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.

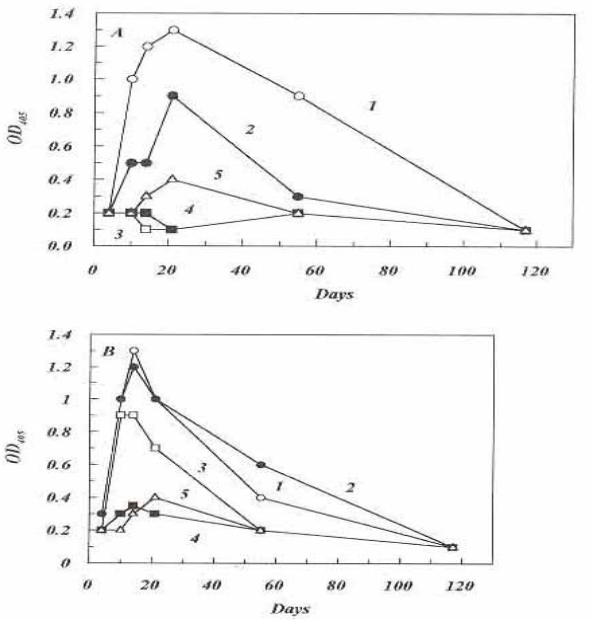


Figure 144. Dynamics of antibody formation as revealed by ELISA. Effect of immunization with complexes of PAA-Cu-BSA of differing protein content at two different Cu^{2-} concentrations. A: n_{Cu}/n_{AA}=0,1; B: n_{Cu}/n_{AA}=0,2; C: n_{BSA}/n_{PAA}=1,0. BSA dose (mg) was: 1, 0,5; 2, 0,25; 3, 0,1; 4,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

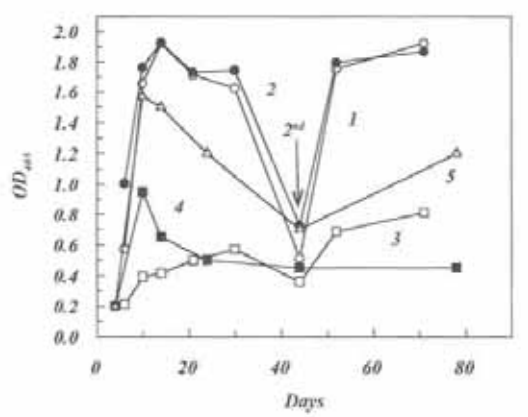


Figure 145. Kinetic of formation of BSA-specific antibodies in the sera of mice immunized with free BSA versus ternary complexes of BSA-Cu^{2+}- PAA at different ratios of the components (n_{PAA}/n_{BSA}). The experimental procedure used was as described in section 2. BSA-specific antibodies were determined 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

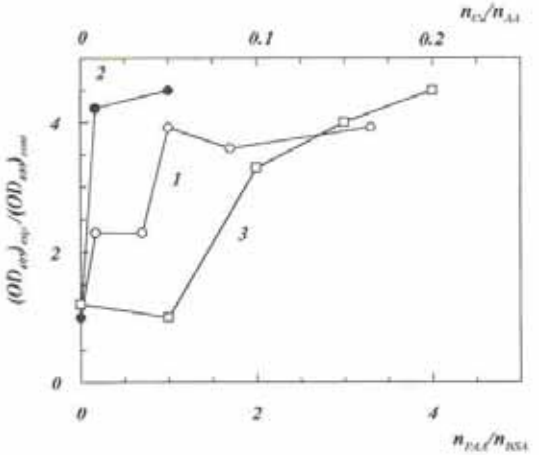


Figure 146. Dependence of relative values of BSA-specific antibodies (OD_{405}exp. /OD_{405}cont.) on n_{PAA}/n_{BSA} and n_{Cu}/n_{AA}. ELISA values determined on the tenth day post immunization were taken as the peak values of the immune response. Data from Figure 145. Plus a series of additional experiments 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

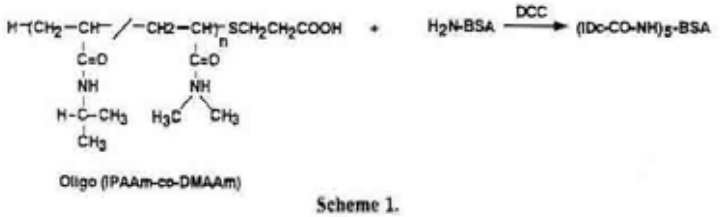
presence of 3-Mercaptopropionie acid as a chain transfer agent in N, N-dimethylformamide as described in the literature [148]. Mole fraction of DMAAm was 11 mol % analyzed by proton NMR spectra and its LCST was 36.5°C. Molecular weight was estimated to be 7000, determined by gel permeation chromatography.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

