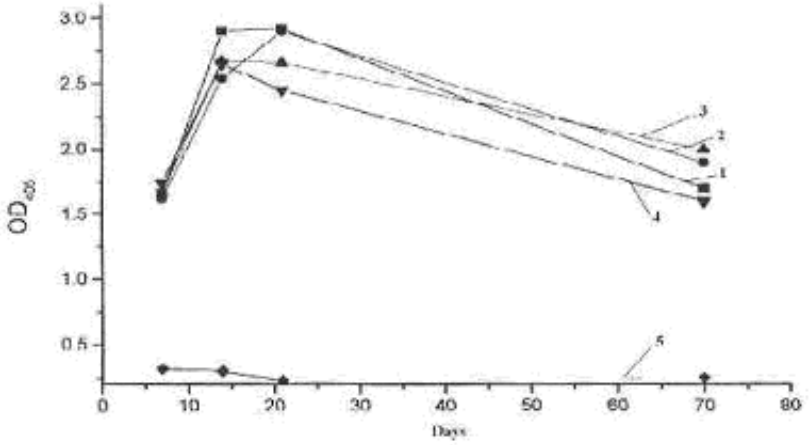


Figure 183. Peptide specific antibody formation dynamics in the mice immunized with biopolymer systems combined by two polypeptides (40-60 and 135-160 sequences) containing PE-peptide conjugates, electrostatic and icon coordination boods (Cu^{2+}) (1■) VACl, (2●) VAC2, (3▲) VAC3, (4▼) VAC4, (5♦) PI-P3

The dynamics of FMDV-specific antibody formation induced by electrostatic and Cu^{2+}-induced polycomplexes are presented in Figure. 7. It can be seen from the data that a single immunization of mice with polycomp1exes prepared with different methods, led to the development of a pronounced primary immune response. In mice, immunized with peptide-comprising Biopolymer Systems, the peptide-specific antibody activity increased in about 7 days and then maintained the very high level up to 70 days. The character of antibody formation is not dependent on the chemical structure of polymer carrier as well as on the method of the formation of Biopolymer Systems.

The physico-chemical mechanism(s) underlying the immunogenic activity of polymer-peptide conjugates may be related to an adjuvant effect of polymeric macromolecules. Free sites

of PE on bioconjugate may have the capacity to interact strongly with the membranes of immunocompetent cells. This may facilitate and stabilize the interaction of polymer bound antigen with specific cell receptors and hence enhance the immune response. The efficiency of such "forced" interactions and high epitope density (binding several peptide molecules to one polymer carrier) are high enough for the immune response.

In conclusion, a method is described for increasing the immune response to polypeptide antigens, which attacks immunological system and is of practical interest. It was found that PE-peptide as well as PE-BSAxPeptide conjugates conferred effective immunoprotection against Foot-and - Mouth Disease Virus.

Polymeric FMDV Vaccine.

Preparation of synthetic vaccine prototype by conjugation of synthetic polyelectrolytes and peptide antigens of 40-60 and 135-160 amino acid sequences of immunogenic VP1 capsid protein of "A" type FMDV which causes epidemics in Turkey was the aim of this project [356]. Thus, by the modification of the immunogenicity of the peptide antigens, development of the new FMD vaccines, diagnostic reagents, pharmaceuticals and biotechnological preparations was considered. In the author's knowledge, this work is the first synthetic peptide vaccine trial in Turkey.

As it was mentioned above, two chemically synthesized peptides corresponding to VP1 protein region of FMDV were firstly conjugated to CP1, CP2 and BSA by using water-soluble carbodiimide. Two polyelectrolyte complexes were prepared by complex formation of BSA-peptide conjugates with cationic PECVP and Cu(II)-induced complex formation with anionic CP1 and CP2.

Table 42. Synthesized amino acid sequences of A Aydın98 FMDV strain [357]

P1	40-60 (21 mere)	Val-Lys-I1e-Asn-Asn-Thr-Ser-Pro-Thr-His-Val-I1e-Asp-Leu-Met-Gln-Thr-His-GIn-His-GIy
P3	135-160(26 mere)	Lys-Tyr-Ser-Ala-Thr-GIy-Glu-Arg-Thr-Arg-GIy-Asp-Leu-GIy-Ala-Leu-Ala-Ala-Arg-Val-Ala-Thr-GIn-Leu- Pro-Ala

Thus, 4 different vaccine compositions were prepared (VAC1, VAC2, VAC3 and VAC4).

Table 43. Composition of the synthetic peptide vaccines.

VAC1	(CP1-P1) + (CP1-P3)
VAC2	(CP2-P1) + (CP2-P3)
VAC3	(PECVP-BSA.PI) + (PECVP-BSA.P3)
VAC4	(CP-Cu^{2+}-BSA.PI) + (CP-Cu^{+2}-BSA.P3)

Side Effects in Guinea pigs*:* 4 animals for each vaccine were inoculated s.c. (2ml/ animal). The local and general reactions were detected and recorded during the 1 month of inspection period.

Side Effects in Cattle*:* VAC2, which passed both guinea pig tests was inoculated to 13 cattle. The local and systemic adverse reactions were inspected clinically for 7 days.

Vaccine Doses*:* Synthetic peptide quantities of vaccines, which used in immunisation and potency trials, are summarized in Table 44.

Table 44: Final synthetic peptide concentration of one vaccine dose for animals

Animal	P1 (μg/dose)	P3 (μg/dose)	P1 + P3 (μg/dose)	Vaccine dose (ml)
Mouse	50	50	100	0.2
Guinea-pig	500	500	1000	2
Cattle	1500	1500	3000	2

Mouse: 8 week old Balb/c mice were immunized intravenous (i.v.) with 4 vaccine candidates. Synthetic peptide combination (P1 + P3) was used as negative control. Animals were bled at weekly intervals and antibody titres were estimated with indirect ELlSA.
Cattle: 10 cattle (in a fattening farm near Ankara) were inoculated with VAC2 s.c. Animals were bled before vaccination (on the day of vaccination), 14^{th} and 21^{st} days pv.. Antibody response against both synthetic peptides and intact virus were tested with indirect ELlSA and LPB ELlSA respectively. Development of neutralizing antibodies against whole virus was also detected with NT with BHK cells.
Protection Test: Guinea pig protection test was applied as described by Barnett, CARABİN 2002 AND Wotzler et al 2002. [358,359]. Groups of four animals were immunized with two fold dilutions of the 4 vaccine candidates and an aluminium hydroxide-saponine adjuvant vaccine prepared with the inactivated A98 virus as control. Immunization was done subcutaneously 2ml vaccine/ guinea-pig where the final synthetic period concentration in each dilution was 1 mg, O.5 mg, 0.25 mg and 0.125 mg. 21 days post vaccination animals were challenged with 400 guinea-pig ID_{50} "A" 98 virus/ animal. A group of four unvaccinated guinea-pigs was also infected with the challenge virus as control.
Vaccine Site Reactions in Guinea-pigs*:* Four vaccine formulations were tested in guinea pigs. There was abnormal reaction in the animals vaccinated with VAC1 and VAC3. A local hyperemia in the inoculation zone was detected for 1-2 days in the animals after vaccination with VAC2. Nevertheless, severe local and systemic reactions appeared just a few minutes after inoculation of VAC4. In coordinated pace, loss of appetite for 1 day, apses with large hyperemia and large swelling zone in the inoculation area. This severe side effects with VAC4 were attributed its CU^{+2} content of the polymer.
Vaccine Site Reactions in Cattle: All of the cattle were inspected for 1 week post vaccination. Mild reactions were detected such as Increase in body temperature of two animals (39.1-40.1) for 1 or 2 days and a small lump with 30-40 mm Φ insensitive to pressure lasting for 7 days maximum.
Immunogenicity:
Mice: The mice immunized intravenously with adjuvant-free polypeptide were not responded to the antigen. Whereas, primary peptide specific immune response increased in the first 7 days and the titers were steady up to 23^{rd} day p.v. in the animals vaccinated intravenously with PE-polypeptide conjugates (Table 45). Since peptides are known as poor immunogens, unresponsiveness to the peptides without adjuvant was expected. The highest antibody level was detected in the sera of the mice inoculated with VAC2 14 days post vaccination and starting from the 21. day pv peptide specific antibody titre decreased gradually.
In fact, the characteristics of antibody development and the level of immune response was not dependant with the preparation method of the biopolymer systems and the structure of the polymer carriers.
Potency in Goinea-pigs: Guinea-pig potency test is stilI an acceptable and reliable method in determination of the poteney of the FMD vaceines. Starting from that point, 4 PE-polypeptide conjugates (VAC1, VAC2, VAC3, and VAC4) which developed the immunogenic activity in the mice were selected to be used in guinea-pig potency test. The conventional vaccine, with $AI(OH)_3$ adjuvant, inactivated virus which contains 8.9μg 146S antigen was protected all of the animals. Guinea-pigs vaccinated with VAC2 which contains 1 mg synthetic peptide/dose was also protected the entire animal. The protection ratio in the animals vaccinated with the same dose (1 mg) of peptide conjugated with different polymers (VAC3 and VAC4) was ¾. However, VAC1 developed weak protection. The importance of the adjuvant in the potency of the inactivated or subunit vaccines is a well known reality [360,361]. For that reason, recently most of the vaccine development studies are targeted to find out more effective adjuvant with minimum side effects. Also in the present study, guinea-pigs vaccinated with the same quantity of synthetic peptides conjugated with different PE's were protected against the same quantity of the virus in different

levels (Table 4).

VAC2 was selected as primary candidate for the further experiments because of its higher protective capacity and lower toxicity in Iab animals.

Vaccine Trial with Cattle: 15-18 months old 10 cattle were vaccinated subcutaneously with VAC2 which contain 3 mg synthetic peptide doses. Animals were bled 14 and 21 days post vaccination. Antibody response to homolog intact virus was evaluated with both LPB-ELISA and NT in BHK21 cell line. Although there was a mild increase in the titres of 8 animals out of 10, none of them passed over the acceptable levels. Since, NI 0.9-1.3 considered uncertain. Only one cattle (ear tag No.99) could reach up to that level with NI 1.2 in 14th day p.v. However, to be honest, this animal was probably primed before vaccination (NI 0.3 at day 0).

Table 45. Summary evaluation of the control tests applied to the synthetic peptide vaccines

TESTS		RESULTS		
Mice	Peptide specific Ab response (indirect ELISA)	VAC1	–	
		VAC2	+	
		VAC3	+	
		VAC4	ND	
Guinea-pigs	Side effects	VAC1	no	
		VAC2	negligible (1-2 days)	
		VAC3	no	
		VAC4	sever	
	Potency	V AC1	– (0/4)*	≤2ml (≤1mg)
		VAC2	+ (4/4)*	2ml (1mg)
		VAC3	+ (3/4)*	2ml (1mg)
		VAC4	+ (3/4)*	2ml (1mg)
Cattle	Side effects	VAC2	no	
	Virus specific Ab response (NT&ELISA)		±	
	Potency		ND	

*number of protected guinea-pigs /number of challenged
ND- not done

In the present study vaccination of the guinea-pigs with 1 mg of PE- conjugated synthetic peptides (40-60 and 135-160 amino acid. residues of VP1) developed complete protection. Antibody response in mice with 100µg of the same peptide vaccine conferred the effectiveness of preparation. Nevertheless, 2 ml vaccine with 2 mg synthetic peptide was not sufficient to develop antibody response against homolog virus.

Antoni et al (1988) showed that the cattle with high antibody titer at 21st day p.v. against synthetic peptide vaccine developed generalized lesions after challenge with 10.000 ID_{50} of the homolog virus [362]. Contrary, in some experiments some of the cattle with insufficient antibody titer could be protected after challenge [363] it is clear that there is some other factors play important role in the protection mechanism of the animals. This can be cellular immunity or other type immunological responses [358].

For this study increasing the quantity of the synthetic peptide per dose could also be a solution to the problem. Tam et al (1989) showed that sufficient protection in cattle could be achieved with 5 mg synthetic peptide [338]. Another alternative is preparation of new vaccine combinations with some additional amino acid residues. Volpina et.al. (1999) declared that besides mice, guinea-pigs and rabbits also sheep and cattle responded to the vaccination with synthetic peptide vaccines contain 170-188 amino acid residues of VP1 protein of A type FMDV [364]. The last solution but not the least is to make some modifications in synthetic polymer composition and coupling mechanisms.

6. BIOPOLYMER SYSTEMS IN RADIOBIOLOGY

Theories of radiation protection can be considered at both the molecular and biochemical-physiological levels. Four molecular level protection hypothesis, radical scavenging, hydrogen

transfer reactions, the mixed disulfide hypothesis and the endogenous nonprotein sulphydryl hypothesis, probably describe different aspects of the actual protection mechanism, although each has inconsistencies [365]. At the biochemical-physiological level, hypothermia induction, hypoxia induction and biochemical shock may be involved in protection of the organism against radiation induced damage and death. It is most likely that no single mechanism can account for the protection offered by a radioprotective drug.

Water-soluble synthetic polyelectrolytes and their various polyelectrolyte complexes (or conjugates) with functionally molecules have potential possibility of radioprotective activity. One of the mechanism of the action of polyelectrolyte in biological systems is the cooperative interaction of polyelectrolyte with the biomacromolecular components of organism. This idea have been based on the results of experiments in polyelectrolyte-protein and polyelectrolyte-cell systems [372]. It is remarkable that the higher immunologically active polyelectrolytes also have radioprotective properties.

As it was mentioned above a relatively new technique involves the use of transition metal (Cu (II)) compounds as a means of activating the polymer carrier and allowing direct coupling of proteins without prior derivatization of the activated polymer, through formation of chelates (Mustafaev and Kabanov, 1981; Mustafaev et al., 1990, 1996). It is known that synthesis and fabrication of polymeric material for biomedical application can be done by radiation techniques such as polymerization, grafting, crosslinking and etching (Swallow, 1973; Spinks and Woods, 1990) [366,367]. Thus, bioreactor, biosensor, artificial organ and drug delivery systems have been studied and developed. Recently, the signal-responsive chemical delivery systems which are a combination of sensor and biofunctional system, prepared by irradiation technology have been studied (Yoshida et al., 1989; Okuda et al., 1999) [368,369].

It is known that superoxide dismutase (SOD), which is present in cytosal of eukaryotic cells is copper-zinc enzyme which catalyses the dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen. Superoxide dismutase is a beta barrel protein with 152 amino acids and consists of two subunits of identical molecular weight joined by a disulfide bond containing two Cu (II) and two Zn (II) atoms per molecule. Studies of enzyme by pulse radiolysis have indicated reduction and reoxidation of the Cu2+ during the catalytic cycle (Mc Cord and Fridovich, 1969; Keele et al., 1971); Forman and Fridovich, 1973). Radio-protective effects on mice of superoxide dismutase have also been reported (Bannister et al., 1971; Akita et al., 1984; Feher et al., 1990). It appears that metal ions in both systems: (PE-Cu2+-BSA) and SOD show similar protective effect against radiation damage.

