Figure 173. The primary immune response of beritary PMC of β-OLH-CP-I + Cu^{2+} +β.OLH: 1 – (CβC =2;

Immunological studies were conducted at the National Institute of Immunology (New Delhi, India) by Drs. Talwar and Arunan with the participation of Mustafaev (Moscow, USSR) [9].

Albino rats were i/v immunized with 0.5 mg and the antibody titers in the blood samples were determined in a radioimmunoassay (ELISA) using a ^{125}I labelled antigen. It may be inferred from these data that all ternary PMC were able to induce strong immune responses to the protein hormone included into PMC. Interestingly the strength of the immune response was either commensurate with or, in some cases, exceeded that of the control nontechnological conjugate. It follows from these data that the strength of the immune response does not depend on the distribution pattern of the carboxyl groups within the composition of CP on the protein/polymer ratio as well as on the transient metal ion concentration in experimental mixtures. Stipulating that ß-OLH possesses no immunogenic activity of its own and that the CP used in this study were nontoxic, technological and had low M_r (10 and 50 kDa), it may be concluded that the proposed method designed to increase the immunogenic activity of protein hormones opens new ways to the chemical synthesis of antifertile vaccines for birth control in animals and man.

5.9.1. Synthetic peptide vaccines.

The need for development of new vaccines and the improvement of currently used ones is defined as one of the prime goals of the World Health Organization. Although vaccine technologies and manufacturing methods have come a considerable distance over the past 50 years, much more development will occur. There will be challenges for biotechnology to arrive at safer, more effective vaccines for an ever-increasing number of antigen targets. Vaccines will remain one of the most cost-effective and logical biomedical technologies of the next century, as diseases are prevented rather than treated. Challenges are also posed in bringing existing vaccines to technologically undeveloped nations, where they are needed most. Vaccines are biologic preparations that elicit immune system responses that protect an animal against pathogenic organisms. The primary component of the vaccines is an antigen, which can be a weakened (attenuated) version of an infectious pathogen or a purified molecule from the pathogen or, more recently, chemically synthesized or recombinantly expressed viral (or bacterial) subunits. However, these antigens are not as immunogenic as live organisms and the vaccines contain as a secondary component of adjuvants to enhance immune response as well as formulation agent to preserve the antigen during storage or upon administration, to provide proper delivery of antigens,

and to minimize side reactions. Therefore, development of new vaccines is an interdisciplinary field that has a broad impact on biotechnology, synthetic chemistry, and immunological developments.

The current vaccines are killed virus vaccines, and they can be hazardous due to contamination by live viruses in the manufacturing process. Taking into account the safety and contamination problems involved in the production of vaccines for living virus and the relatively short-term storage possibility in addition to the narrow protection range, the development of synthetic peptide vaccines is a challenge. Synthetic peptide vaccines are expected to be stable and inexpensive to produce. They also provide two possible methods for dealing with antigenic variation. First, since linear synthetic epitopes are fairly easily prepared, mutations, once located, can be readily incorporated into the synthesis. Second and perhaps of greater importance, invariant antigenic sequences of the pathogen can be utilized to promote longer-lasting immunity [320,321].

Investigation of synthetic peptide vaccines has largely centered on antiviral agents, such as foot and mouth disease virus (FMDV), hepatitis B, influenza virus, poliovirus, and human immunodeficiency virus(HIV). It should be noted that antibacterial peptide vaccines are also of current interest, such as for diphtheria and cholera toxins, as well as antiparasitic immunogens for prevention of malaria [320-324].
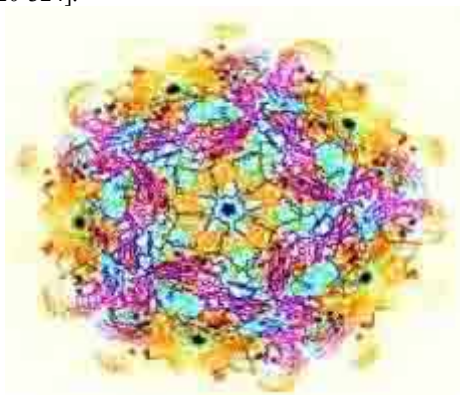
Figure 173. Virus of Foot and Mouth Disease.