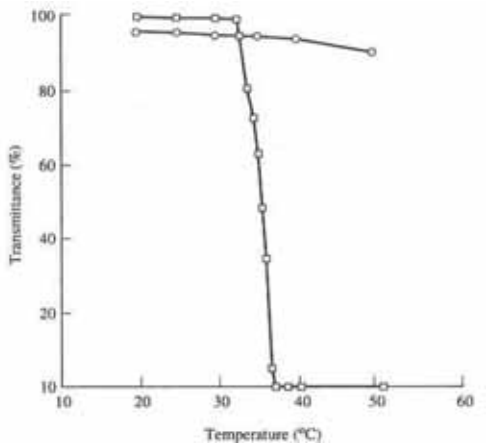


Figure 147. Temperature dependence of transmittance for aqueous solutions of IDc-conjugated. O: native BSA; Í: IDc-BSA.

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.

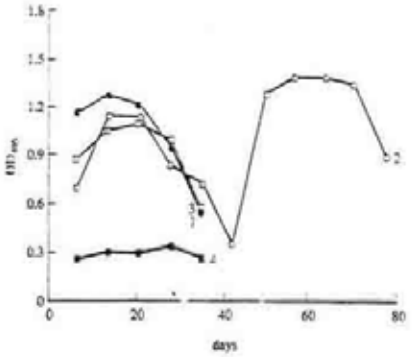


Figure 148. The kinetics of formation of primary and secondary BSA-specific antibodies (OD_{405}) in the blood sera of mice immunized intravenously with IDc-BSA conjugates at different protein dose (mg): 0.2 (1), 0.1 (2), 0.05 (3), 0.01 (4).

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.

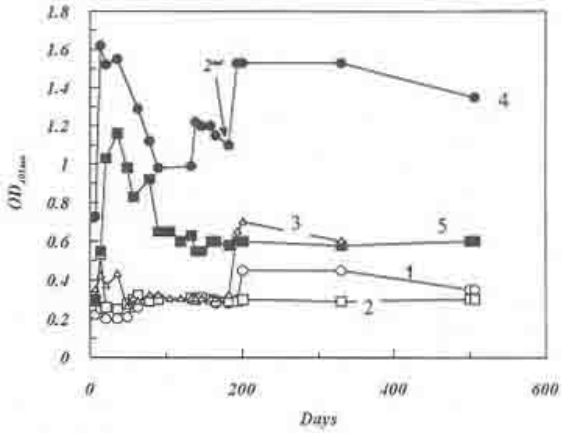


Figure 149. The kinetics of primary and secondary BSA-specific antibody (OD_{405}) formation in mice immunized intravenously with BSA (1), CP1-BSA (2) and (CP1-Cu^{2+}-BSA) mixtures in water (3) and in 0.154 M NaCl (4,5). n_{Cu}/n_{AA}=0.2; pH 7.0; n_{BSA}/n_{CP}=1.0 (4), 2.0 (5). Free BSA and BSA in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

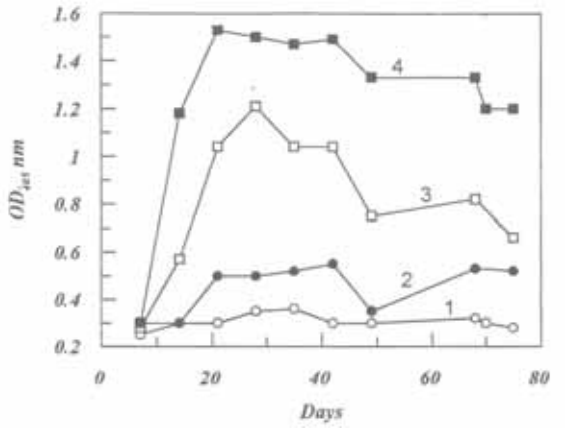


Figure 150. The kinetics of primary BSA-specific antibodies (OD_{405}) formation in mice immunized intravenously with CP1-Cu^{2+}-BSA complexes prepared in 0.154M NaCl. Solution was prepared by dissolving in 0.154M NaCl solvent of the precipitate obtained after heating (37˚C) of soluble ternary polycomplexes 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.