Recently, the effects of Cu on stability and composition of water-soluble ternary polyelectrolyte-Cu-protein complexes against radiation damage was studied before evaluating their possible usage as a radioprotector [121,370,371].

Fraction of polyacrylic acid (240 kDa), BSA and superoxide dismutase (SOD) [378-382] were used as a components for preparation of polycomplexes.

To produce polymer-protein mixtures, BSA and SOD solutions (l g/l) were added to PAA (l g/l), dissolved in phosphate buffer, pH = 7.2. The ternary (PAA-Cu^{2+}-protein) mixtures were, in turn, prepared by different methods: by adding protein solutions to the polymer-metal complex (PMC), by adding polymer solutions to the protein-Cu^{2+} complexes and by adding Cu^{2+} ions ($CuS0_4.5H_2O$, pH = 4.0) to the polymer-protein mixtures. The pH values were adjusted with 1 M NaOH to the desired pH. BSA/PAA ratios (n_{BSA}/n_{PAA}) were calculated using the equation $n = cN_A/M$ where n is the number of molecules in 1 ml; M is the molecular weight of components; N_A is the Avagadro number; c is the concentration in g/l. The heterogenecity of polymers and proteins and the fraction compositions of the mixtures were estimated by using two HPLC systems.

γ-radiolysis of the aqueous solutions of PAA, BSA, SOD, PAA-BSA, PAA-Cu^{2+}-BSA and PAA-SOD mixtures, open to air, was performed by using a ^{60}Co γ-source (Picker 9 V). 5 ml solutions of samples were put in bottles. The samples were irradiated at a position of 10 cm from

the source. The dose rate was measured to be 54.5 Gy/h as determined by Fricke dosimetry. A Shimadzu UV-2401 PC spectrophotometer was used for spectroscopic analyses.

The spectrophotometric results of the irradiation experiments are presented in Table 46. As it can be seen from comparisons of the results of optical densities of irradiated and unirradiated solutions in aerated conditions, unlike the solutions of free components (PAA, BSA, PAA-Cu) for the ternary mixture PAA-Cu-BSA the values of %OD in the dose up to 1.2 kGy are changed insignificantly. A significant decrease in the radiation change of the values of %OD of these mixtures was observed for solutions containing N_2. A percentage change of optical densities in N_2O saturated solutions of BSA was higher than aerated and N_2 saturated solutions (Table 46).

Table 46. Percentage changes in optical density values {%OD = [(ΔOD/OD)*100]} in γ-radiolisis (medium: aerated)

Dose(Gy)	PAA	PAA-Cu^{2+}	BSA	PAA-Cu^{2+}- BSA
33.34	0.04	0.65	2.28	0.02
100.03	2.12	1.92	5.71	0.09
133.37	5.16	4.09	8.24	0.12
471.94	11.69	6.51	11.41	0.53
655.12	15.37	9.01	26.35	8.50
675.28	16.49	10.2	38.01	8.81
1036.66	17.08	-	49.43	9.74
1044.91	17.19	-	51.40	10.35
*655.12	10.25	3.31	18.41	2.23
**655.12	16.40	9.12	56.76	6.59

*Medium in N_2 ** Medium in N_2O

Although the radiation –chemical changes were measured by UV-Vis spectrophotometry, more detailed information was obtained by the method of HPLC. The HPLC results of the irradiated and unirradiated PAA, BSA, PAA-Cu^{2+}, PAA-BSA and PAA-Cu^{2+}-BSA solutions in O_2 atmosphere are shown in Figure 184. No change was observed in BSA solutions irradiated at low doses (up to 0.655 kGy). However, the values of retention time (RT) and form of these peaks (heterogenecity) of BSA solutions irradiated at 1.044 kGy significantly differ from unirradiated protein solutions. For the PAA and PAA-Cu^{2+} solutions deformation of the peaks was observed at the higher irradiation dose (1.2 kGy).

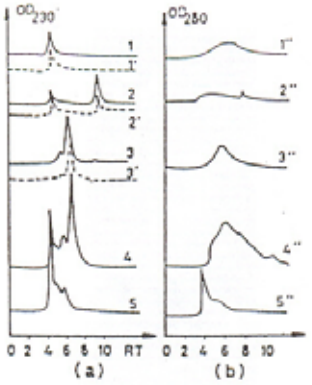


Figure 184. HPLC results of unirradiated and irradiated solutions of PAA (1), PAA-Cu^{2+} (2), BSA (3), PAA-BSA (4), PAA-Cu^{2+}-BSA (5) in the presence of O_2; 1, 2, 3, 4, 5 (Unirradiated samples); 1′, 2′, (1.044 kGy); 3′ (0.675 kGy) 1′′, 2′′, 3′′, 4′′, 5′′ (1.2 kGy) [Cu^{2+}] = 1.388 x 10^{-3} M, $C_{PAA} = C_{BSA} = 0.1$ g/dl

On the bases of these results, as well as other from earlier investigations [372,373], it can be proposed that BSA and PAA undergoes degradation and crosslinking at this dose. Similarly, denaturation and aggregation have been obtained with irradiation of proteins such as bovine and human serum albumines, egg albumin, casein and β -lactoglobulin [373].

Figure 184(b) compares HPLC results of the irradiated and unirradiated PAA, PAA-Cu^{2+} and BSA solutions with those of PAA-BSA and PAA-Cu^{2+}-BSA mixtures at the high irradiation dose (1.2 kGy). The chromatograms obtained for the irradiated solutions of PAA-BSA mixture clearly demonstrate the formation of a new form of the protein and macromolecules at this dose. The behaviour of BSA upon irradiation in the presence of PAA macromolecules do not change essentially. The denaturation and aggregation by the crosslinking way of macromolecules in mixture takes place as in the case of individual components. At the same time, as can be seen from the results (Figure 184(b)), the behavior of ternary PAA-Cu^{2+}-BSA mixture after irradiation was not significantly different from unirradiated solutions. Although the shapes of the peaks and RT values remained the same, the peak areas decreased upon irradiation.

Addition of Cu^{2+} ions to PAA-BSA mixture reduces the extent of radiation–induced change of the protein and PAA macromolecules in the particles of ternary polycomplexes. This phenomenon can be considered to "protect" (or stabilization) of the macromolecules against radiation damage. Preservation of native structure of BSA in ternary PAA-Cu^{2+}-BSA complexes upon irradiation was observed and this was confirmed by the immunological methods recently. Injection of irradiated ternary PAA-Cu^{2+}-BSA complexes to animals resulted on the production of BSA-specific antibodies.

Studies of the fraction composition of polymer-protein mixtures at different irradiation doses by HPLC permit to elucidate some important features characterizing the obtained "protection" phenomenon.

6.1. PAA-BSA Systems:

The HPLC results of the unirradiated and irradiate PAA-BSA mixtures at different irradiation dose are shown in Figure 185.

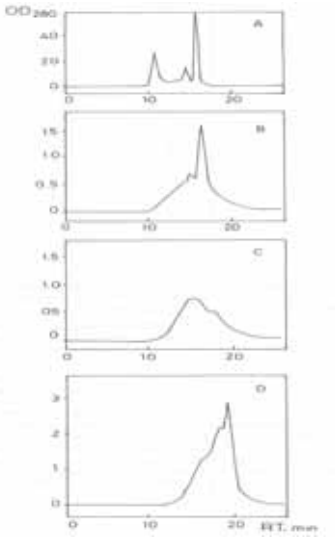


Figure 185. HPLC results of the unirradiated (A) and Irradiated (B-D) PAA-BSA mixture at different irradiation doses (Gy): 100 (B), 300 (C), 1200 (D), $C_{BSA} = C_{PAA} = 0.01$ g/l

As it is seen from this figure, the unirradiated PAA-BSA mixture is characterized by a multimodal distribution of components. The comparison of the values of retention times (RT) corresponding to the peaks of the mixture and of the individual components (RT of the protein and PAA under the similar conditions are equal to 15.77min (monomer), 14.63min (dimer), l3.8min (trimer) and 10.8 min (PAA), respectively) shows that the interaction between BSA and PAA at the pH 7.0 was weak, if not negligible. The preexisting electrostatic repulsive forces between similarly (negatively) charged PAA and BSA prevent the formations of polycomplexes. The results are consistent with the results obtained by sedimentation and HPLC analysis of PAA-BSA systems (Kabanov et al., 1978; Mustafev et al., 1996, 1998). Stable bioconjugation of PAA with BSA took place, however, upon irradiation of the mixture PAA-BSA (B, C, D). The peak with the RT of pure PAA is absent in chromatograms and the value s of RT, the distribution of compounds and the shape of the peaks (heterogenecity) of irradiated PAA-BSA solutions significantly differ from unirradiated polymer-protein mixtures.

The increase of RT values and narrowing of the heterogenecity of reaction products were observed at the higher irradiation dose. On the basis of the results, it can be proposed that in the mixture of PAA-BSA, the macromolecules undergo degradation and crosslinking under these conditions. Degradation and crosslinking reactions are reported for the γ-radiolysis of powdered PAA and polymethacrylic acid (Afanas'ev et al., 1985; Hill et al., 1990) (dose fare of approximately 10 kGy) [374,375]. Denaturation and aggregation have been obtained with irradiation of proteins such as bovine and human serum albumins, egg albumin, casein and β-lactoglobulin (Urbain, 1977).

Figure 186 compares HPLC results of the irradiated and unirradiated PAA-Cu^{2+}-BSA mixtures at different irradiation doses. As suggested by the change in chromatograms (Figures 187A and 186B (1)) stable complexation of PAA with BSA via Cu^{2+} took place upon addition of copper ions into PAA-BSA mixture. It is remarkable that the character of the distribution of compounds in ternary mixture in contrast to PAA-BSA mixtures practically does not change during irradiation up to 1.2 kGy. At the high irradiation dose (2.5 kGy) the areas of the peak with law RT decreased and the distribution of compounds and the heterogenecity of solutions significantly differ from solutions irradiated at ≤ 1.2 kGy. This may cause the radiation-induced covalent crosslinking of particles. Therefore, the addition of Cu^{2+} ions to PAA-BSA mixture protects the PAA and BSA components of ternary PAA-Cu^{2+}-BSA complexes against radiation damage. The mechanism underlying the protection effect might be related to the conversion of superoxide anion (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) via Cu^{2+} ions (Mustafaev et al., 1996).

One can as time that in the case of the PAA-BSA mixtures containing Cu^{2+}, the reaction of radiation-induced crosslinking starts after the complete oxidation of Cu^{2+} ions in composition of polycomplex particles with superoxide anions (O_2^-).

Figure 186b compares the results of HPLC analysis of unirradiated and irradiated solutions of ternary PAA-Cu^{2+}-BSA mixtures prepared at different initial n_{BSA}/ n_{PAA} ratios and more high concentration of Cu^{2+}. When PAA-Cu^{2+} solution is titrated with protein solution ($n_{BSA}/ n_{PAA} < 0.1$), BSA is complexed with the polyion via copper ions (A-D). The particles of ternary polycomplex moved in chromatograms as a pure PAA (peaks 1) and some free BSA molecules (or BSA-Cu^{2+} complexes) remain in solution (peaks 2). The pattern changes significantly on further increase of the ratio, $n_{BSA}/ n_{PAA} \geq 1$ (E). Under this condition, a further increase in BSA content ($n_{BSA}/ n_{PAA} = 2.0$) led than to the decrease of peak 1, the intensity of peak 2 corresponding to free (or BSA-Cu^{2+}) BSA increased. Notice that the intensity of peak with RT between peaks 1 and 2 corresponding to the dimer form of BSA content increased considerably. Thus, it can be proposed that a further increase in BSA content to breakdown some of the polycomplex as in mechanism (1) by the formation of BSA-Cu^{2+}-BSA and BSA-Cu^{2+} complexes and free PAA-Cu^{2+} or (PAA): (Mustafev et al., 1996):

$$[PAA - Cu^{2+} - BSA] \xrightarrow{BSA} [(BSA)_2\text{-}Cu^{2+}] + PAA(Cu^{2+}) \quad (1)$$

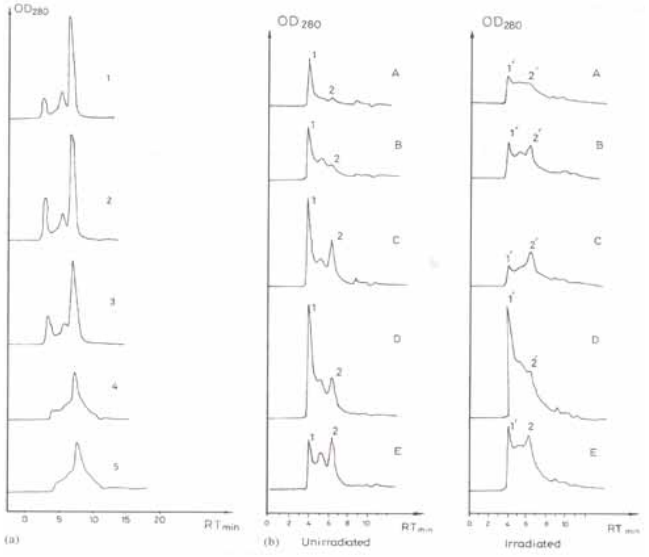


Figure 186. (a) HPLC results of the unirradiated (1) and irradiated (2-5) PAA-Cu^{2+}-BSA mixtures at different irradiation dose (Gy): 300 (2), 1200 (3), 2500 (4), 3000 (5); $C_{BSA} = C_{PAA}$= 0.01 g/l. $[Cu^{2+}] = 1.4 \times 10^{-4}$ g mol/l. (b) HPLC results of the PAA-Cu^{2+}-BSA mixtures, prepared at different initial n_{BSA}/n_{PAA} : 0.1 (A); 0.2 (B); 0.5 (C); 1.0 (D); 3.0 (E); $[Cu^{2+}] = 1.4 \times 10^{-4}$ g mol/l; irradiation dose: 1200 Gy

The higher capacity of BSA in complex formation with Cu^{2+} than PAA (Lau and Sarkar, 1971; Dixon and Sarkar, 1974) is consistent with this proposal.

An analysis of the irradiated ternary PAA-Cu^{2+}-BSA mixtures at different initial n_{BSA}/n_{PAA} ratios of components deserves some consideration. On the whole, the addition of Cu^{2+} ions protects the components of PAA-BSA mixture, prepared at different n_{BSA}/n_{PAA} ratios, against radiation damage, although some difference on the heterogenecity of the solution after irradiation took place. Taking into account the fact of the radiostability of fraction (2′), under these can one may consider that fraction 2 in the mixture (Figure 186b unirradiated solutions) contain BSA-Cu^{2+} complexes.