Peptides manifest a variety of physiological and therapeutically properties. However after administration into living organism they are very often subjected to biodegradation (e.g. by proteolytic enzymes) or they are of immunogenic nature and they may start the corresponding immune reaction of the organism. Besides, the weak immunogenecity of them prevent immune protection from infection. Thus, they lifetime and the magnitude of the immune response *in vivo* depends on the nature of these bioactive compounds and have to be regulated and optimized in each case. The project proposes to study a novel approach to solve this problem by including of such polypeptide epitops of viruses of different disease to Biopolymer systems.

The use of peptide epitops of viruses particles as vaccines have several potential advantages over whole viral or bacterial preparations [322-325,338]. However, to elicit the maximum immunogenic response from such antigens, they have to be used with traditional adjuvant, which limit their practical applications. Besides, it is generally necessary to bind the peptide molecules also to a carrier protein, which may initiate a corresponding immune (e.g. allergic) reaction of the organism.

The peptides (the 140-160 fragment of VP1 protein of FMDV [326], immunogenic peptide of human hepatitis B virus pres (120-145) [339,340], rabies virus polypeptide antigens (130-141) [341]), prepared by chemical synthesis, and coupled to large carrier proteins like

keyhole limpet hemocyanin (KLH) were elicited neutralizing antibodies and protected guinea pigs in the mixture of incomplete Freund's adjuvant. Cattle that received a high dose of peptide (5 mg) or that had been vaccinated twice with a smaller dose, developed high levels of neutralizing antibodies. However, full protection against challenge was not obtained. Such immunizations, at the same time with disadvantageous of classical adjuvants, suffer from difficulties in producing conjugates of reliable composition, and from unwanted anti-carrier immune responses. Also, the protein carrier can induce hypersensitive, allergenic side reactions in the patient after repeated inoculations. These difficulties can lead to irreproducibility in the immune response [342]. To circumvent these problems, two new protocols in vaccine design are now emerging that is based entirely on synthetic peptides. The multiple antigenic peptide (MAP) concepts were recently introduced by Tam [343]. Such systems were obtained by stepwise solid-phase synthesis of a MAP in which the final multibranching MAP core bound with eight copies of the antigenic peptide. The dense peaking of so many copies of a highly antigenic epitope has been shown to produce a strong immunogenic response.

To elicit full immunogenic activity, another current thinking suggests that a vaccine should consist of B at T cell epitops to be most effective. This theory was first supported by Francis and co-workers [344], who noted that the carrier- free FMDV vaccine derived from residues 141 to 160 of VP1 did not protectively immunize the H-2^d strain of mice. By coupling this B cell antigen to known T cell epitops of ovalbumin and sperm whale myoglobin, high levels of cross-selective antibodies were invoked, which neutralized FMDV in subsequent challenge experiments.

Utilizing the template-assembled synthetic protein engineering techniques of Mutter [345], Kobbs-Conrad and coworkers [342] designed a totally synthetic vaccine consisting of single or multiple copies of B and T cell epitopes built into a β-sheet template peptide. They observed high titers of antibodies in response to this template-assembled vaccine bearing B cell epitopes of LDH-C and T cell antigens of tetanus toxoid. When a chemically synthesized peptide, bearing hepatitis B virus α-determinant specificity, was conjugated to a dipalmityllysine moiety (enhancement by conjugation to a fatty acid carrier), a significant improvement in anti-hepatitis B surface antigen response was obtained, in comparison to the corresponding peptide conjugate [346].

Novel low-molecular-weight synthetic vaccine against FMD containing a patent B-cell and macrophage activator (T-cell epitope) was obtained by conjugation of peptide fragment (135-154) of VP1 protein of FMDV to tripalmitoly-s-glyseryl-cysteinylserylseryl [347], which shows protection in guinea-pigs again FMD viruses. However, the MAP system has not yet been proved to be an effective vaccination vehicle, although it does offer exciting possibilities for the future. Gel filtration experiments suggest that the above-mentioned conjugates form large aggregates, possible micelles, which may play a significant role in the enhancement of the anti-peptide response.

It is known that Lactide-co-glycolide polymer microsphere technology is feasible and holds great promise for improving human vaccines [349]. Peptides carrying an immunodominant T-helper delineated from the rabies virus nucleoprotein either alone or in combination with liner B-cell epitope was incorporated into poly(DL-Lactide-co-glycolide)(PLG) microspheres and stimulated a peptide-specific T-cell line[348]. Such formulations of PLG upon subcutaneous immunization of mice induced the best immune response, in magnitude comparable or even superior to that induced by peptide emulsified in complete Freund's adjuvant. Despite the potential of microencapsulated vaccines, a number of unsolved questions persist. Some of them: residinal solvents and monomers in the microspheres, adverse reactions with slowly released antigen, control of allergic reactions, the size of microspheres, etc.