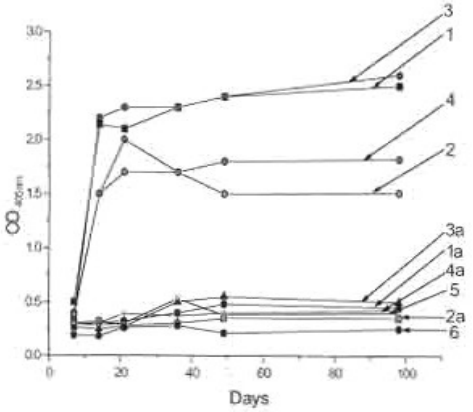


Figure 151. The dynamics of formation of BSA-specific antibodies (OD_{405}) in the blood sera of mice immunized with CP-BSA conjugate at dilute solutions. [0.1 (1a), 0.05 (2a), 0.03 (3a); 0.01 (4a) g/dl] and thermally precipitated concentration [2.286 (1); 1.346 (2); 0.673 (3); 0.448 (4) g/dl] preparing 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%

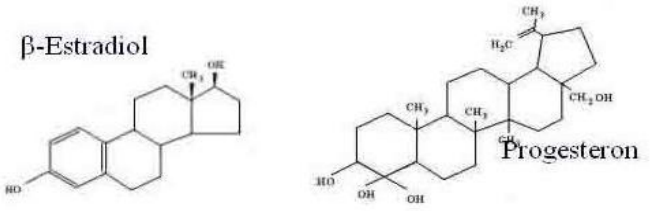
immunogen	IgE HAT (log_2 1/T), M ± m 10th day	18th day	IgG (M ± m) 10th day	18th day
OVA	0	0	7.0 ± 0.3	6.2 ± 0.6
PVP(R_2, R_{16})*-OVA	7.25 ± 0.14	4.5 ± 0.3	7.8 ± 0.5	10.5 ± 0.5
CP-OVA	0	0	7.3 ± 0.2	7.4 ± 0.2
CP-Cu^{2+}-OVA	0	0	7.5 ± 0.4	7.1 ± 0.3

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

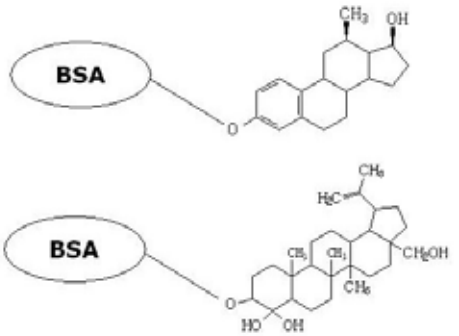
5.8.1. Steroid Hormones

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].



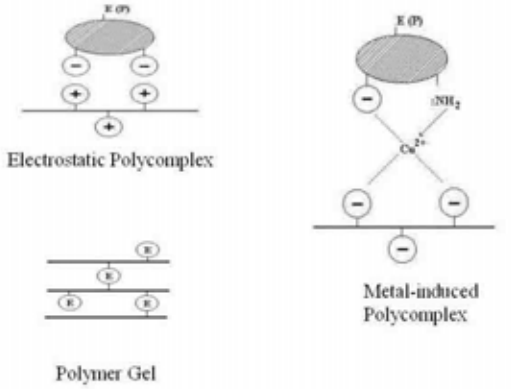
Using azocoupling techniques, Erlanger et al [263] have prepared steroid-bovine serum albumin (BSA) conjugates that elicited production of antisteroid antibodies. Later, amino phenyl derivatives were used for conjugation of steroids. Niswender et al. pointed out that the ratios of carrier/protein were 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

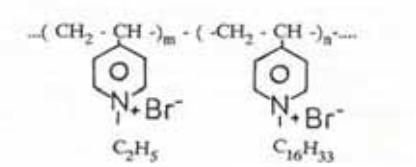
complexes containing steroid hormones (or anticancer betulin hapten) and protein antigens, monoclonal antibodies produced against steroid hormones in regard to association constants, specificity and immunoglobulin subclass obtained by author's colleagues and collaborators are summarized in this chapter. 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.

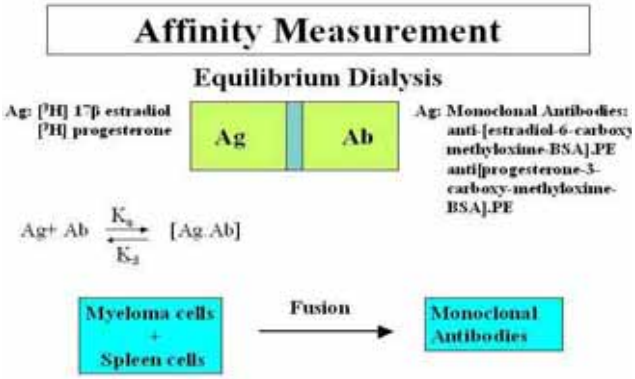
Estradiol containing PEC. The polyelectrolyte complexes chosen was the copolymer of 4-vinyl-N-ethylpyridine bromides PVP(R_2, R_{16}), where *(n/m* + n)xl00 = 10 mol%. PE was obtained by quaternization of narrow fractions of poly-4-vinylpyridine (PVP) *(Pw* = 10^3) with ethyl (R_2) and cetylbromides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