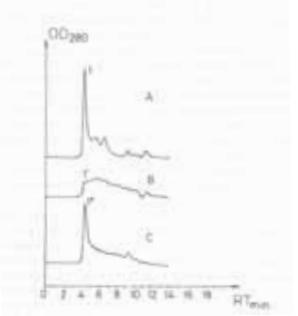


Figure 187. HPLC results of the unirradiated PAA-Cu^{2+}-BSA mixtures prepared in water (A) and in 0.15gmol/l NaCl solution (B); (C) After irradiation of mixture A and adding 0.15gmol/l NaCl; C_{BSA}/C_{PAA}= 0.01 g/l; $[Cu^{2+}] = 1.388 \times 10^{-3}$ g.mol/l

The suggested mechanism $Cu^{2+} \rightarrow Cu^{1+}$ by O_2^- may led to the weakening of ternary polycomplexes, that exert influence on the redistribution of solution components after irradiation. Figure 187 compares the results of HPLC analysis of unirradiated solutions of ternary PAA-Cu^{2+} mixtures prepared in water and in 0.154 g mol/l NaCl solution. As it can be seen, the heterogenecity of the mixture prepared in the presence of law molecular salts, significantly differs from those, which do not contain a specially added NaCl. Besides, the OD_{280} values in salt-containing mixture are essentially lower than in water system. On the basis of these results, it can be proposed that a soluble ternary polycomplex is not stable under physiological conditions (pH = 7.0; 0.154 g mol/l NaCl) and the interaction becomes a complicated character. The charge neutralization of particles (free section PAA, etc.) in mixture by NaCl leads to the decrease the size of particles, (broad peaks in chromatograms by high-diffusion coefficient of compact particles). Figure 187c corresponds to the HPLC results of the PAA-Cu^{2+}-BSA mixtures preparing in the presence of 0.154 g mol/l NaCl after irradiation at 2.5 kGy. Addition of NaCl to this irradiated ternary mixture does not lead to essential change in the character of the chromatograms of the solution. This phenomenon can be explained due to radiation-induced covalent crosslinking of protein and polymer macromolecules in ternary polycomplex after irradiation at 2.5 kGy, which lead to the additional stabilized polycomplex particles against NaCl.

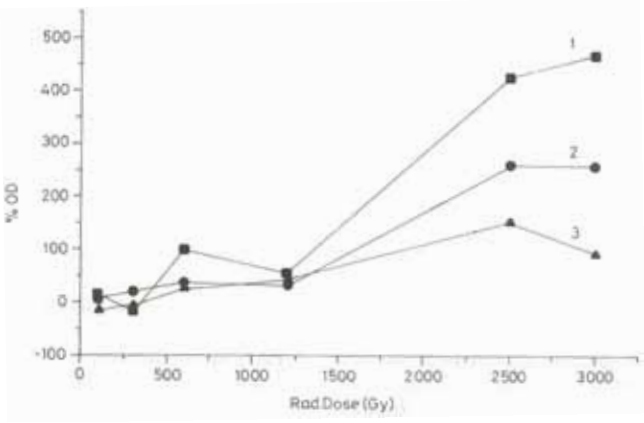


Figure 188. Percentage changes in optical density values %OD [%OD = (ΔOD / OD) x 100] %OD versus radiation dose (Gy). 1 – PAA; 2 - (PAA - SOD); 3 - SOD; $C_{BSA} = C_{PAA} = 0.01$ g/l

The preparation order of irradiated and unirradiated mixtures does not affect composition. The results have shown that the character of the formation of ternary complexes is same. Initially, addition of BSA into (PAA-Cu^{+2}) the mixture or addition of PAA into (Cu^{+2}-BSA) is practically same. One can assume that process of Cu^{2+} -induced complex formation between PAA and protein molecules is the equilibrium reaction.

6.2. Poly(NIPAAm)-BSA Systems

Poly(N-isopropylacrylamide) homopolymer do not contain the corresponding functional groups for protein covalent binding and complex formation in neutral water solution. The covalent binding of poly (NIPAAm) with BSA was carry out by irradiation method recently [371]. The solutions of poly (NIPAAm) and BSA were irradiated at different doses with a ^{60}Co γ-source. The change of the Tripthophan fluorescence intensity of polymer-protein mixture with increasing irradiation dose and temperature is shown in Figure 189.

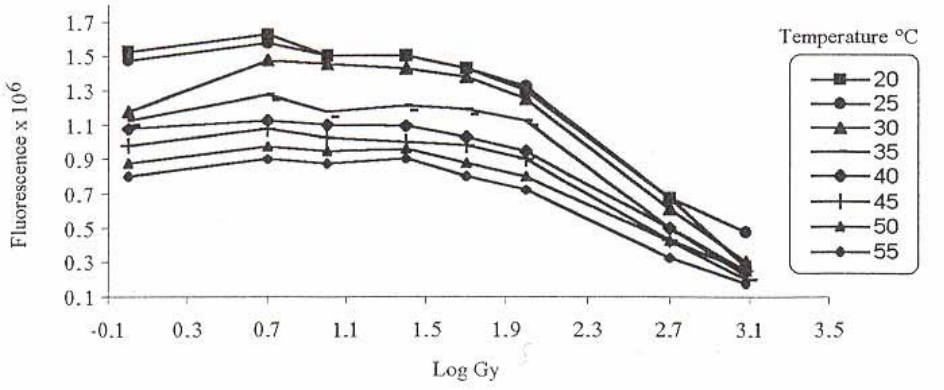


Figure 189. Fluorescence intensity of poly(NIPAAm)-BSA conjugates at different radiation dose and temperature

It was observed that the fluorescence intensity of polymer-protein mixture decreased with increasing irradiation dose and temperature. At the same time it was observed that the fluorescence intensity showed a very little change for absorbed dose of up to 0.1 kGy, but it decreased considerably for doses higher than 0.1 kGy. It was suggested that covalent conjugates occurs under this condition and this phenomenon was caused with the structural alteration of protein molecules in the composition of covalent conjugates. Formation of radiation-induced covalent conjugates in poly(NIPAAm)-BSA mixtures was confirmed by the HPLC and immunological methods recently. Injection of irradiated poly(NIPAAm)-BSA mixtures to animals resulted on the production of BSA-specific antibodies (Mustafaev et al. unpublished results).

6.3. PAA-SOD Systems

Figure 188 illustrates the dependence of the percentage changes in the optical density values %OD of the solutions of free PAA, SOD and equimolar mixture, PAA-SOD on the irradiation dose. As can be seen from comparison of these results, a significant increase in the values of %OD was observed for solutions of free PAA. Percentage change of optical density in solutions of PAA-SOD was lower in solutions of free PAA and higher in solutions of free SOD. The OD results obtained after irradiation indicate that all systems undergo some change in chemical structure (degradation -COOH groups, hydrogen abstraction from the polymer chain and crosslinking reactions (Hill et al., 1992) [375].

Figure 190a shows the results of HPLC analysis of unirradiated and irradiated solutions of free SOD, PAA and PAA-SOD mixtures at different irradiation doses. The irradiated solutions of PAA were characterized in chromatograms only by one peak and the retention time corresponding to the peaks practically does not change and remains equal to that of unirradiated PAA. The values of areas did not change considerably over a wide range of irradiation dose (up to 2.0 kGy). However, there exists a critical irradiation dose in the system (> 2.0 kGr) at which the areas of these peaks increased and reached a maximum although the values of RT did not change. (We cannot analyze the degradation and crosslinking of polymer chains after irradiation by HPLC methods because PAA macromolecules before and after irradiation is the polyelectrolytes with unfold structure and do not separate on molecular weight in HPLC). On the basis of these results, as well as from earlier investigations (Urbain, 1977; Pietrzak, 1995) [373,376], it can be proposed that the increase of the optical density with the dose is probably due to an unidentified radiolytic product which absorbs at the same wavelength. This might be due to organic peroxide formation.

Other results obtained for the irradiation of SOD solutions are shown in Figure 190a (B). It can be seen from the results that the SOD solutions in chromatograms were characterized by one peak (up to 0.6 kGy) at the low irradiation dose, but for the dose > 0.6 kGy, a new peak appears in chromatograms; the new peak appears with increasing values of RT and the irradiated SOD solutions were characterized by bimodal distribution. The bimodal distribution of components was obtained up to 3.0 kGy irradiation dose and change in the areas of these peaks was weak, if not negligible, with increasing irradiation dose.

Bovine erythrocyte SOD was subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulfate ± β-mercaptoethanol (Keele et al., 1971) [377]. It was shown that sodium dodecylsulfate was able to cause the dissociation of the enzyme only in the presence of β-mercaptoethanol. On the basis of these results Keele et al., concluded that SOD is composed of two subunits of equal size, the association of two subunits does involve at least one disulfide bridge. With radiation changes in the primary structure of proteins involved, oxidation of SH groups, partial deamination, decarboxylation and oxidation of phenol radicals and radicals of the heterocyclic amino acids. The principal reaction of oxygenated aqueous solutions of proteins is degradation and crosslinking by the peroxides (Swallow, 1973) [367]. We may conclude that the peroxide was able to destroy the disulfide bonds which is the most weak covalent bonds in protein structure.

HPLC results of PAA-SOD mixtures at different irradiation doses are given in Figure 190a (C). The mixture of PAA-SOD up to 0.6 kGy irradiation dose was characterized in chromatograms by two peaks corresponding to PAA (RT= 10.58-10.96 min) and SOD (RT= 16.96-17.24 min). The area of these peaks in the mixtures analogous to free polymer and enzyme systems did not change with increasing irradiation dose. Moreover, at irradiation doses ≥ 0.6 kGy, the peak corresponding to the SOD fraction doubled and behaved as in the free SOD solutions by the increasing irradiation dose. This result demonstrates that SOD is a scavenger of superoxide radicals and prevents the covalent conjugate formation. The effect of transition from unimodal to bimodal distribution of SOD fraction by irradiation is probably conditioned by destruction of the disulfide bond and separation of the two subunits of the enzyme. The superoxide anion (O_2^-) which is formed by the univalent reduction of O_2 by ionizing radiation will be captured with transient metal ions of the SOD molecule. Both copper and zinc ions might take part in catalysis of the reaction $O_2^- + O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2$ and protect the macromolecules against radiation damage.

6.4. Mechanism of the radiation-induced conjugation

Our results indicate that water-soluble PAA-BSA bioconjugates are formed at natural pH upon irradiation. The preexisting electrostatic repulsive forces between PAA and BSA (pI = 4.9) do not prevent the formation of covalent conjugates in the radiolysis of PAA-BSA mixtures.

The unirradiated mixture of PAA-SOD was characterized by two peaks corresponding to free PAA and SOD fractions. At neutral pH, PAA and SOD (the isoelectric point of the enzyme is 4.95) have negative charges and are incapable of binding to each other.

This protein consists of two subunits of identical molecular weight joined by a disulfide bond and contains two Cu(II) and two Zn (II) atoms per molecule. Zn^{++} plays a structural role and lends it enhanced stability whereas Cu^{++} is directly involved in the catalytic activity and binds two histidine residues. It can be proposed that the higher capacity of SOD in complex formation with copper-zinc ions than PAA prevents the ternary PAA-metal-enzyme complex formation.

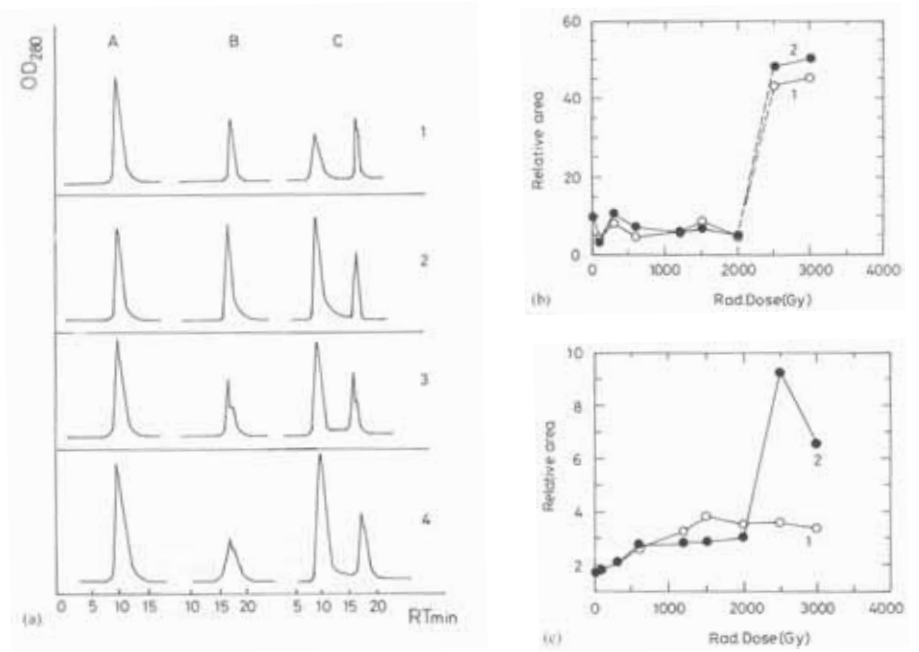


Figure 190. (a) HPLC chromatograms of unirradiated and irradiated solutions of PAA (A), SOD (B), PAA + SOD (C); 1 - unirradiated solutions; irradiated solutions at different irradiation dose (Gy): 600 (2); 2500 (3); 3000 (4); $C_{BSA} = C_{PAA} = 0.01$ g/l (b) Relative area of the peaks, corresponding free PAA (1) and PAA in mixture PAA-SOD versus radiation dose (2). (c) Relative area of the peaks, corresponding free SOD (1) and SOD in mixture PAA-SOD versus radiation dose (2)

Mechanism of protection

The effect of high energy radiations on water [367] may be summarized as:

$$H_2O \rightarrow 2.7\ (e^-_{aq}) + 2.7\ (OH\bullet) + 0.55\ (H\bullet) + 0.45\ (H_2) + 0.71\ (H_2O_2)$$

where the numbers before chemical symbols represents G-values. Larger yields of HO_2 and O_2^- are formed in aerated solutions by reaction of e^-_{aq} and H with oxygen [366]

$$e^-_{aq} + O_2 \rightarrow O_2^- \qquad \text{and} \qquad H\bullet + O_2 \rightarrow HO_2\bullet$$

With radiation, changes in the primary structure of proteins involved oxidation of SH groups, partial deamination, decarboxylation and oxidation of phenol radicals of the heterocyclic amino acids. The principal reaction of oxygenated aqueous solutions of protein is:

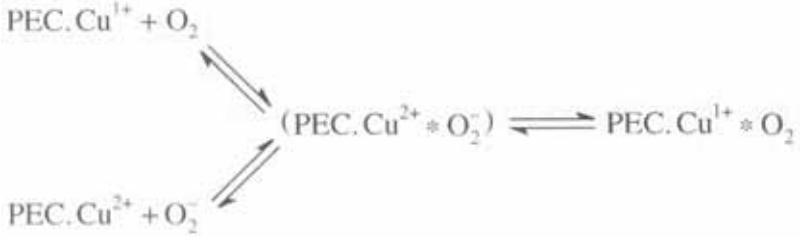
$$RCONH\text{-}CHR_2 + O_2 + H_2O \rightarrow RCONH_2 + RCOR + H_2O_2$$

Irradiation of polymers in the presence of oxygen can give rise to peroxides, which may subsequently lead to degradation and crosslinking of the polymer chain. The higher change in optical density of BSA solutions saturated with N_2O could be due to the increased yield of OH radicals in N_2O saturated solutions, where e^-_{aq} is converted to an equivalent amount of OH radical:

$$e^-_{aq} + N_2O \xrightarrow{+H_2O} OH\bullet + OH^- + N_2$$

According to Mustafaev [121], the mechanism underlying the protection effect in the

mixture of PAA-Cu^{2+}-BSA might be related to the complexation of copper in polyelectrolyte complex (PEC) with superoxide anion (O_2^-) and the formation of the following equilibrium:



Measurement of Cu(I) ions in irradiated solutions of 0.1% PAA-Cu(II) and 0.2% PAA-Cu(II) by neocuproine showed that the concentration of Cu(I) increases with increasing radiation dose.(Figure 191)

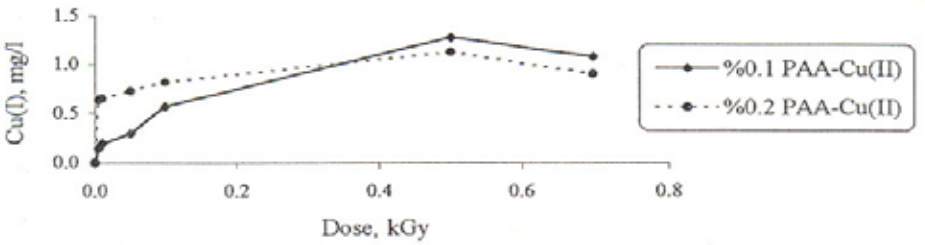


Figure 191. Effect of radiation dose on formation of Cu (I) in PAA-Cu(II) system; $[Cu^{2+}] = 1.388 \times 10^{-3}$ M, [PAA] = 0.1 g/dl and 0.2 g/dl, $n_{Cu^{2+}}/n_{PAA} = 0.05$

Copper ions may also react with OH and HO_2:

$$OH\bullet + Cu^{2+} \rightarrow Cu^{3+} + OH^- \text{ and } HO_2\bullet + Cu^{2+} \rightarrow Cu^{1+} + O_2 + H^+$$

Besides, one can notice that when polyanion macromolecules reacts with the protein globules to mask a group that is practically radiation sensitive and may act as a "sacrificial" protective agent. Both physical (charge and energy transfer) and chemical (reacting the amino acids with carbonyl compounds and Cu, recombination of forming radicals, "repair effect", etc.) protection can occur in this system. Stabilization of PAA chains in ternary polycomplexes was partially realized by Cu^{2+}. As shown earlier, the degradation of aqueous solutions of high molecular weight polymetacrylic acid caused by HO_2 radicals formed during irradiation is also inhibited by the protective SH compounds, amines, alcohols, cyanides, etc.

We can propose that in the mixture of PAA-SOD, Cu-Zn-superoxide dismutase is the scavenger of the superoxide anion by the following equlibrium:

$$Me^{2+}\text{-SOD} + 2\,O_2^- + 2\,H^+ \rightleftharpoons Me^{1+}\text{-SOD} + H_2O_2 + O_2$$

Metal ions act as protective agents of the protein globules against radiation damage and prevent the radiation-induced covalent conjugation of PAA macromolecules with SOD molecules. In conclusion, the presented results show the preparation of water-soluble covalent conjugates in the mixture of polyanions with similarly (negatively) charged proteins by radiation-induced method. Injection of irradiated PAA-BSA mixtures to animals resulted on the production of BSA-specific antibodies (Mustafaev et al., unpublished results), which open the new possibilities for the creation of immunogenic biopolymer systems by these methods. A considerable interest exists for the establishment of the influence of transient metal ions on these processes. Comparison of these results with the radioprotective activity of polymeric compounds will be of interest for the elucidation of the mechanism of the action of PE in the organism. In addition, such reactions may

simulate, for instance biomolecule reactions in the presence of transient metal ions.

7. CONCLUSION

The data presented in the monography provide factual evidence concerning the mechanism of binding of linear polyelectroleytes to proteins in aqueous solutions and the structure of soluble polycomylexes formed thereby. Some peculiarities and regularities of complex formation are discussed, and structural models of the synthetic compounds are proposed. An attempt has been made to classify the specific effects underlying the interactions between linear and globular macromolecules. It has been demonstrated that the use of fractions with predetermined molecular mass and a narrow range of molecular mass distribution as complex forming polymers males it possible to elaborate adequate approaches to the study of complex formation reactions in such systems and to apply a wide variety of present day physico-chemical methods for their investigation.

The experimental results testify to the fact that various proteins, regardless of their functional activity and physico-chemical properties, form soluble complexes with polyelectrolytes according to a common mechanism. Depending on the chemical nature of the polymeric carrier and environmental conditions, two types of soluble polymer-protein complexes may be constructed.

Complexes of the first type are formed in those systems which display a marked cooperativity of binding and a tendency towards self-organization. One characteristic feature of these complexes is the uneven distribution of protein molecules between the adsorbing polyions, i.e., the alternation of maximally loaded with protein polyelectrolytes and unloaded macromolecules. The components of such complexes have rod-like structure: the core of the rod is made up by protein globules that are closely linked together; the linear macromolecule is twisted around the protein globule, the length of the rod, i.e., the number of protein globules on it, being directly proportional to the degree of polymerization of the linear macromolecule. Under conditions when the compactization of polyions occurs as a result of nonpolar interactions of lateral hydrophobic radicals, the structural organization of the polymeric complexes is characterized by complex interactions between the polyion and the protein. The compact polyelectrolyte macromolecule forms the particle core on which protein molecules are situated, in many sites polymerized regions come to the surface.

Complexes of the second type are formed, as results of the uniform loading of protein globules between the polyelectrolyte chains, i.e., the protein molecules are randomly distributed between the adsorbing polyions. In this case the structure of soluble complexes being formed retains the conformation of a statistical coil of the polyelectrolyte carrier. By changing the chemical composition of the complex-forming polymer and environmental conditions (ionic strength, pH) one may induce an intermediate transition from the distribution of the "all or nothing" type to the uniform distribution. Possible causes of the existence of two types of soluble polycomplexes differing in their structural organization have been described. It has been supposed that such aggregates may be regarded as models of specific natural biocomplexes (nucleoprotein and polysaccharide-protein complexes), many of which may also be formed from proteins and naturally occurring polyelectrolytes as a result of self-organization.

The self-assembly reactions described herein are accouip1ished via relatively simple and nonspecific physico-chemical interactions between linear and globular macromo1ecules. The general structural and thermodynamic principle of self-assembly of highly ordered aggregates from chemica1ly complementary linear and globu1ar macromolecules has been proposed. Quite probably, the ‘minimum’ requirements concerning the nature and dynamics of macromolecular interactions are sufficient for such a self-assembly to be implemented in systems of different chemical nature, including those containing no biopolymers. One may assume that these results provide compelling evidence of the simplicity and uniformity of fundamental physico-chemical

principles underlying the occurrence and functioning of living systems.

The approaches and methodology used in the study of two-component polymer-protein systems play a very important role during the analysis of interactions occurring within multicomponent systems. The results cited herein suggest that the mode of distribution of protein fractions between the complex-forming polymer (both in artificial serum protein mixtures and in whole sera) is, in a greater degree, regulated by the possibility of 'positive' interactions between the identical subunits (globules) of the complex components which allows the synthesis of both individual and mixed (chimeric) polycomplexes. It has been shown that in solution self-organization, macromolecular exchange and substitution are also inherent in complex mixtures. Under these conditions the reaction equilibrium is reached rather quickly, almost at a rate of the reagent mixing. Specifically, the modes of formation, structure and composition (and, as a matter of consequence, the reactivity of the complex components) depend on the ionic strength and pH of the medium.

These data testify to the high practical utility of synthetic polymer-protein complexes in immunological, chemical and enzymological studies as well as m clinical medicine.

Very encouraging result were obtained in immunologic studies employing the use of polyelectrolyte complexes of natural antigens for the construction of polymer-subunit immunogens; these complexes served as a basis for creating effective vaccinating compounds aimed at the protection of animals from various infections. Thus, it has been shown that conjugation of model (BSA, BGG, OVA, etc), microbial and viral antigens (B. tuberculosis antigen, influenza virus, H-antigen of Salmonella, α-fetoprotein, etc.) with polyelectrolytes of different origin results in stable complexes endowed with an exceedingly high immunological activity. Joint investigations conducted in collaboration with our Indian colleagues at the National Institute of Immunology in New Delhi have culminated in the synthesis of highly immunogenic polyelectrolyte complexes of gonadotropic hormones whose application will open promising perspectives for the construction of anti fertile vaccines designed for birth control in animal and man. Noteworthy, these polycomplexes elicit thymus-independent immune responses, stimulate immunological memory (secondary immune responses) and, as was revealed by animal studies, afford rather an effective immunological protection.

One of the most perspective trends in modern-day immunology is the search for ways and means of lowering the immunogenecity of protein preparations (hormones, plasma components, etc.) injected to man curative purposes. For instance, multiple injections of insulin to patients with diabetes mellitus induce sensitization which, in turn, diminishes the biological activity of the drugs and evokes side reactions, e.g., allergy. Therefore the lowering of immunogenicity of protein preparations seems to be a very perspective approach. The working models of polymer-antigen complexes proposed herewith demonstrate that depending on the "architecture" of the antigen-polyelectrolyte particles the same polymeric carriers can be used for the construction of both highly immunogenic and weakly immunogenic preparations.

Hence a natural question arises as to what is the further fate of the polymeric carrier entering the organism. Marked progress has been attained in the synthesis of polymeric carriers capable of being split off and exported from the organism. As far as the choice of effective carbo-chained polymeric carriers is concerned, it does not present any serious problem. Thus, the construction of highly immunogenic preparations through the binding of nontoxic polyelectrolytes with natural antigens via transient metal ions (ternary polyelectrolyte-metal-antigen complexes) provides an illustrative example. This method is universal and allows the synthesis of polyelectrolyte complexes on the basis of a vast variety of polymers irrespective of their molecular mass (involving those, with universally accepted values of molecular masses) and composition, thus significantly expanding the range of polymers of immunological and clinical purpose.

Studies of complex formation between synthetic polyelectrolytes and biopolymers have culminated in the synthesis of highly immunogenic artificial antigens capable of affording

effective immunological protection from various infections as well as of radiopritector preparations displaying a high specific activity and stability. Analysis of physico-chemical capabilities of such polycomplexes in vitro and under conditions of the whole organism makes them a helpful tool in theoretical immunology and radiobiology studies as well as in other areas of the biological science. Thus, a direct correlation has been found between the ability of unnatural polyelectrolytes and their polycomplexes to enter multipoint interactions with proteinaceous and cellular components of the blood in model systems, on the one hand, and their physiological activity in vivo, on the other. This finding sheds additional light on the mechanisms underlying various effects of polymeric compounds in vivo. These results, however, do not indicate that the mechanisms underlying the physiological effects of such complexes should necessarily be based on polyelectrolyte interactions with blood serum proteins, e.g., serum albumin or other biosystem components selected as model compounds. No doubt, the observed correlation between the results obtained in vitro and in vivo implicates a similarity of mechanisms underlying the multipoint interactions inherent in polyelectrolytes as biphilic cooperative systems. These results may also be interpreted as being due to the lowered toxicity of polyelectrolytes without any alterations in their physiological activity. The latter circumstance is of key importance for practical medicine.

Further continuation of in depth studies in this field will inevitably lead to the solution of a vitally important task, namely, the synthesis of polyelectrolyte complexes of low and high molecular weight natural compounds having a predetermined structure and composition. These studies will develop along several lines namely further elaboration of theoretical concepts of polyreactions biomodelling and construction of ,radioprotectors, artificial polydeterminant antigens, drugs and vaccines of the future.

REFERENCES

[1] Tsuruta, T., Adv. Polym. Sci., 126, 1, 1996.
[2] Voycheck, C.L., and Tan, J.S.,"Ion containing polymers and their biological interactions" In: “Polyelectrolytes”, Science and Technology, Hara, M (Ed.), New York, 299-388, 1992.
[3] Ito, H., Miyamato, T., Inagani, H., Noshiki, Y., Iwata, H., and Matsuda, T., J. Appl. Polym. Sci., 32, 2, 3413-3418, 1986.
[4] Kataoka, K., Tsurata, T., Akaike, T., and Sakurai, Y., Macromol. Chem., 181, 1363-1369, 1980.
[5] Duncan, R. and Kopecek, J. Adv. Polym. Sci., 57, 53-101, 1984.
[6] Osada, Y., Advances in Polymer Science (S.Olive and G.Henrici, eds.), vol. 82, Springer, Berlin, 1987, 1-46.
[7] Okano, T., Yui W., Yokoyama, M. and Yoshida, R., “Advances in polymeric systems for drug delivery”, Japanese Technology Rewiews: Biotechnology, Gordon and Breach, vol. 4., No 1, Yverdon, Switzerland, 1994.
[8] Ogata, N., Kim, S.W., Feijen, J., Okano, T. (Eds.), “Advanced Biomaterials in Biomedical Engineering and Drug Delivery Systems”, Springer-Verlag, Tokyo, 381, 1996.
[9] Petrov, R.V., Mustafaev, M.I., and Norimov, A.Sh.,, Sov. Med. Rev. D. Immunol., Harwood Academic Publishers GmbH, UK, 1992, 1-113.
[9a] Mustafaev, M.I., Norimov, A.S., Petrov, R.V., “Synthetic immunomodulators", eds., Petrov, R., Nauka, Moscow, 1992.
[10] Ikada, Y., Suzuki, Y., Tamada, Y., In: Hoffman, A.S., Ratner, B.D., Horbett, T.A. (eds), “Polymer as biomaterials”, Plenum, New York, 135, 1985.
[11] Ottenbrite, R.M., Regelson, W., Kaplan, A., Carchmen, R., Morahan, B., and Munson, A., Donaruma, L.G., Vogl, O. (Eds.), Academic Press, New York, 1978, 262-304.
[12] Bamford, C.H., Cooper, S.L., Tsuruta, T., J. Biomater. Sci. Polymer Edn., 1, 1, 1989.