Biodegradable water-soluble polyelectrolytes developed over the past decade for the activation of the immune system (immunostimulants) have significant potential for the creation of highly immunogenic preparations for human and veterinary medicine. It was shown that the

quaternary polycondine salts, which contain biodegradable N-C bonds in structure, increase the immunogenecity of weakly antigenic biomolecules by several times and can effect the immune system avoiding T-helpers [9]. The Cu^{2+}-complexes of copolymers of piperazin with metilenbisacrylamide possess own sufficiently immunostimulant (adjuvant) activity in models of mice and have broad physico-chemical potential for the preparation of stable polycomplexes with different antigens [185].

Recently, vaccine "Grippol"-trivalent polymer-subunit vaccine containing the sterile conjugate of influenza virus surface proteins, types A and B, bound with copolymer polyoxidonium (polyconidine derivatives) has been developed [318]. The administration of "Grippol" to children of school age (6-18 years) demonstrated low reactogenicity of the vaccine, its safety and sufficient prophylactic effectiveness, and no side effects produced by "Grippol" were registered.

In the light of these findings it was very important to establish the probability of inducing synthesis of peptide containing immunogenic polyelectrolyte conjugates. The synthetic peptide analog HA_2^- docapeptide of HA_2^- subunit of hemagglutinin influenza virus was turn into highly immunogenic preparations by the covalent cross-link with synthetic carbochain PE [9]. By the incorporation of synthetic peptide analogous (decapeptide) of utilizing hormone releasing hormone (LHRH) into polymer-metallic (PE-Cu^{2+}) complexes synthesized ternary PE-Cu^{2+} peptide polycomplexes which were able to induce strong peptide-specific immune response in the experiments on rats [9].

Foot-and-Mouth Disease Virus (FMDV) Vaccine. Recently, we report a novel approach to a totally synthetic vaccine, which consists of a Hb_SAg and food-and-mouth disease virus(FMDV) VP1 peptides, prepared by chemical synthesis and nonimmunogenic membrane active carbochain polyelectrolytes[355,356].
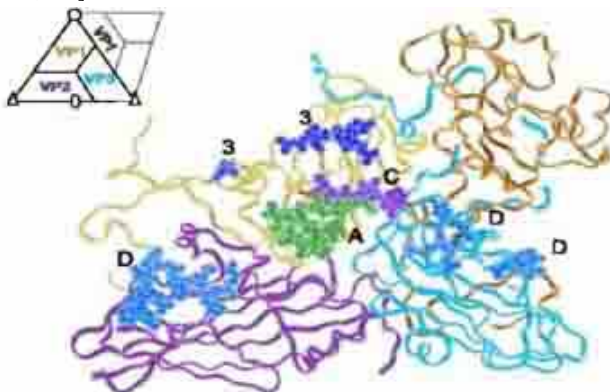

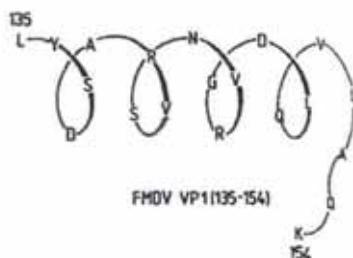**Figure 173.** The structure of FMDV VP1 protein