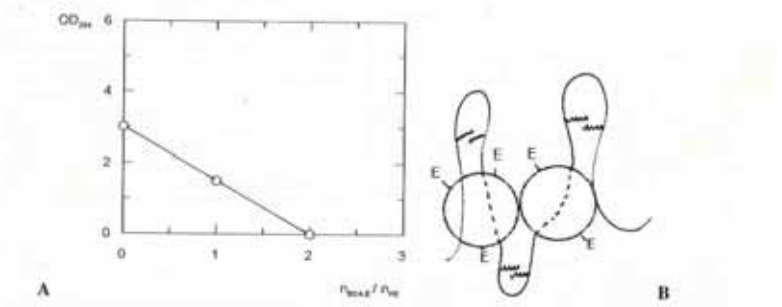


Figure 152. (a) Dependence of values of optical density (OD_{254}) of matrix solution of mixture PVP ($R_2.R_{16}$)-BSA.E obtained by UV spectrophotometric analysis at 254 nm on n_{BSA}/n_{PE}- (b) A schematic presentation of the structure of BSA.E complexes with PVP ($R_2.R_{16}$). For explanations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

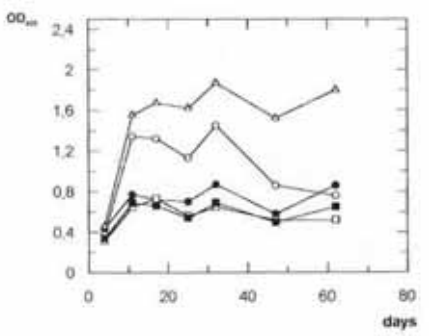


Figure 153. The dynamics of estradiol-specific (BSA.E-specific) antibody formation [as assayed by ELISA (OD405)], induced by free BSA.E and by polycomplexes of PVP (R2.R16)-BSA.E at different protein doses: 100 µg (○), 50 µg (●), 10 µg(□)50 µg free BSA.E (■), prepared by dilution of polycomplex solution,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

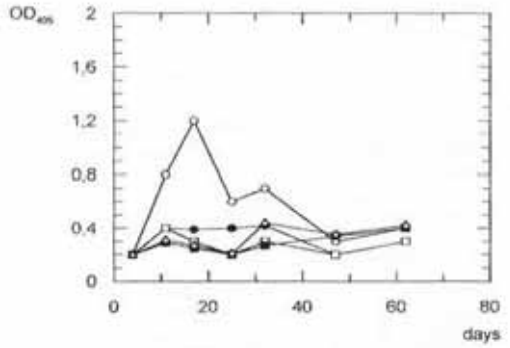


Figure 154. The dynamics of formation of BSA-specific antibodies (OD405) induced by free BSA.E polycomplexes of PVP ($R_2.R_{16}$)-BSA.E and mixture of BSA.E + IFA at different protein doses. For PVP ($R_2.R_{16}$)-BSA 100 μg (○), 50 μg (●), 10 μg (□); for BSA.E 50 μg (■); and for BSA.E + IFA mixture,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

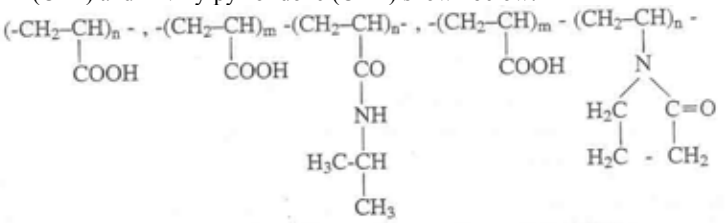
Monoclonal antibody	K_d *(M)*
PE–BSA.E	
2F3	6.5×10^{-7}
4F1	1.2×10^{-7}
8F9	8×10^{-6}
6B8	3.8×10^{-8}
6B11	7×10^{-8}
IFA–BSA.E	
8H7	3.3×10^{-8}
8H9	2×10^{-8}
10F4	1.6×10^{-7}
15D4	5.5×10^{-8}
14D2	3.8×10^{-6}
16G4	1.2×10^{-7}