[13] Walter, H., in: Catsimpoolas (ed) "Method of cell separation", Plenum, New York, vol. 1, 307, 1977.
[14] Albertsson, P.Å., "Partition of cell particles and macromolecules", 3rd Ed.Wiley, New York, 1986.
[15] Strege, M.A., Dubin, P.L., West, J.S., Daniel Flinta, C.D., In: Ladisch, M., Willson, R.C., Painton, C.C., Builder, S.E. (eds), "Protein purification: from molecular mechanisms to large-scale processes", American Chemical Society, Washington, DC, chap. 5, 1990.
[16] Evdakov, V.P., Kabanov, V.A., Kozhinova, E.V., Petrov, R.V., Savinova, I.V., Fedoseeva, N.A., Khaitov, R.M., Khaustova, L.I., Dokl. Akad. Nauk. SSSR, 224, 464, 1976.
[17] Mustafaev, M.I., "Biopolymers (Biyopolimerler)", Gebze, Türkiye, 1996.
[18] Mustafaev, M.I., Saraç, S.A., "The polymeric material encyclopeptide: Synthesis, properties and applications", Solomone, Ed., CRC press: Boca Rato, FL, 1996, 5771-5777.
[18a] Mustafaev, M.I., Turkish Journal of Chemistry, 20, 126-138, 1996.
[19] Abuchouwski, A., van Es., T., Pelczul, N.C. and Davis, F.F., J. Biol. Chem., 252, 3578-3581, 1977.
[20] Braatz, J.A., Yasuda, Y., Olden, K., Yamada, K.M., and Heifetz, A.H., Bioconjugate Chem., 4, 262-267, 1993.
[21] Nathan, A., Zalipsky, S., Ertel, S.I., Agathes, S.N., Yarmush, M.L, and Kohu, J., Bioconjugate Chem., 4, 54-62, 1993.
[22] Morris, W., Steinhoff, M.C., Russel, P.K., Vaccine, 12, 1, 5-11.
[23] Kohn, J., Niemis, S.M., Albert, E.C., Murphy, J.C., Langer, R. and Fox, J.G., J. Immunol. Methods, 95, 31-38, 1986.
[24] Lee, W.Y. and Sehon, H., Natur., 267, 618-619, 1977.
[25] Sarac, A.S., Özeroğlu, C., and Mustafaev, M.I., J. Bioact. Compet. Polym., 10, 121, 1995.
[26] Kabanov, V.A., Mustafaev, M.I., Norimov, A.Sh., Petrov, R.V., and Khaitov, R.M., Dokl. Acad. Nauk SSSR, 243, 1130, 1978.
[27] Mustafaev, M.I., Manìco, V.M., Sokolova, E.A. and Gadzhiev, R.I., Immunology (Moscow), 6, 48, 1990.
[28] Mustafaev, M.I. and Norimov, A.Sh., Biomed. Sci., 1, 274-278, 1990.
[29] Mustafaev, M.I., Babakhin, A.A., Popov, A.N., Litvinov, I.S., Merkushov, A.V. and Gushin, I.S., Molek. Biol. (Moscow), 24, 358, 1990.
[30] Morawetz, H. and Hughes, W.L., J. Phys. Chem., 56, 64, 1952.
[31] Morawetz, H. and Zimmering, P.E., J. Phys. Chem., 58, 753, 1954.
[32] Morawetz, H., Sage, H., Arch. Biochem. Biophys. 56, 103, 1955.
[33] Mustafaev, M.I., Goncarov, V.V., Evdakov, V.P., and Kabanov, V.A., Dokl. Acad. Nauk SSSR, 225, 721, 1975.
[34] Kabanov, V.A., Evdakov, V.P., Mustafaev, M.I., and Antipina, A.D., Mol. Biol. (Moscow), 11, 582, 1977.
[35] Kabanov, V.A., Zezin, A.B., Mustafaev, M.I., and Kasaikin, V.A., "Polymeric Amines and Ammonium Salts", Goethals, E. J., Ed., Pergamon Press, Oxford, 1980, 173.
[35a] Berdick, M., Morawetz, H., J. Phys. Chem., 57, 959, 1953.
[36] Kabanov, V.A., Mustafaev, M.I., Belova, V.V., and Evdakov, V.P., Mol. Biol. (Moscow), 12, 1264, 1978.
[37] Kabanov, V.A., Mustafaev, M.I., Belova, V.V., and Evdakov, V.P., Biophysics (Moscow), 23, 789, 1978.
[38] Kabanov, V.A., Mustafaev, M.I., Bloxhina, V.D., and Agafeva, V.S., Mol. Biol. (Moscow), 14, 64, 1980.
[39] Kabanov, V.A., Mustafaev, M.I., and Goncarov, V.V., Dokl. Akad. Nauk SSSR, 244, 1261, 1979.

[40] Sacco, D., Bonneaux, F., Dellacherie, E., Int. J. Biol. Macromol., 10, 305, 1988.
[41] Petrak, K., "Polyelectrolyte complex" In: Polyelectrolytes: Science and Technology, Hara, M. (Ed.), Marcel Dekker, Inc., New York, 1992, 265-297.
[42] Samsonov, G.V., Panamareva, R.B., Luchko, R.G., Biofizika, 14, 634, 1969.
[43] Lenk, T., Thies, C., In: Eisenberg, A., Bailey, F.E. (eds), "Coulombic Interactions in Macromolecular Systems", American Chemical Society, Washington, DC, chap 8, 1987.
[44] Dubin, P., Ross, T.O., Sharma, I., Yegerlehner, B., In: Hinze, W.L., Armstrong, D.W., (eds), "Ordered media in chemical separations", American Chemical Society, Washington DC, chap. 8, 1987.
[45] Veis, A., Am. Chem. Soc., Div. Polym. Chem. Prepr., 32, 1, 596, 1991 and references therein.
[46] Burgess, D.J., Carless, J.E., J. Colloid. Interface Sci., 98, 1, 1984.
[47] Nguyen, T.Q., Makromol. Chem. 187, 2567, 1986.
[48] Sternberg, M., Hershberger, C., Biochim. Biophys. Acta, 342, 195, 1974.
[49] Kokufuta, E., Shimizu, H., Nakamura, I., Macromolecules, 14, 1178, 1981.
[50] Berdick, M., Morawetz, H., J. Biol. Chem., 206, 959, 1954.
[51] Bozzano, A.G., Andrea, G., Glatz, C.E., J. Membr. Sci., 55, 181, 1991.
[52] Clark, K.M., Glatz, C.E., Biotechnol. Prog., 3, 241, 1987.
[53] Fisher, R.R., Glatz, C.E., Biotechnol. Bioeng., 32, 777, 1988.
[54] Shieh, J., Glatz, C.E., Am. Chem. Soc., Div. Polym. Chem. Prepr., 32, 1, 606, 1991.
[55] Burgess, R.R., Jendrisak, J.J., Biochemistry, 14, 4634, 1975.
[56] Margolin, A., Sheratyuk, S.F., Izumrudov, V.A., Zezin, A.B., Kabanov, V.A., Eur. J. Biochem., 146, 625, 1985.
[57] Park, J.M., Muhoberac, B.B., Dubin, P., Xia, J., Macromolecules, 25, 290, 1992.
[58] Shaner, S.L., Melancan, P., Lee, K.S., Burgess, R.R., Gold Spring Harbor Symp. Quant. Biol., 47, 463, 1983.
[59] Kokufuta, E., Shimizu, H., Nakamura, I., Macromolecules, 15, 1618, 1982.
[60] Mustafaev, M.I., and Kabanov, V.A., Pharmacol. Toxicol. (Moscow), 45, 395, 1980.
[61] Mustafaev, M.I., and Kabanov, V.A., Vysocomol. Soedin Ser A, 23A, 271, 1981.
[62] Dubin, P., Ahmed, L., Xia, J., J. Macromol. Sci., A31, 17, 1994.
[63] Kabanov, V.A., Mustafaev, M.I., and Goncarov, V.V., ibid, 23A, 2275, 1981.
[64] Kabanov, V.A. and Mustafaev, M.I., ibid, 23A, 2255, 1981.
[65] Mustafaev, M.I., Proceedings of the Second All-Union Symposium on Interpolymeric Complexes, USSR, Riga, 1969, 326.
[66] Izumrudov, V.A., Kasaikin, V.A., Ermakova, L.N., Mustafaev, M.I., Zezin, A.B., Kabanov, V.A., Visokomolek. Soed., 23A, 1365, 1981.
[67] Dubin, P.L., Murrell, J.L., Macromolecules, 21, 2291, 1988.
[68] Anufrieva, Y.V., Pautov, V.D., Kuznetsova, N.P., Lushchik, V.B., Krakovyak, M.O., Polymer Science USSR, 29, 663, 1987.
[69] Güney, O., Saraç, A. S., Mustafaev, M., J.Bioact. Compet. Polym., 12, 231, 1997.
[70] Strelzowa, S.A., Tolstogusow, W.B., Colloid & Polymer Sci., 255, 1054, 1977.
[71] Papissov, I.M., Baranovski, V.Yu., Sergieva, E.I., Antipina, A.D. and Kabanov, V.A., Vysokomol. Soedin., SerA, 16, 1133, 1974.
[72] Papissov, I.M., Baranovski, V.Yu., Chernyak, V.Ya., Antipina, A.D. and Kabanov, V.A., Dokl. Akad. Nauk SSSR, 199, 1334-1337, 1974.
[73] Olins, D.E., Olins, A.H. and van Hippel, P., J. Mol. Biol., 24, 157, 1967.
[74] Cha, H.J., Izumi, T., Kokufuta, E., Frank, C.W., Am. Chem. Soc., Div. Polym. Chem. Prepr., 33, 872, 1992.
[75] Mustafaev, M.I., Kabanov, V.A., Berezin, I.V., Dikov, M.M., Solid Phase Chem., 1, 1980.
[76] Dikov, M.M., Osipov, A.P., Egorov, A.M., Berezin, I.V., Mustafaev, M.I., Kabanov,

V.A., Biochemistry (Moscow), 49, 8, 1113-1121, 1984.
[77] Dikov, M.M., Karulin, A.Y., Osipov, A.P., Egorov, A.M., Berezin, I.V., Mustafaev, M.I., Kabanov, V.A., Biochemistry (Moscow), 49, 9, 1219-1227, 1984.
[78] Berezin, I.V., Mustafaev, M.I., Dikov, M.M., Egorov, A.M., Pat. No: 2835139/28-04/150959, 25.10.1980.
[79] Xia, J., Dubin, P., Dauzenberg, H., Langmuir, 9, 2015, 1993.
[79b] Xia, J., Dubin, P.L., "Protein-polyelectrolyte complexes", in: Macromolecular complexes in chemistry and biology, Dubin, P., Bock, J., Davis, R., Schulz, D.N., Thies, C., (Eds.), Springer-Verlag, London, 1994, 247-272.
[80] Kokufuta, E., Takahashi, K., Polymer, 31, 1177, 1990.
[81] Kabanov, V.A., Mustafaev, M.I., Vysokomol. Soed. (High Polym. Comp.), 23A, N2, 1128-1136, 1981.
[82] Tristam, G., "Proteins", ed. Neurath,G., Bailey, K.M., IL, Moscow, 1, 244, 1956.
[83] Hiemenz, P.C., "Principles of colloid and surface chemistry", Marcel Dekker, New York, 1986.
[84] Baranovsky, V.Y., Litmanovich, A.P., Papisov, I. M., and Kabanov, V.A., Eur. Polym. J., 17, 696, 1981.
[85] Kabanov, V.A., Mustafaev, M.I., Goncarov, V.V., Petrov, R.V. and Khaitov, R.M., Dokl. Acad. Nauk SSSR, 250, 1504, 1980.
[86] Ohno, H., Abe, K. and Tsuchida, E., Makromol. Chem., 182, 1253, 1981.
[86b] Cha, H.J., Izumi, T., Kokufuta, E., Frank, C.W., Am. Chem. Soc., Div. Polym. Chem. Prepr., 33, 872, 1992.
[87] Kokufuta, E., Takahashi, K., Polymer, 31, 1177, 1990.
[87a] Kokufuta, E., "Complexation of proteins with polyelectrolytes", in: "Macromolecular complexes in chemistry and Biology", Dubin, P.L., et. al. (eds.), Springer-Verlag, London, 1994, 300.
[87b] Xia, J., Dubin, L.P., Morishita, Y., Sato, T., Muhaberac, B.B., Biopolymers, 35, 411-418, 1995.
[87c] Park, J.M., Muhoberac, B.B., Dubin, P., Xia, J., Macromolecules, 25, 290, 1992.
[88] Teramoto, A., Watanabe, M., Izuka, E., Abe, K., J. Macromol. Sci. Pure Appl. Chem., A31, 1, 53-64, 1994.
[89] He, X.M., Carter, D.C., Nature, 358, 209-315, 1993.
[90] Brown, J.W., Ed. Serum albumin; Pergamon press, New York, 1976.
[91] Peters, I.T., Adv. Protein Chem., 37, 161, 1985.
[92] Burstein, E.A., Vedenkina, N.S., Ivkova, M.N., Photochem. Photobiol., 18, 263-279, 1973.
[93] Peters, T., "All about albumin: Biochemistry, genetics and medical applications", Academic press: San Diego, 1996.
[94] Burstein, E.A., Biophysica, 13, 433-442, 1968.
[95] Peters, T., Jr., Biochim. Biophys. Acta, 39, 546-550, 1969.
[96] Peters, T., Jr., Bumenstock, F.A., J. Biol. Chem., 244, 1574-1580, 1967.
[97] Neurath, H. and Bailey, K. (Eds.), "The proteins", Acad. Press Inc., New York, 1953, 630.
[98] Zaitsev, V.S., Izumrudov, V.A., Zezin, A.B., Polymer Science USSR, 34, 54, 1992.
[99] Zaitsev, V.S., Izumrudov, V.A., Zezin, A.B., Kabanov, B.A., Dokl. Akad. Nauk. USSR, 332, 319, 1992.
[100] Cantor, C. and Schimmel, P., Biophys. Chem. (Russian translation) Mir, Moscow, 2, 7, 1984.
[101] Mustafaev, M.I., Tsareva, E.A., Evdakov, V.P., Vysokomol. Soedin. (Highpolym. Compounds.), AXVII, N10, 2226-2230, 1975.
[102] Mustafaev, M.I., Goncarov, V.V., Evdakov, V.P., Kabanov, V.A., Dokl. Akad. Nauk