Figure 174. Amphipathic α-helix in VP1 protein of FMDV

FMDV terminally afflicts domestic livestock and has had devastating economic effects on the agricultural industry. FMDV consist of four protein subunits, VP1-4, of which only VP1 (1D) showed immunogenic activity when the individual proteins were used [325,326]. However, the antigenicity of VP1 was noted to be considerably reduced relative to the whole intact virus, which suggests that the other subunits are required for the correct folding of VP1. Strohmaier at al. [327] and Bittle at all [328] indicated that the peptide residues 141 to 160 of VP1 was the most effective immunogen. This segment has been found actually more antigenic than whole protein VP1 and to be cross selective for several stereotypes of FMDV [325]. The peptides that consist of the three regions of VP1, 138-154, 140-160 and 200-213, are able to induce neutralizing antibodies against the homologous virus type and protected guinea-pigs [328,329]. Further studies undertaken to locate antigenic determinants on FMDV suggested the presence of several antigenic regions. Residue at positions 40-60, 130-171, 141-160 of VP1 (1D) of different stereotypes of O, A and C have been shown as antigenic [330,333]. Analysis of crystallographic X-ray diffraction data suggest that type O amino acids 41 to 50 could form a surface loop close to the 141 to 160 region [334]. Also it was suggested that the 41-50 loop may either have insufficient amino acids exposed to the virus surface for recognition by antipeptide antibodies, but those that are alternatively, its proximity to the epitope may indirectly effect the conformation [335]. Recently, 43-49, 135-151, 166-170 and 195-206 amino acid sequences of VP1 protein were also determined as antigenic sites of type C [336,337].

One of the promising alternatives to classical adjuvants is the use of nonimmunogenic synthetic polyelectrolytes (PE) that are negatively or positively charged polymers, as carrier for antigens [9,19,21-24,28,125,240,242]. We, as well as the others, have previously shown that the attachment of weak microbial and viral protein antigens to various charged polymers allows the modulation not only of their immunogenicity, but also protective activity.

In the present study, polypeptide antigens corresponding to amino acid sequences predicted from the nucleotide sequence of Foot-and-Mouth disease virus (FMDV) VP1 protein were synthesized chemically, the polyelectrolyte-polypeptide conjugates were prepared and their immunogenic properties were investigated and discussed in terms of a novel immunogenic system. The polypeptide-comprising Biopolymer Systems were obtained by two methods: 1) inclusion of polypeptides in polyelectrolyte complexes (PEC) of PE with weak protein antigen (bovine serum albumin - BSA) which were stabilized by electrostatic and/or Cu^{2+}-induced interaction of compounds; 2) covalent cross-linking of peptides with PE directly. Immunogenecity of Biopolymer Systems without traditional adjuvants and recognition of antibodies in blood sera were investigated.

The peptides were synthesized by using the solid-phase methods developed by Merrifield, with Millipore's Automated Peptide Synthesizer. The characterization steps include chromatographic, spectroscopic, and fluorometric analyses while the purification step includes gel electrophoresis techniques. After coupling of all desired amino acids in the chain, the product was cleaved from the support with TFA cocktails. Each synthetic peptide was subjected to acid hydrolysis at low pressure (6 M HCl, 110 °C, 72 h) and its amino acid composition was determined. In this study, 40-60 (P1), 135-160 (P2), and 140-160 (P3) fragments of FMDV VP1 antigens were synthesized.

40-60 residues
TRP -VAL-LYS-ILE-ASN-ASN-THR-
SER-PRO-THR-HIS-VAL-ILE-ASP-LEU
MET-GLN-THR-HIS-GLN-HIS-GLY

135-160 residues
TRP -LYS-TYR-SER-ALA-THR-GLY-
GLU-ARG-THR-ARG-GLY-ASP-LEU-
GLY-ALA-LEU-ALA-ALA-ARG-VAL-
ALA-THR-GLN-LEU-PRO-ALA-CYS

The cationic polyelectrolytes (PE) are the copolymers of 4-vinylpyridine with 4-vinyl-N-ethylpyridine (PEVP) and 4-vinyl-N-cetylpyridine (PECVP). PE was obtained by quaternization of narrow fractions of poly-4-vinylpyridine ($P_n = 10^3$) with ethyl and cetylbromides by the method previously described.

The anionic PEs are polyacrylic acid (PAA), copolymers of acrylic acid (AA) with N-isopropylacrylamide (NIPAAm) (CP1) and N-vinylpyrolidone (VP) (CP2):
To carry out PE-peptide (PE. Pep) and BSA-peptide (BSA. Pep) conjugation reactions, we used carbodiimide activation method [355].