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

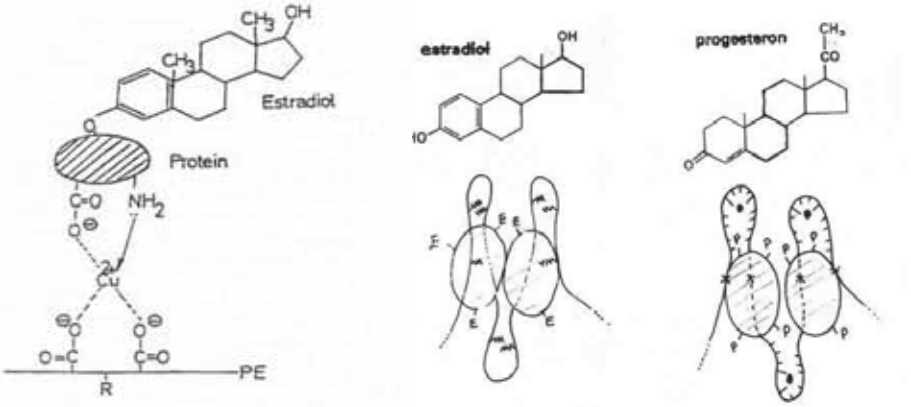


Figure 155. Hypothetical scheme of the structure of BSA.E conjugates with Polymer-metal complex. For more explanation see text

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.

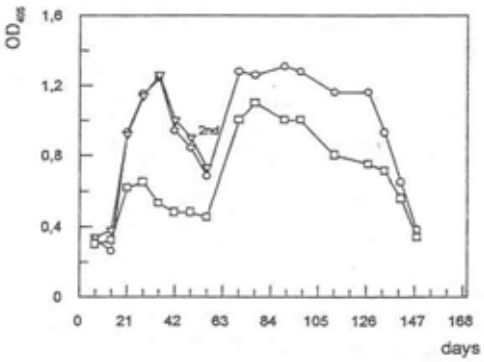


Figure 156. Estradiol-specific antibody response to PAA-Cu^{2+}-BSA.E complex and BSA.E conjugates. Mice were immunized with 100μg PAA-Cu^{2+}-BSA.E (0); 50μg PAA-Cu^{2+}-BSA.E (Δ); 100μg BSA.E (ف). Second immunizations were performed with BSA.E on day 56. Anti-estradiol antibody activity was measured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

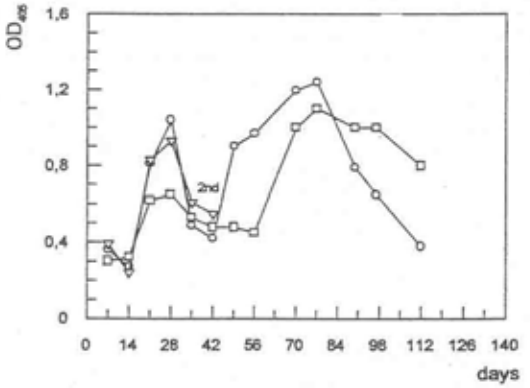


Figure 157. Estradiol-specific antibody response in mice immunized with CP1-Cu^{2+}-BSA.E complexes at different doses: 100μg CP1-Cu^{2+}-BSA.E (0); 50μg CP1-Cu^{2+}-BSA.E (Δ); and 100μg BSA.E alone (ڤ). Second immunizations were performed with BSA.E on day 42 Anti-estradiol antibody activity was measured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

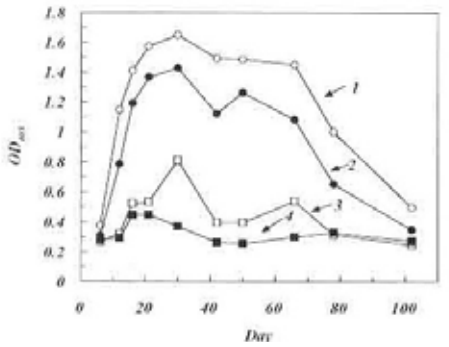


Figure 158. The dynamics of formation of P-specific (BSA.P-specific) antibodies (OD_{405}) in the blood sera of mice immunized with free BSA.P and PAA-Cu^{2+}-BSA.P complexes at different protein doses prepared by dilution of polycomplex solutions: 100μg (1); 50μg (2); 10μg (3) BSA-P in PAA-Cu^{2+}-BSA.P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

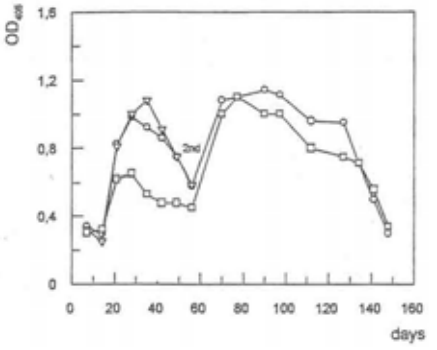


Figure 159. Estradiol-specific antibody response to CP2-Cu^{2+}-BSA.E complex and BSA.E conjugates. The groups of mice were given with 100μg CP2-Cu^{2+}-BSA.E (0); 50μg CP2-Cu^{2+}-BSA.E (Δ); and 100μg BSA.E (ڤ). Second immunizations were performed with BSA.E on day 56 Anti-estradiol antibody activity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