SSSR (Proceedings of the USSR Academy of Sciences), N 225, 721, 1975.
[103] Kabanov, V.A., Mustafaev, M.I., Goncharov, V.V., Petrov, R.V., Khaitov, R.M., Dokl. Akad. Nauk SSSR (Proceedings of the USSR Acad.of Sci.), 250, 6, 1504-1507, 1980.
[104] Kabanov, V.A., Mustafaev, M.I., Goncarov, V.V., Vysokomol. Soed. (High polym.Comp.), 23(A), N2, 1121-1128, 1981.
[105] Yaskin, P.N., Mustafaev, M.I., Goncarov, V.V, Bull. Experim. Biol. and Medicine, N1, 136, 1982.
[106] Kennedy, Y.F., Chem. Soc. Rev., 8, 221, 1979.
[107] Sarkar, B. and Wigfield, Y., Can. J. Biochem., 46, 148, 1968.
[108] U.S. Pat. No.0097003 (02.06.83).
[109] U.S. Pat. No.9678667 (07.07.87).
[110] U.S. Pat. No.0109688 (30.05.84).
[111] U.S. Pat. No.0145359 (23.10.84).
[112] U.S. Pat. No.730276 A261 T 31/74,1972
[113] Wöhrle, D., Tsuchida, E., Ciardelli, F. (Eds.), "Macromolecule-Metal Complexes", Springer-Verlag, New York, 1995, 71.
[114] Kabanov, V.A., Kozhevnikova, V.A., Kokorin, A.I., et. Al., Vysokomol Soedin, 21A, 209, 1979, 19, 118, 1977.
[115] Zezin, A.B., Kabanov, V.A., Usp. Khim., 51, 1447, 1982.
[116] Subramanian, R., Natarajan, P., J. Polym. Sci. Polym. Chem. Ed., 22, 437, 1984.
[117] Saegusa, T., Kobayashi, S., Jamada, A., J. Appl. Polym. Sci., 21, 2481, 1977.
[118] Mustafaev, M.I., Abramenko, T.V, Filatova E.D., Immunologiya (Immunology), N1, 53, 1986.
[119] Mustafaev, M.I., Norimov, A.Sh., Goncarov, V.V., Zavgorodny, S.G., Immunologiya (Immunology), 6, 13-17, 1988.
[120] Sarac, A.S., Özeroğlu, C., and Mustafaev, M.I., J. Bioact. Compet. Polym., 10, 121, 1995.
[121] Mustafaev, M.I., Saraç, S.A., Erkol, A., Bayülken, S., Dinçer, B., Sezer, E., Polyme. Bull., 1996, 36, 623-627.
[122] Özeroğlu, C., Güney, O., Saraç, A.S., Mustafaev, M.I., J.of Appl. Polm.Sci., 60, 759-765, 1996.
[123] Özeroglu, C., Namazova, N., Mustafaev, M.I., Sarac, A.S., Colloid. Polym. Sci., 274, 418-427, 1996.
[124] Mustafaev, M.I., Çırakoğlu, B., Saraç, S.A., Öztürk, S., Yücel, F., Bermek, E., J. Appl. Polym. Sci., 62, 99-109, 1996.
[125] Mustafaev, M.I, Yücel, F., Öztürk, S., Çırakoğlu, B. and Bermek, E., J.lmmunol. Methods, 197, 31-37, 1996.
[126] Saraç, S.A., Yavuz, Ö., Sezer, E., Mustafaev, M.I., Polym. News, 22, 258-261, 1997.
[127] Mustafaev, M.I, Mustafaeva, Z., Bermek, E., Osada, Y. , J. Bioact. Compat. Polymers, 13, 33-49, 1998.
[128] Filenko, A., Demchenko, M., Mustafaeva, Z., Osada, Y., Mustafaev, M., Biomacromolecules, 2, 1, 270, 2001.
[129] Filenko, A., Demchenko, M., Mustafaeva, Z., Mustafaev, M.I., Physics of the alive, , 8, 2, 72-81, 2000.
[130] Tropsha, Y.G., Polinskii, A.S., Yaroslavov, A.A., Pshezhetskii, V.S. and Kabanov, V.A., Polym. Sci. USSR, 28, 7, 1527, 1986.
[131] Strauss, U.P. and Begala, A., J. Am. Chem. Soc., Polym. Preprints, 19, 255, 1978.
[132] Polinskii, A.S., Phezhetskii, V.S. and Kabanov, V.A., Dokl. Acad. Nauk SSSR, 256, 129, 1981.
[133] Klotz, I.M., and Curme, H.G., J. Am. Chem. Soc., 70, 939-943, 1948.
[134] Peters, Jr., T., Biochim. Biophys. Acta, 39, 546-550, 1960.
[135] Peters, Jr., T. and Bumenstoek, F.A., J. Biol. Chem., 242, 1574-1580, 1967.

[136] Izumrudov , V.A., Zezin, A.B., Kabanov, V.A., Dokl. Akad. Nauk SSSR, 274, 1156, 275, 1120, 1984.
[137] Lakowich, J. R., "Principles of Fluorescence Spectroscopy of Proteins", Plenum Press, New York and London, 1986, 496.
[138] Demchenko, A. P., "Ultraviolet Spectroscopy of Proteins", Springer-Verlag, Berlin-Heidelberg, 1986, 312.
[139] Laussae, J. P. and Sarkar, B., J. Biol. Chem., 255, 7563-7570, 1980.
[140] Fabry, T.L., Simo, C. and Yavaherian, K., Biochim. Biophys. Acta, 160, 188-195, 1968.
[141] Bae, Y.H., Okano, T., Hsu, R., Kim, S.W., Makromol. Chem. Rapid Commun., 8, 481-485, 1987.
[142] Scranton, A.B., Rangarajan, B., Klier, J., "Biomedical applications of polyelectrolytes" in: "Peppas, N.A., Langer, R.S. (Eds.), "Biopolymers II", Springer, Berlin, 1995, 3-54.
[143] Putnam, D., Kopecek, J., in: Peppas, N.A., Langer, R.S. (Eds.), "Biopolymers II", Springer, Berlin, 1995, 57-123.
[144] Hunkeler, D., Prokop, A., Powers, A., Haralson, M., di Maris, Wang, T.A., Polym. News, 22, 232-240, 1997.
[145] Monji, N., Hoffman, A.S., Appl. Biochem. Biotechnol., 14, 107, 1987.
[146] Bae, Y.H., Okano, T., Hsu, R., Kim, S.W., Macromol. Chem. Rapid Commun., 8, 481-485, 1987.
[147] Yamada, N., Okano, T., Sakai, H., Karikusa, F., Sawasaki, Y., Sakurai, Y., Makromol. Chem. Rapid Commun., 11, 571-576, 1990.
[148] Okano, T., Yamada, N., Sakai, H., Sakurai, Y.A., J. Biomed. Mater. Res., 27, 1243-1251, 1993.
[149] Plate, N.A., Vasiliev, A.E., "Physilogical active polymers", Khimia, Moscow, 1986.
[150] Wong, S.S., "Chemistry of protein conjugation and cross-linking", London, CRC Press Inc., 1993.
[151] Li, Y., Mattison, K.W., Dubin, P.L., Havel, H.A., Edwards, S.L., Biopolymers, 38, 527-533, 1996.
[152] Schild, H.G., Prog. Poly. Sci., 17, 163-249, 1992.
[153] Dilgimen, A.S., Mustafaeva, Z., Demchenko, M., Kaneko, T., Osada, Y., Mustafaev, M., Biomaterials, 22, 2383-2392, 2001.
[154] Hames, B.D., Rickwood, eds., "Gel electrophoresis of proteins", New York, Oxford Uni. Press, 1981.
[155] Laemmli, U.K., Nature, 227, 680-685, 1970.
[156] Chen, J.P., Yang, H.J., Hoffman, A.S., Biomaterials, 11, 625-630, 1990.
[157] Cole, C.A., Schriner, S.M., Priest, J.H., Monji, N., Hoffman, A.S., in: Russo, P. (Ed.), "Reversible polymeric gels and related systems", ACS Symp. Series, Washington DC, American Chem. Soc., 1987, vol. 350, 245.
[158] Petrov, R.V., Khaitov, R.M., Norimov, A.Sh., Kabanov, V.A., Mustafaev, M.I., Filatova, E.D., Dokl. Akad. Nauk SSSR (Proceedings of the USSR Academy of Sci.), 249, 1, 249-252, 1979.
[159] Petrov, R.V., Kabanov, V.A., Khaitov, R.M., Mustafaev, M.I., Norimov, A.Sh., Filatova, E.D., Zh. Mikrobiol. Epidemiol. Immunobiol., J. of Microbiology Epidem. and Immunobial., 2, 58-63 , 1981.
[160] Petrov, R.V., Kabanov, V.A., Mustafaev, M.I., Norimov, A.Sh., Immunologiya (Immunology), N6, 1982.
[161] Vinogradov, I.V., Kabanov, V.A., Mustafaev, M.I., Norimov, A.Sh., Petrov, R.V., Dokl. Akad. Nauk SSSR (Proceedings of the USSR Acad. of Sci.), 263, 1, 228-230, 1982.
[162] Mustafaev, M.I., Petrov, R.V., Khaitov, R.M., Immunologiya (Immunology), N6, 1982.
[163] Petrov, R.V., Mustafaev, M.I., Khaitov, R.M., Immunologiya (Immunology), N6, 52, 1982.

[164] Petrov, R.V., Mustafaev, M.I., Norimov, A.Sh., Immunologia (Immunology), N3, 56, 1983.
[165] Abramenko, T.V., Vinogradov, V.V., Kabanov, V.A., Mustafaev, M.I., Petrov, R.V., Khaitov, R.M., Zh. Mikrobiol. Epidemiol., Immunobiol.,Journal of Microbiology Epidemiol. and Immunobiol., 11, 86-89,1983.
[166] Manko, V.M., Mustafaev, M.I., Kompanietz, N.A., Zh.Mikrobiol., Jour. of .Microbiology, N2, 1983.
[167] Skorodinskaya, A.M., Kemenova, V.A., Efimov, V.S., Mustafaev, M.I., Kasaikin, V.A., Zezin, A.B., Kabanov, V.A., Khim. Farm. Zh., 18, 3, 283, 1984.
[168] Khaitov, R.M., Mustafaev, M.I., Norimov, A.Sh., Savgorodnyi, S.G., Abramenko, T.B., Immunologiya, 2, 22-25, 1986.
[169] Mustafaev, M.I, Norimov, A. Sh., Zavgorodny, S.G., Filatova , E.O., lmmunology (Moscow), 4, 88-89, 1989.
[170] Mustafaev, M.I., Manko, V.M., Socolova, E.A., Gadziev, R.I., Immunologia (Moscow), 6, 48-51, 1990.
[171] Petrov, R.V., Mustafaev, M.I., Norimov, A.Sh., Eivazova, E.S., Dokl. Akad Nauk SSSR (Moscow), 312, 2, 505-509, 1990.
[172] Kabanov, V.A., Mustafaev, M.I., Nekrasov, A.V., Norimov, A.Sh., Petrov, R.V., Dokl. Akad. Nauk SSSR , Proceedings of the USSR Academy of Sciences, 274, 4, 998-1001, 1984.
[173] Khaitov, R.M., Mustafaev, M.I., Norimov, A.Sh., Zavgorodny, S.G., Abramenko, T.V., Bull. Eksp. Biol. Med., Bull. of Exper. Biology and Medicine Moscow, 100, 11, 597-600, 1985.
[174] Petrov, R.V., Kabanov, V.A., Mustafaev, M.I., Norimov, A.Sh., Dokl. Akad. Nauk SSSR, Proceedings of the USSR Academy of Sciences, 283, 3, 744-748, 1985.
[175] Petrov, R.V., Kabanov, V.A., Khaitov, R.M., Mustafaev, M.I., Norimov, A.Sh., Gen Microbiol. Virusol. (Moscow), 6, 30-35, 1986.
[176] Mustafaev, M.I., Norimov, A.Sh., Immunologiya (Immunology), 7217-B87, Moscow: VINITI Press, 1988.
[177] Manko, V.M., Mustafaev, M.I., Gadzhiev, R.I., Immunologiya (Immunology), 6, 52-55, 1990.
[178] Petrov, R.V. Mustafaev, M.I., Norimov A.Sh.. Biomed.Sci., 1, 5, :531-533, 1990.
[179] Mustafaev, M.I., Popov, A.N., Immunologiya (Immmunology), N1, 28, 1990.
[180] Mustafaev, M.I., Romanova, R.Y, .Norimov, A.Sh, Filatova, E.D., Rusak, A.F., Immunologiya (Immunology), 4, 28-31, 1992.
[181] Mustafaev, M.I., Romanova, R.Y., Norimov, A.Sh., Filatova, E.D., Rusak, A.F., Immunologiya (Immunology), 4, 28-31, 1992.
[182] Mustafaev, M.I., Kabanov, V.A., Nikolaenko, V.V., Norimov, A.Sh., Petrov, R.V., Pat. No:3254917/23- 05(030105) 27.02.1981.
[183] Mustafaev, M.I., Kabanov, V.A., Khaitov, R.M., Topchiev, D.A., Pat. No: 4224471; 30.09.87.
[184] Mustafaev, M.I., Kabanov, V.A., Kargina, O.V., Pat. No: 4224481; 20.09.087.
[185] Mustafaev, M.I., Kabanov, V.A. Kargina,O.V. Sehtepenko O.B. Pat. No: 4224470; 12.08.87.
[186] Mustafaev, M.I., Alekseeva, N.Y., Khaitov, R.M., Abramenko, T.V., Pat. No: 4123973; 30.09.87.
[187] Hanks, E.G., Ainsworth, E.J., Rad. Res., 32, 367, 1967.
[188] Vorob'ev, A.A., Vasil'ev, N.N., Adjuvanti Medetsina, Moscow, 1969.
[189] Zemscov, V.M., Zhurn. Mikrobiol. Epidemiol. i Immunologii, No.3, 16, 1972.
[190] Cone, E.R., Pharmac. Ther., 8, 321, 1979.
[191] Petrov, R.V., Gvozdetskii, A.N., Gorokhov, A.A., Evdakov, V.P., Kabanov, V.A.,