To prepare PE-BSA.Pep electrostatic complexes, various concentrations of the BSA.Pep conjugate solutions were added to PEVP (or PECVP), dissolved in phosphate buffer (PBS), pH 7.2. In practice, 1,2 and 5 mg/ml BSA.Pep conjugate solutions which were mixed with 1 mg/ml PE solution and 200 /µl of this mixed solution were centrifuged at 10000 rpm for 10 min. The supernatant was taken and diluted to 4 ml in PBS and investigated by different methods. The concentrations of free PE were obtained from the calibration curve of $OD_{254} = K.C$ (C is the concentration of PE). The protein / PE ratio (nBSA.Pep/nPE) was calculated using the equation n = $C.N_A/M$, where n is the number of the molecules in 1 ml, N_A and M are the Avogadro's number and molecular weight correspondingly. To produce the PE-Cu^{2+} complex, the $CuSO_4.5H_2O$ (pH 4) solution was added to PE, dissolved in PBS. The desired pH values were adjusted with 1 M NaOH. The ternary PE-Cu^{2+}-protein complexes were, in turn, prepared by adding BSA.Pep conjugate solution to the PE-Cu^{2+} solution.

The heterogenicity of PE, proteins, peptides and the fraction compositions of the reaction products were estimated by using gel filtration chromatography (SIL-10Ai HPLC).
PEVP-BSA.Pep, PECVP-BSA-Pep, PAA-Cu^{2+}-BSAPep, CPI-Cu^{2+}-BSA(Pep, CP2-Cu^{2+}-BSA-Pep complexes, PE-Pep and BSA-Pep. covalent conjugates were used as the immunogen. Eight week-old BAIB/c mice were immunized with each of the complexes by intravenous injections. All groups were followed for development of antibody activity for polypeptides (FMDV VP1) for a period of 50-150 days after primary immunization.

The indirect enzyme-linked immunoadsorbent assay (ELISA) was used to detect antibody activity for polypeptides.

PE-peptide conjugates. HPLC analysis of the free components and reaction products in the mixture of PAA-Pep, CP1-Pep and CP2-Pep with water-soluble carbodiimide are provided. HPLC results of the reaction products prepared under this condition are shown in Figure 175. The solution of the reaction products between PE and peptide molecules in all cases was characterized in the chromatograms practically by one peak at the RT region corresponding to RT of peptide solution. The peak, corresponding to free PE was absent in chromatograms around RT = 1O min. Thus, under conditions where PE and polypeptide molecules are incapable of binding to one another, the WSC promoted the formation of water-soluble polymer-peptide covalent conjugates.

The conjugates were studied by ion exchange HPLC method (Figure 176). The solution of free P2 peptide as well as CP2-P2 reaction products is characterized by a bimodal distribution of elution components on ion-exchange chromatograms. On the other hand, conjugation induces an increase in the values of RT as compared with pure EP, a slight decrease of RT and width of the peaks as compared with pure peptide molecules. This indicates that in the CP-P conjugates formed, conjugate particles possess *more* friable structures in which *more* of the reactive groups are open for interaction with column materials.

The yield of conjugation should be directly proportional to the concentration of ε-aminolysil groups of Lys amino acid of polypeptide molecules. The ε-aminolysil contents of the FMDV and polymer-peptide conjugates were studied by fluorescamine, which interacts with the primary amino groups of samples. it was shown that the number of free amino group s of FMDV and FMDV in reaction products significantly decreased ($N_{exp.}/N_o = 0.8$, N_o and $N_{exp.}$ - the bound number of fluorescamine molecules with free polypeptides (N_o) and peptides after conjugation reaction ($N_{exp.}$)) One can assume that the ratio $N_{exp.}/N_0 = 0.8$ (or 80 percent) also characterized