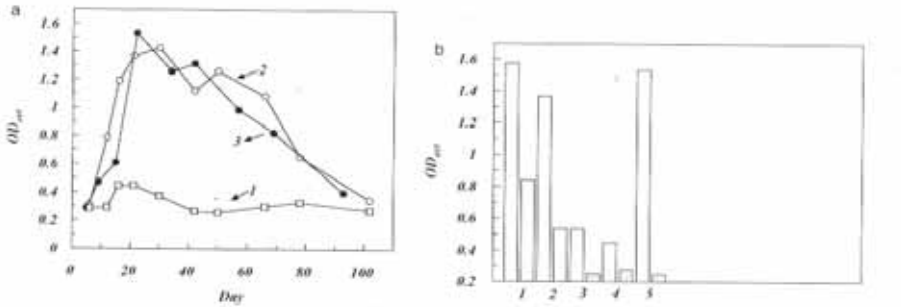


Figure 160. (a) Comparison of the dynamics of formation of P-specific antibodies (OD405) in the blood sera of mice, immunized with free BSA.P (1), PAA-Cu2 + BSA.P (2) and BSA.P +I.F.A. (3) mixtures $n_{BSA.P}$ n_{PAA}= 2.0. n_{Cu}/n_{AA}=0.20; protein dose is 50 μg, (b) Comparison of P- and BSA-specific 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

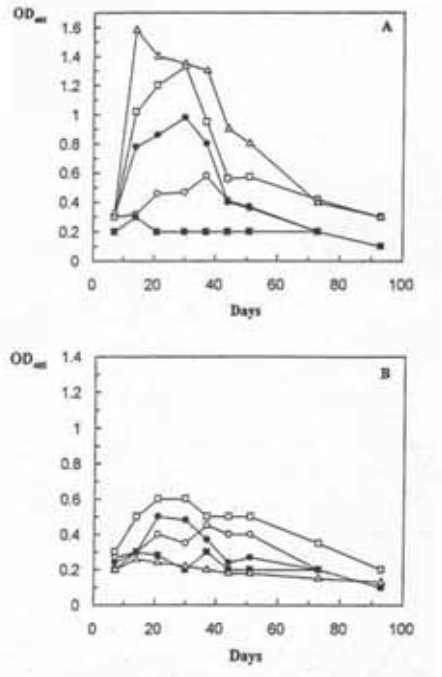


Figure 161. The dynamics of pcogestercee-specific (BSA.P-specific) and BSA-specific setibody formation [as assayed by ELISA (OD_{405})], induced by free BSA.P, BSA.P-IFA, and by polycomplexes of BSA.P-Cu_2*-CPI at different protein doses; 10 μg (antigen dose 3 μg) (○), 50 μg (antigen dose 15 μg) (●), 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.

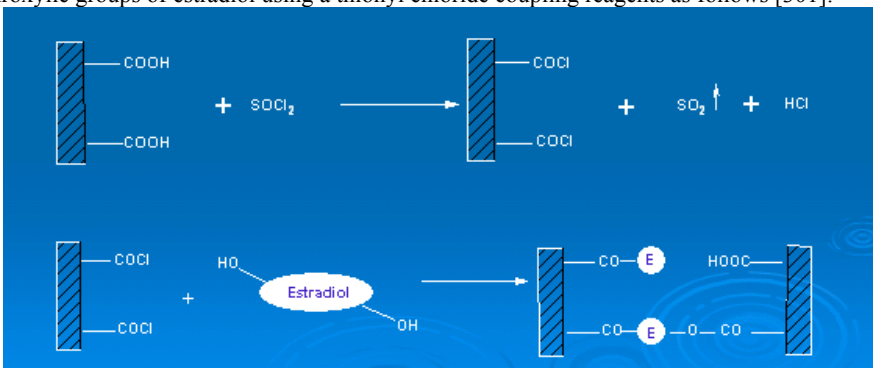
It should be noted that these polycomplexes stimulate the production of polyclonal antibodies. At the same time, the technique of raising monoclonal antibodies provided the possibility to determine the specificity and affinity of the antibodies to estradiol and progesterone produced by using synthetic 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

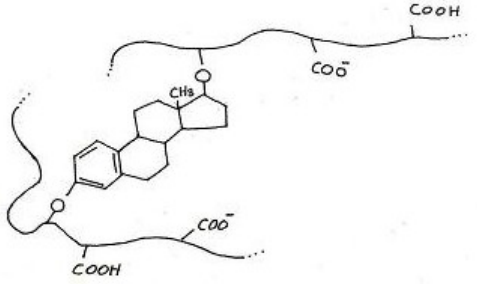
response to both protein and hapten when the antigens (BSAxHapten) are administered with polymeric systems. These results may suggest that polymeric carriers may be promising adjuvants in approaches to combined vaccines. On the contrary, injection of estradiol and progesterone with protein carrier or 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.