Kabanova, E.A., Zhurn. Mikrobiol. Epedemiol. i Immunologii, No.II, 37, 1974.
[192] Batirbekov, A.A., Evdakov, V.P., Kabanov, V.A., Kozhinova, E.V., Petrov, R.V., Savinova, I.V., Fedoseeva, N.A., Khaitov, R.M., Khaustova, L.I., Tsitologia, 18, 1259, 1976.
[193] Kargin, V.A., V.A., Kabanov, Mustafaev, M.I., Patrikeeva, T.I., Vysokomol.Soed.(Highpolym. Comp.) AIX, N2, 1967.
[194] Kabanov, V.A., Mustafaev, M.I., Aliyev, K.V., Vysokomol.Soed.(Highpolym. Comp.), 12, 4, 855, 1970.
[195] Mustafaev, M.I., Aliev, K.V., Kabanov, V.A., VINITI , 1970.
[196] Petrov, R.V., Khaitov, R.M., Kozhinova, E.V., Evdakov, V.P., Gvozdetskii, A.N., Tsitologia., 17, 321, 1975.
[197] Petrov, R.V., Khaitov, R.M., Kozhinova, E.V., Evdakov, V.P., Tsitologia, 17, 1172. 1975.
[198] Petrov, R.V., Khaitov, R.M., Norimov, A.Sh., Nazhmetdinov, A.M., Diskant, P., Zavgorodnii S.G., Korijakin, S.A., Immunologia, No.2, 39. 1981.
[199] Petrov, R.V., Khaitov, R.M., Norimov, A.Sh., Zavgorodnii, S.G., Bul. EXT. Biol. i Med., No.4, 461, 1981.
[200] Kabanov, V.A., Zezin, A.B., Pure and Appl. Chem., 56, 343, 1984.
[201] Kabanov, V.A., Zezin, A.B., Soviet Scientific Reviews, Sec. B., Chemistry, 4, 207, Harwood Acad. Pub.GmBH and OPA, Arnsterdam, 1982.
[202] Kabanov, V.A., Papissov, M., Visokomolek. Soed., 21A, 243, 1979.
[203] Savinova, I.V., Fedoseeva, N.A., Evdakov, V.P., Kabanov, V.A., Visokomolek. Soed., I8A. 2050, 1976.
[204] Kabanov, V.A., Zezin, A.B., Mustafaev, M.I., Kasaikin, V.A., "Soluble interpolymeric complexes of polyamines and polyammonium salts" In: Polymeric Amines and Ammonium Sallts .Ed.Goethals, Ghent, Belgium, 173-192, 1979,
[205] Kabanov, V.A., Mustafaev, M.I., Blokhina, V.D., Agafeva, V.S., Mol. Biol. (Mosk), 14, 1, 47-57, 1980.
[206] Mustafaev, M.I., Kabanov, V.A., Farmakol. Toksikol. (Pharmacology and Toxicology), 43, 4, 395-399, 1980.
[207] Mustafaev, M.I., "Complexes of non-natural polyelectrolytes and proteins" (Doctor of Chemical Sciense thesis), Moscow, 1981, 417.
[208] Katchalsky, A., Danon, D., Nevo, A., De Vries, A., Biochem. Biophys. Acta., 33, 120, 1959.
[209] Lalezary, P., Spaet, T.H., J. Lab. and ed., 57, 868, 1961.
[210] Jenkins, C.S.P., Packhan, M.A., Kilough-Rathbone, R.L., Mustard, J.F., Blood, 37, 395, 1971.
[211] Efimov, V.S., Men'shova, G.A., Gulyaeva, Zh.G., Farmakol. . i ToksikoloRia, No.4, 409, 1978.
[212] Efimov, V.S., Usmanov, D.M., Musaev, U.N., Farmakol. i Toksikologia, No.6, 628, 1978.
[213] Efimov, V.S., Gulyaeva, Zh.G., Men'shova, S.I., Rozvodovskii, E.F., Zezin, A.B., Lakin, K.M., Farmakol. i Toksikologia, No.6, 688, 1979.
[214] Kabanova, E.A., Kokorin, I.N., Krasnova, L.N., Gvozdetskii, A.N., Dokl. Akad. Nauk SSSR, 242, 490, 1978.
[215] Khaitov, R.M., Ataulakhanov, R.I., Immunogia, No.4, 30, 1982.
[216] Kabanov, V.A., Petrov, R.V., Khaitov, R.M., Zhurn. Vsesousn. Khim. Obchestv. im. Mendeleeva, 27, No.4, 417, 1982.
[217] Kabanov, V.A., Mustafaev, M.I., Blokhina, V.D., Agafieva, V.S., Molek. Biologia, 14, 64, 1980.
[218] Mustafaev, M.I., Kabanov, V.A., Farmakol. i Toksikologia, 43, 64, 1980.
[219] Kabanov, V.A., Polinskii, A.S., Yaroslavov, A.A., Chechik, O.S., Dokl. Akad. Nauk SSSR, 283, No:6, 1985.

[220] Kabanov, V.A., Zezin, A.B., Makromol. Chem., Suppl. 6, 259, 1984.
[221] Petrov, R.V., Evdakov, V.P., Khaitov, R.M., Filatova, E.D., Alekseeva, N.Yu., Savinova, I.V., Kozhinova, E.V., Vorontsov, E.D., Dokl. Akad. Nauk SSSR, 236, 1260, 1977.
[222] Sela, M., Science, 166, 1365, 1969.
[223] Benacerraf, B., Ann. Immunol., 125-C, 143, 1974.
[224] Petrov, R.V., Kabanov, V. A., Khaitov, R.M., Immunologia, No.2, 120, 1983.
[225] Sela, M., Science, 166, 1365, 1969.
[226] Benacerraf, B., Ann. Immunol., I25-C, 143, 1974.
[227] Petrov, R.V., Kabanov, V. A., Khaitov, R.M., Immunologia, No.2, 120, 1983.
[228] Petrov, R.V., Khaitov, R.M., "Artificial antigens and vaccines (in Russian)", Moscow, Medicina Press, 1988, 191-231.
[229] Kalita, S.A., Abramenko, T.V., Mustafaev, M.I., Alekseeva, N.I., "Immunogenic and immunostimulating properties of plague microbe fractional complexes with synthetic polyelectrolytes", in: "Proceeding of the All-Union Conference on Immunology", USSR, Taitu, 1989, 23.
[230] Goncharov, V.V., Mustafaev, M.I. and Frolov, T.V., "Scanning electron microscopic studies of the mechanism of synthetic polyelectrolyte interaction with sheep red blood cells", in: "Proceeding of the Twelfth All-Union Conference on Electron Microscobe", USSR, Sunny, 1982, 27.
[231] Tsuchida, E., "Interaction of polycations with erythrocyte membrans and its application as a reagent for cell fusion", in: "Polymeric Amines and Ammonium Salts", Ghent, 1979, 127-130.
[232] Williams, D., "Metals of life", Inostr. Liter. Press., Moscow, 1975, 236-242.
[233] Yatsimirsky, K.B., "Biological aspects of coordinate compounds", Khimiya Press, Kiev, 1979, 281.
[234] Prohoska, I.R. and Zukasowycz, O.A., Science, 213, 559-561, 1983.
[235] Fracex, P.I. and Haas, S.M., J. Nutrition, 107, 1889-1892, 1977.
[236] Hart, D.A., Cellular Immunology, 71, 159-164, 1982.
[237] Hart, D.A., ibid, 71, 169-182, 1982.
[238] Hughes, M., "Inorganic Chemistry of Biological Processes", Inostr. Liter. Press, Moscow, 1983, 220.
[239] Başalp, A., Bermek, E., Çirakoğlu, B., Çoka, V., Mustafaev, M., and Saraç, A.S., Hybridoma, 15, 233-238, 1996.
[240] Mustafev, M.I, Yücel, F., Çırakoğlu, B. and Bermek, E., Immunol. Lett., 52, 63-68, 1996.
[241] M. I. Mustafaev, Y. Osada, A. Başalp, Z. Mustafaeva, B. Çırakoğlu and E. Bermek, "Review, New Temperature-responsive immunogens by Poly(N-isopropyl-acrylamide)-modified bovine serum albumin", In: Recent Advances in Peptide and Protein Delivery, Editions de Sante, Paris, 1998, 145-162.
[242] Yücel, F., Çırakoğlu, B., Mustafaeva, Z., Mustafaev, M., Hybridoma, 20, 1, 11-15, 2001.
[243] Başalp, A., Mustafaeva, Z., Mustafaev, M.I., Hybridoma and Hybridomics, 21, 1, 45-51, 2002.
[244] Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T. & Sakurai, Y., *Bioconjugate Chem.,* 4, 42-46, 1993.
[245] Matsukata, M., Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Sakurai, Y. & Okano, T., J. *Biochem.,*116, 682-686, 1994.
[246] Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y. & Okano, T., *Bioconjugate Chem.,* 796-101, 1996.
[247] Dong, L. C. & Hoffman, A. S., J. *Control/ed Release,* 4, 223-227, 1986.
[248] Matsuda, K., Orii, H., Hirata, M. & Kokufuta, E., *Polymer Gels and Networks,* 2, 299-305, 1994.
[249] Heskins, M., Guillet, J. E. & James, E., *J. Macromol. Sci. Chem.,* A2, 1441-1455, 1968.

[250] Shild, H.G., *Prog. Polym. Sci.,*17, 163-24912, 1992.
[251] Mustafaev, M., Osada, Y., Matsukata, M., Başalp, A., Çırakoğlu, B., Bermek, E, Polymer Gels and Networks. 4, 363-372, 1996.
[252] Engwall, E., *Meth. Enzymol.,* 70, 409, 1980.
[253] Kabanov, V.A., "Basic properties of soluble interpolyelectrolyte complexes applied to bioengineering and cell transformations", in: Macromolecular complexes in chemistry and biology, Dubin, P., Bock, J., Davis, R., Schulz, D.N., Thies, C., (Eds.), Springer-Verlag, London, 1994, 151-174.
[254] Popov, A.N., Mustafaev, M.I., Voitenko, V,G., Gushin, I.S., "Allergenicity of two types of water-soluble complexes of OVA with polyelectrolytes and their ability to induce the production of homocytotropic antibodies in mice", in: "Proceedings of Second All-Union Symp. on Interpolymeric Complexes", Riga, USSR, 1989, 327-341.
[255] Vaz, E.M., Vaz, N.M. and Levine, B.B., Immunology, 21, 11-15, 1971.
[256] Dephenghi, R. and Mason, A.J., In: "Medicinal Chemistry", Burger, A., (Ed.), Wiley-Interscience, New York, 1970, 911.
[257] Dean, P.D., Rowe, P.H., and Exley, D., Steroids Lipids Res., 3, 82-89, 1972.
[258] Garza, G.A., and Rao, P.N., Steroids. 42, 469-474, 1983;
[259] U.S. Patent No:3940475, 1976.
[260] Wa1ker, C.S., Clark, S.J., and Wotiz, H.H., Steroids 21, 259-283, 1973.
[261] Da Re, P., Valenti, P., Braga, P.C., and Ferri, S., Arch. Pharm (Weinheim), 308, 981-982, 1975.
[262] U.S. Patent No:4952569 A (Simons), Estradiol Derivatives, 1990.
[263] Erlanger, B.F., Methods Enzymol., 70, 85-104, 1980.
[264] Niswender, G.D., and Midgley, A.R., "Immunological Methods in Steroid Determination", Chap. 8, Peron, F.G., and Caldwell, B.F. (Eds.), Appleton, New York, 1970,
[265] Harlow, E., and Land, D., "Antibodies", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988.
[266] Fantl, V.E., Wang, D.Y., and Whitehead A.S., J. Steroid Biochem., 14, 405, 1981.
[267] Fantl, V.E., Wang, D.Y., and Knyba, R.J., J. Steroid Biochem., 17, 125, 1982.
[268] Fantl, V.E. and Wang, D.Y., J. Endocrinol., 100, 367, 1984.
[269] Weliky, N., and Weetall, H.H., Immunochemistry, 2, 293-322, 1965.
[270] Sauer, M.J., Foulkes, J.A., Worsfold, A., and Morris B.A., J. Reprod. Fertil., 76, 37-39, 1986.
[271] Goto, N., Kato, H., Maeyema, J.I., Shibono, M., Saito, T., Yamaguchi, J., and Yoshihara, S., Vaccine, 15, 1364-1371, 1997.
[272] Grupta, R.K., and Rost, B., In: Vaccine Adjuvant, O'Hagan, D.T. (Ed.)., Humana Press, Totawa, 2000, 65-89.
[273] Lindblad, E.B., In: Vaccine Adjuvants, O'Hagan, D.T. (Ed.), Humana Press, Totawa, 2000, 49-63.
[274] Lei, Y., and Lu, B., Yao Hsueh Hsueh Pao., 28, 269-301, 1993.
[275] Ye, W.P., and Chien, Y.W., Pharm. Dev. Technol., 1, 1-19, 1996.
[276] Robaire, B., Duron, J., and Hales, B.F., Biol. Reprod., 37, 327-334, 1987.
[277] Elsaesser, F., Hayashi, S., Parvizi, N., and Ellendorf, F., Steroids, 54, 159-168, 1989.
[278] Brannon-Peppas, L., J. Biomater. Sci. Polymer, 5, 339-351, 1994.
[279] Sitruk-Ware, R., J. Steroid Biochem. Mol. Biol., 53, 247-251, 1995.
[280] Casanas-Roux, F., Nisolle, M., Marbaix, E., Smets, M., Bassil, S. and Donnez, J., Human Reprod., 1, 357-363, 1996.
[281] Nowak, M., Buntner, B., Bero, M., Gorski, J., Kajdanuik, D., GlogowskaSzelag, J., and Plewka, A., Acta Pharm. Hung., 66, 153-156, 1996.
[282] Irion, G.D., Garrison, M.D., and Abraham, W., Pharm. Res., 12, 1618-1622, 1995.