the yield of conjugation reaction between CP1 and FMDV, polypeptide.
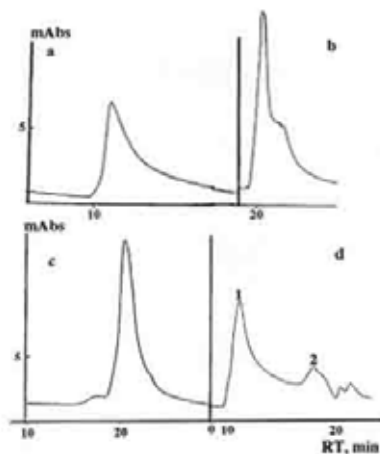
Figure 175. Gel filtration HPLC chromatograms of free CP (a), P2 (b), reaction products of CP2-P2 prepared in HRM systems (c) and mixture CP2-P2 (d). RT-retention times, UV-280 nm C_{P2} = 2mg/ml; 40μl. Concentrations of CP2, conjugate and P2: 3mg/ml; 40μl injection, 1ml/min; 25°C
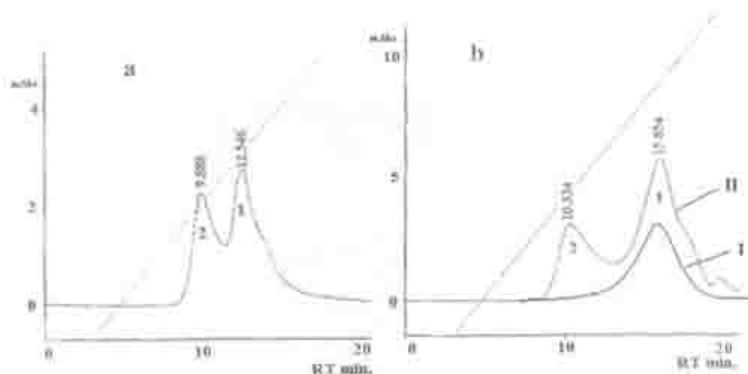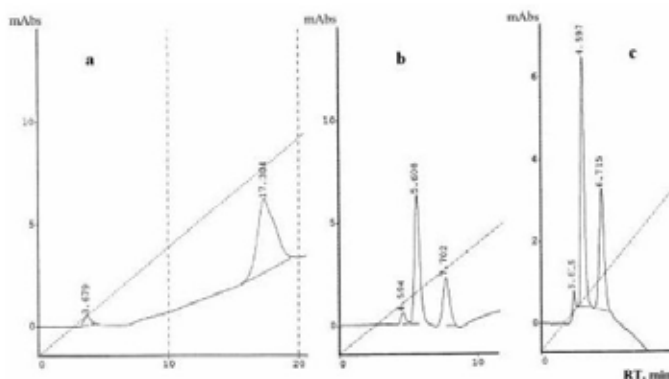

Figure 176. Ion-exchange HPLC results of Poli(VP-AA)-CDI-Peptide mixtures

BSA - peptide conjugates. Figure 177 presents the HPLC results of the covalent conjugation of the reaction products of BSA with FMDV by the activation of dicyclohexylcarbodiimide (DCC). Analysis of reaction products by use of Ultra free- CL high flow filters showed that polypeptide molecules were covalently bonded to BSA, resulting in the formation of bioconjugates with complicated structure. One can speculate that at the same time with monomer and dimer form of BSA.peptide (BSA.Pep) conjugates, the formation of water-soluble bioconjugate aggregates takes place. These aggregates were obtained as a single peak in the free eluent volume (Figure. 3, RT = 10.748 min).

However, in contrast to DCC carbodiimide conjugation reaction of BSA with polypeptides, the activation of water-soluble carbodiimide (WSC) lead to the formation of bioconjugate molecules with more essentially homogenous composition (and structure). A typical HPLC result of BSA-FMDV conjugate prepared by WSC is given in Figure. 4. The conjugate

solution was characterized in chromatograms by a single peak. Moreover, free BSA macromolecules were absent in the solution as indicated by HPLC (see Figure. 177a RT = 16.374 min for free BSA). These findings indicated that, under these conditions water-soluble carbodiimide promoted the covalent cross-linking of the BSA globules with polypeptide molecules with sufficiently increased yield and homogenous composition.

Figure 177. Ion-exchange HPLC results of BSA-CDI-Peptide mixtures

Preparation of PE-BSA.Pep complexes. BSA.Pep molecules were found to interact with polycations and to form soluble or insoluble protein-PE complexes. Starting with very low BSA.Pep/PE ratios, that is $n_{BSA.p}/n_{PE} = 0.1$, a phase separation took place in this system: PEVP-BSA.Pep and PECVP-BSA.Pep. Analysis of the matrix solution of insoluble mixtures shows that at the $n_{BSA.p}/n_{PE} = 1$, free fractions of PE remained in the matrix solution (Figure 178a). The existence of free PE under these conditions indicates a nonrandom distribution of the conjugate (BSA.P) molecules between the coils of polycations. The number (Ni) of the protein molecules bonded by a single chain of PEVP as well as PECVP of a given degree of polymerization under given conditions equal Ni = 2, i.e., two molecules of BSA.Pep. bound by one chain of polycation in composition of water-soluble polycomplexes: PEVP-(BSA Pep) and PECVP-(BSA Pep).
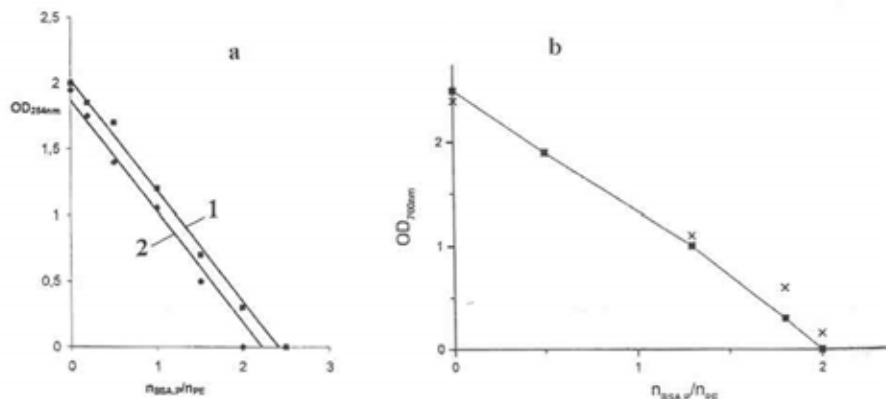