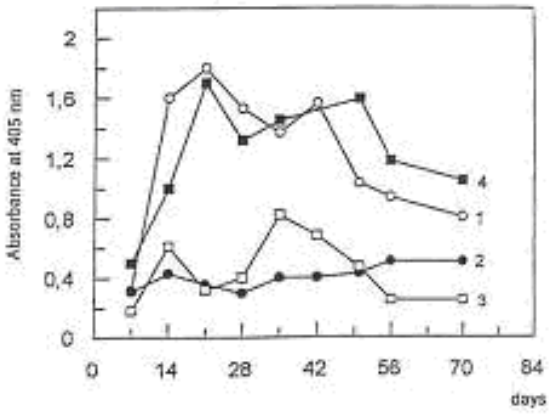


Figure 162. The dynamics of formation of 17β-estradiol specific antibodies (OD_{405}) in the blood sera of mice immunized with subcutaneously implanted PAA-estradiol gels (I), BSA.E (2), E + IFA mixtures (3), and BSA.E + IFA (4). Hapten dose is 100 μg

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].

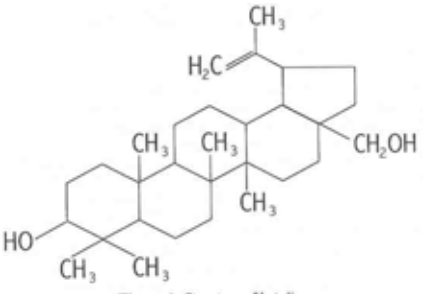
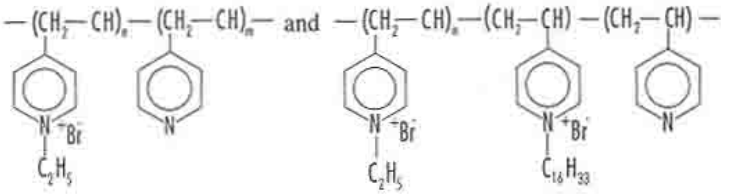


Figure 163. Structure of betulin

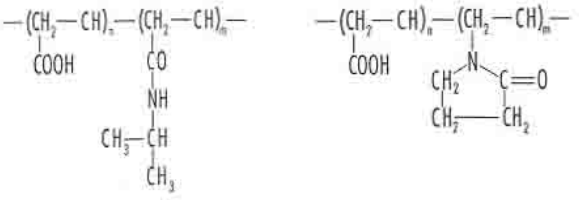
Nitroaromatic betulin derivates as redox cycling agents were single-electron reduced by ferrodoxin: NADP + reductase and flavocytochrome b2 at rates comparable with their simple structured analogs [309]. Poly(vinylpyrrolidone) and Proxanol polymers solubilize betulinic acid from betulin with the same 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.

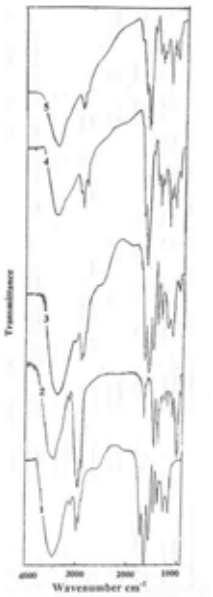


Figure 164. FT-IR spectra of CP1 (1), betulin (2), CP1+betulin physical mixture (3), and the products obtained from the CP1+betulin+$SOCl_2$ mixture at different contents of $SOCl_2$ 1 mL (4) and 0.183 mL (5)

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.

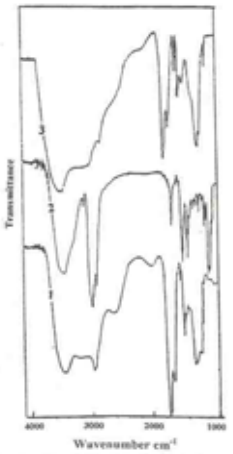


Figure 165. FT-IR spectra of CP2 (1), betulin (2), and the products obtained from the CP1+betulin+$SOCl_2$ mixture (3)

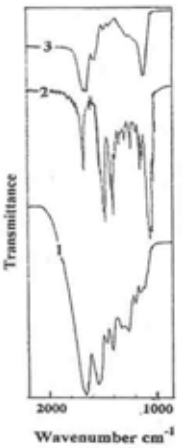


Figure 166. FT-IR spectra of BSA (1), betulin (2), and the products obtained from the BSA + betulin + carbodiimide mixture (3).

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.