[283] Plotka, E.D., Eagle, T.C., Vevea, D.N., Koller, A.L., Sniff, D.B., Tester, J.R. and Seal, U.S., J. Wildl. Dis., 24, 507-514, 1988.
[284] Plerdaeu, A., Rabadex, J.C., Gueniffey, H., and Lenuz, C., Eur. Polymer J.,17, 801-805, 1980.
[285] Yolles, S., Morton, J.F., and Sartori, M.F., J. Polymer Sci. Polymer Chem. Ed., 17, 4111-4113, 1979.
[286] Kenny, J.S., Hughes, B.W., Masada, M.P., and Allison, A.C., J. Immunol. Meth., 121, 157-166, 1989.
[287] Gonzalez, S., Nazabal, C., Vina, L., and Caballero, E., Dev. Biol. Stand., 92, 269-276, 1998.
[288] Ellis, R.W., Vaccine, 17, 1635-1642, 1999.
[289] Andre, F., Vaccine, 17, 1620-1627, 1999.
[290] Rao, P.N., Wang, Z., Cessac, J.W., and Moore, P.H., Jr., Steroids, 63, 141-145, 1998.
[291] U.S. Pat. No: 4 952569. 28.08, Estradiol Derivatives, 1996.
[292] Allcock, H.R., and Fuller, T.J., Macromolecules, 13, 1345-1350, 1980.
[293] Zupon, M.A., Fang, S.M., Christensen, S.M., and Petersen, R.V., J. Pharm. Sci., 72, 1323-1326, 1983.
[294] Ritter, H., Makromol. Chem. Rapid. Commun., 3, 431-435, 1982.
[295] Pinazzi, C., Rabadeux, J., and Plerdeau, A., Eur. Polymer. J., 14, 205-209, 1977.
[296] Pinazzi, C., Menil, A., and Plerdeau, A., Bull. Socie'te' Chimique de France, 3, 667-670, 1974.
[297] Galfre, G., and Milstein, C., Methods Enzymol., 73, 3-46, 1983.
[298] Lietzke, R. and Unsicker, K., J. Immunol. Methods, 76, 223-228, 1985.
[299] Buntner, B., Nowak, M., Kaspercyzk, J., Ryba, M., Grieb, P., Walski, M.M., Doryzinski, P., and Bero, M., J. Controlled Release, 56, 159-167, 1998.
[300] Ye, W.P., and Chien, Y.W., Pharm. Dev. Technol., 1, 1-9, 1996.
[301] Başalp, A., Mustafaeva, Z., Mustafaev, M.I., Bermek, E., Hybridoma, Vol.19, 6, 19, 495-499, 2000.
[302] Mustafaev, M.I., Başalp, A., Yücel, F., Çırakoğlu, B., Bermek, E., Patent: 97/ 00395; TUBİTAK-Marmara Araştırma Merkezi, 1997.
[303] Mustafaev, M. I., Başalp, A., Mustafaeva, Z., Bermek, E., Patent Başvurusu: TUBİTAK-Marmara Araştırma Merkezi, 1999.
[304] Sun, I.C., Wang, H.K., Kashiwada, Y., Shen, J.K., Cosentino, L.M., Chen, C.H., Yang, L.M., Lee, K.H., J, 41, 23, 4648-4657, 1998.
[305] Niura, N., Matsumoto, Y., Miyairi, S., Nishiyama, S. And Naganuma, A., Mol. Pharmacol., 56, 6, 1324-1328, 1999.
[306] Steele, J.C., Warthurts, D.C., Kirby, G.C. and Simmonds, M.S., Phytother. Res., 13, 2, 115-119, 1999.
[307] Flekhter, O.B., Karachurina, L.T., Poroikov, V.V., Nigmatullina, L.R., Baltina, L.A. and Zarudii, F.S., Boorg. Khim., 26, 3, 215-223, 2000.
[308] Sun, I.C., Shen, J.K., Wang, H.K., Cosentino, L.M. and Lee, K.H., Bioorg. Med. Chem. Lett.,8, 10, 1267-1272, 1998.
[309] Miskiniene, V., Dickancaite, E., Nemeikaite, A. And Cenas, N., Biochem. Mol. Biol. Int., 42, 2, 391-397, 1997.
[310] Son, L.B., Kaplun, A.P., Spilevskii, A.A., Andia-Pravdivyi, I.E., Alekseeva, S.G., Griboev, V.B. and Shvetz, V.I., Bioorg. Khim., 24, 10, 787-793, 1998.
[311] Mustafaev, M., Mustafaeva, Z., Ergen, E., Uraki, Y., Sano, Y., J. Bioact. Comp. Polymers, 17, 251–269, 2002.
[312] Filenko, A., Demchenko M., Mustafaeva, Z.,.Mustafaev, M.I., Physics of the alive, 8, 2, 72-81, 2000.
[313] Petrov, R.V., Kabanov, V.A., Khaitov, M., et al., immunologia, 5, 125, 1983.

[314] Petrov, R.V., Zhdanov, M., Kabanov, V.A., Khaitov, R.M., Norimov, A.Sh., Sinyakov, M.S., Nekrasov, A.V., Kharetonenkov, I.G., Bul. Exp. Biol. i Med., No.2, 184, 1985.
[315] Sinyakov, M.S., Norimov, A.Sh., Nekrasov, A.B., Khaitov, R.M., Petrov, R.V., First All-Union Congress of Biophysics, Moscow, Abstracts, 3, 55, 1982.
[316] Petrov, R.V., Khaitov, R.M., Zhdanov, V.M., et. al., Vaccine, 3, 392-400, 1985.
[317] Norimov, A.Sh., Mustafaev, M.I., Romanova, R.Y., Abramenko, T.V., Bull. Eksp Biol Med., Moscow, 111, 2, 180-181, 1991.
[318] Elshina, G.A., Gorbunov, M.A., Shervali, V.I. et. al., Zh Mikrobiol Epidemiol Immunobiol., Moscow, 3, 48-53, 1998.
[319] Talwar, G.P. (Ed.), "Contraception Research for Today and the Nineties", Springer-Verlag, New York, 1986.
[320] Hruby, V.J., Sharma, S.O., Collins, N., Matsunaga, T.O., Russel, K.C., Applications of Synthetic Peptides. In:Grant GA, editar. SyntheticPeptides. New York, W.H. Freeman and Company, 1992:261-349.
[321] Lerner, R.A., Nature 1982; 299:592-595.
[322] Audibert, F., Jolivet, M., Chedid, L., Alanf, J.E., Boquet, P., Ruaille, P. and Sifferto, O., Nature, 289, 593-599, 1981.
[323] Jacobs, C. O., Sela, M., Arnon, R., Proc. Natl. Acad. Sci., 80, 7611-7619, 1983.
[324] Richman, S.J., Thomas, V., Sharma, P., Flint, J., Ardeshir, F., Grass, M., Silverman, C. and Rcese, R.T., "Construction of carrier-free synthetic peptide antigens capable of stimulating biostable antibody responses to a 75 kDa malarial parasite protein", in: "Synthetic Peptides:Approaches to biological problems", Tam, J.P. and Kaiser, E.T., eds, Lis, A.R., Inc., New York, 1989, 1-113.
[325] Brown, F., "The next generation of Foot and Mouth Disease Vaccines, in: Tam, J.P., Kaiser, E.T., eds., "Synthetic peptides: Approcjes to biological problems", New York, Alan R. Liss Inc; 1989, 127-142.
[326] Barteling, S.J. and Vreesweijk. J., Vaccine, 9, 75-88, 1991.
[327] Strohmaier, K., Franze, R. and Adam, K.H., J Gen Viral., 59, 295-305, 1982.
[328] Bittle, J.L., Houghton, R.A., Alexander, H. et. AI. Nature, 298, 30-34,1982.
[329] Pfaff, E., Mussgay, M., Bohm, H.A., Shulz, G.E. and Scaller, H., EMBO J., 1, 869-874, 1982.
[330] Weddll, G.N., Yansura, O.G., Dowbenko, D.J. et. al., Proc. Nathl. Acad. Sci., USA 82, 2618-2622, 1985.
[331] Brown, F., "Chemical basis of antigenic variation in foot-and-mouth disease virus", ln: Regenmortel M.H.V. and Neurath A.R., editors, lmmunochemistry of Viruses.The basis for serodiagnosis and vaccines, EIsevier Sci. Publ. B.V., 1984, 265-279.
[332] Beck, E., Feil, G. and Strohmaier. K., EMBO J. 2, 555-559, 1983.
[333] Xie, O.C., McCahon, D., Crowther, J.R., Belsham, G.J. and McCullough, K.C., J. Gen Vir., 68, 1637-1647, 1987.
[334] Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. and Brown, F., Nature, London,; 337, 709-716, 1989.
[335] Parry, N.R., Barnett, P.V., Ouldrigde, E.J., Rowlands, D.J. and Brown, F., J. Gen Vir., 70, 1493-1503, 1989.
[336] Feigelstock, D.A., Maten, M.G., Valero, M.L., Andreu, D., Domingo, E. and Palma E.L., Vaccine; 14, 97-102, 1996.
[337] Domingo, E., Mateu, M.G., Escarmis, C., et. al., Virus Genes, 23, 197 -207, 1996.
[338] Grant, G.A., "Synthetic Peptides", ed., USA, 1992, 283-289.
[339] Neurath, A.R., et. al. Nature, 315,154,1985.
[340] Neurath, A.R., et. al. Synthetic Peptides: Approach_s to Biological Problems, UCLA Symposia on Molecular and Cellular Biology, New Series, Alan R.liss New York, 1989, v.86, p.143.

[341] Wang H.H., Dietzschold B., Koprowski H., Chin. Med. J. (Ehgl), , 102, 11, 885-9, 1989
[342] Kobbs-Conrad, S., Gerdon, A., and Kaumaya, P.T., "Multivalent B- and T-cell epitope vaccine desing" ln: Proceedings of the Twelfht American Peptide Symposium, Smith, J. and River, J., eds., Ercom, Leiden, 1991.
[343] Tam, J.P., "Multiple antigenic peptide systems: A novel desing for synthetic peptides vaccines and immunoassay", ln: Synthetic Peptides: Approaches to Biological Problems, Tam, J.P. and Kaiser, E.T., eds., Alan R.liss, Inc., New York, 1989, 3-18.
[344] Francis, M.J., Hasting, G.Z., Syred, A.D., Mc Ginn, B., Brown, F. and Rowlands, D.J., Nature, 330, 168-170.
[345] Mutter, M. Synthetic proteins with a new three-dimensional architecture.ln Proceedings of the Tenth American Peptide Symposium.G.R. Marshall, ed. ESCOM, Leiden, 1988, 349-353.
[346] Hopp T.P., Mol. Immunol., 21, 1, 13-6, 1984.
[347] Wiesmuller K.H., Jung G., Hess G., Vaccine, 7, 1, 23-33, 1989.
[348] Erte H.C., Vargal, I., Xiang, Z.Q., Kaiser, K., Stephens, L, Otvos, L.Jr., Vaccine, 14, 9, 879-85, 1996.
[349] Morris, W., Steinhoff, M.C., Russell, P.K., Vaccine, 12, 1, 5-11, 1994.
[350] Dimarchi, R., Brooke, G., Gale, C., Cracknell, V., Doel, T., and Nowat, N., Science, 232, 639-642, 1986.
[351] Petrov, R.V., Khaitov, R.M., Zhdanov, V.M., et. al., Vaccine, 3, 392-400, 1985.
[352] Schulz, R. C., Schmidt, M., SchwarzenBach, E., Zöller, I., Macromol. Chem. Macromol. Symp., 26, 221-231, 1989.
[353] MühlBach, K., Schulz, R. C., Macromol. Chem., 189, 1267, 1988.
[354] Schulz, R.C., Mühl Bach, K., Perner, Th., Ziegler, P., Polymer Preprints, 27, 2, 25. 1986.
[355] Mustafaev, M. and Mustafaeva, Z., Int. .Journal of Health Care Engineering, Tech. and Health Care, v 10n 3,4, 217-227, 2002
[356] Deliloğlu Gürhan, S.I., Mustafaev, M., Mustafaeva, Z., Aynagöz, G., Ünver, G., Ünal, N. and Çelik, G., In:Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Appendix41, 349-357, FAO, UN2002.
[357] Aktaş, S., "Moleculer epidemiology of Foot and Mouth Disease Types O and A in Turkey", Thesis R7871, Ph.D. Thesis, University of Reading, 1998.
[358] Barnett, P. V., Carabin, H., Vaccine, 20, 1505-1514, 2002.
[359] Wang, C.Y., Chang, T.Y., Walfield, A.M., Ye, J., Shen. M., Chen, S.P., Li, M.C., Lin, Y.L., Jong, M.H., Yang, P.C., Chyr, N., Kramer, E., Brown, F., Vaccine 20, 2603-2610, 2002.
[360] Francis, M.J., Hosting, G.Z.,- Syred, A.D., Mc Ginn, B., Brown, F. and Rowlands, D.J. Nature 330, 168-170, 1987.
[361] Oldstone, M.B., J.Virol., 69, 12,7423-7429, 1995.
[362] Antoni, F., Soos, T., Varga, J., et. al. Magyar Allatovosok Labja, 43,9, 561-566, 1987
[363] Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., Weigele, M., Science, 178, 1972.
[364] Vasantha, S., Antony, A. And Lal, S.M., Acta virol., 31, 1091-15, 1987.
[365] Copeland, S.E., Photochemistry and photobiology, 28, 839-844, 1978.
[366] Spinks, J.W.T., Woods, R.T., "An Introduction to Radiation Chemistry", Wiley, New York, 1990.
[367] Swallow, A.J., "Radiation Chemistry", Longman, London, 1973.
[368] Yoshida, M., Asano, M., Morita, Y., Kaetsu, I., Imai, K., Mashimo, T., Yuasa, H., Yamanaka, H., Kawaharada, U., Suziki, K., Biomaterials, 10, 1, 16-22. 1989.
[369] Okuda, T., Waikita, K., Tsuchiya, N., Hatsuoka, K., Koga, Y., Kaetsu, I., Mausi, 48, 2, 141-145, 1999, (in Japanese).

[370] Mustafaev, M.I., Bayülken, S., Ergen, E. Et.al., Radiation Physics and Chemistry, 60, 567-575, 2001
[371] Bayülken, S., Yüce, G., Erkol, A.Y., Mustafaeva, Z., Mustafaev M.I., J.of Radioanalitical and nuclear chemistry, 259, 2, 315-319, 2004.
[372] Alexander, P., Fox, M., Hitch, S.F., Trans Farad.Soc. 49, 330-355, 1953.
[373] Urbain, W.M. Radiation Chemistry of major Food Proteins, Elias P.S., Cohen, A.J., ed., USA, 1977.
[374] Afanas'ev, A.M., Barakova, V.A., Demishev, V.N., Novozhilov, V.A., High Energy Chem., 19, 342. 1985.
[375] Hill, D.J.T., O'Donnell, J.H., Winzor, C.L., Winzor, D.J., Polymer, 31, 538, 1990.
[376] Pietrzak, M., J. Radioanal. Nucl. Chem., 198, I, 191-202, 1995.
[377] Keele, B, Mc Cord, J., Fridovich, I., J. Biol. Chem., 246, 2875, 1971.
[378] Akita, S., Nagayama, M., Kaneda, T., Oka, T., Ohishi, N., Yagi, K., J. Appl. Biochem., 6, (1-2), 64-69, 1984.
[379] Bannister, J., Bannister, W., Wood, E., Eur. J. Biochem., 18, 178, 1971.
[380] Dixon, J.W., Sarkar, B., J. Biol. Chem., 249, 18, 5872, 1974.
[381] Feher, J., Lang, I., Nekam, K., Gergely, P., Muzes, G., Tokai J. Exp. Clin. Med., 15, (2-3), 129-134, 1990.
[382] Forman, H.J., Fridovich, I., J. Biol. Chem., 248, 2645, 1973.