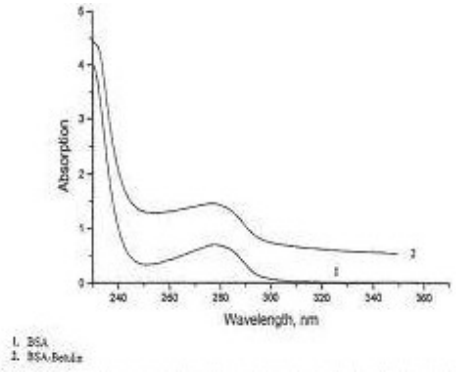


Figure 167. The adsorbance spectra of solution of BSA (1) and the product obtained frost the BSA+ betulin + carbodiimide mixture (2) at pH 7. BSA concentration in the both cases 1mg/1ml

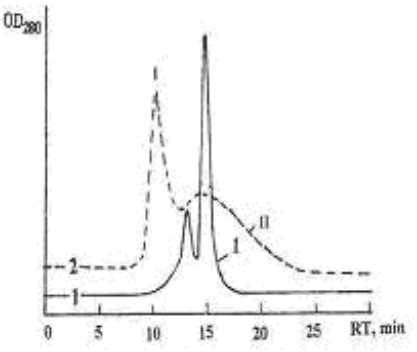


Figure 168. HPLC analysis of the solutions of pure BSA (1) and the product obtained from the BSA+betulin+carbodiimide mixture (2) prepared in phosphate buffer (pH 7); Uv – 280nm; 0.7mg/mL; 40gLc 1mL/min

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].

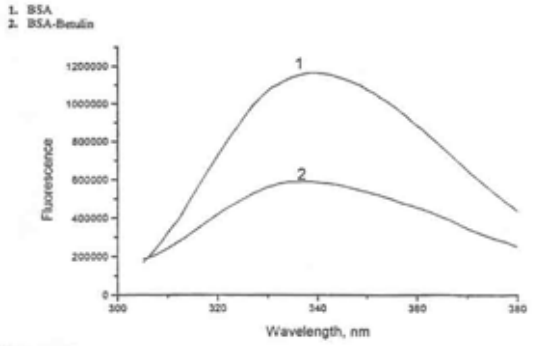


Figure 169. Fluorescence spectra of pure BSA (1) and the product obtained from the BSA+betulin+carbodiimide mixture (2), prepared in phosphate buffer (pH 7), BSA concentration in both solution were 0.71 mg/ml

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.

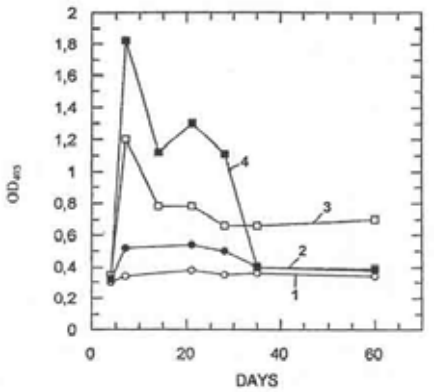


Figure 170. The kinetics of betulin-specific antibody formation (OD_{405}) in blood of sera of mice immunized subcutaneously with betulin (1), CP1-B (2) and CP2-B conjugates (3); 1-control, nonimmunized mice; Dose 1 mg conjugate/mouse

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).

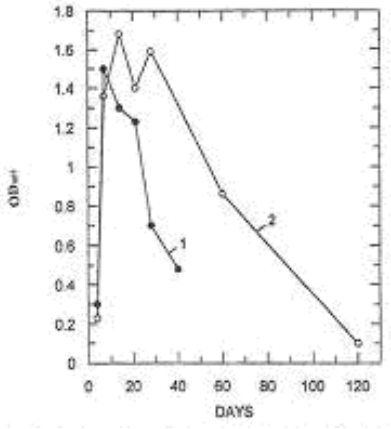


Figure 171. The kinetics of betolin-specific antibody formation (OD_{405}) in blood of mice immunized intravenously with CP2-Betulin (1) and BSA-Betulin (2) conjugates. The results by immunization with pure betulin (Figure 7) (2); Dose 1 mg conjugates

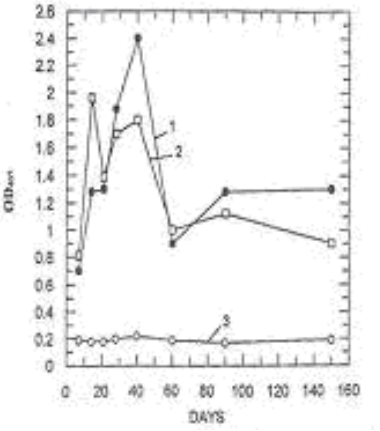


Figure 172. The kinetics of betolin-specific antibody formation (OD_{405}) in mice serum immunized intravenously with PEVP-BSA B (1) and PECVP-BSA B (2) diseases. 3 – nonimmunized mice; dose 700 μg for coch mice

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).