Figure 178. Dependence of optical density (OD_{405} and OD_{700}) of matrix solution of mixture PEVP-BSA*P2 (a,2), PECVP-BSA*P2 and CP2-Cu^{2+}-BSA*P2 (b) obtained by UV spectrophotometric analysis at 254nm and 700nm on the n_{BSA*P2}/n_{PE}

Ternary PE-Cu^{2+}-BSA .Pep complexes. As shown by HPLC analysis, complex cannot be formed between BSA.Pep and anionic PE at pH 7,0 in the absence of copper ions. However, water-soluble and insoluble complexes are formed upon addition of divalent copper ions to the solution of the mixtures PAA – BSA.Pep, CP1 - BSA.Pep and CP2-BSA.Pep. The extent of complex formation was dependent on the amount of Cu^{2+} added and was nearly quantitative at $n_{Cu}/n_{AA} = 0.25$ (Figure 178b).

The CP1-(Trp+(135-160)) conjugate was studied by fluorescence method. The results in Figure 179 indicate that peptide Trp+(135-160) solution shows discrete (structured) emission spectra at $\lambda_{max} \approx 315$ and 325 nm. Such Trp residues were attributed to class S in the hypothesis of discrete states. Therefore, peptide tryptophanyls exist in the hydrophobic environment of polypeptide chain and completely isolated from water solution.
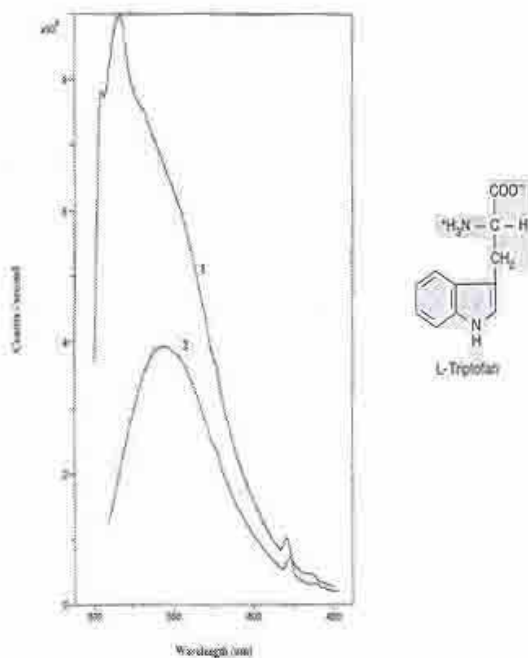

Figure 179. Fluorescence spectra of pure FMDV peptide and P50-peptide conjugate in water solutions. Peptide concentrations 0.07 mg/ml; phosphate buffer (pH 7.0), 25°C. Quanta Master spectrofluorometer (Photon Technology International, Canada) The excitation wavelength 280nm

Previously, the large hydrophobicity and amphypatic α-helical structure of 135-160 fragments was demonstrated by Pfaff and co-workers [329]. The fluorescence intensity (I_{max}) of peptide after conjugation with CP1 decreases (quenching) which testify conjugate formation. On the other hand, conjugation of peptide with copolymer macromolecules induces a marked red shift of λ_{max}. This indicates that in the CP1-peptide conjugates, peptide Trp as compared with pure peptide molecules are essentially exposed to the solution.

We can assume that the conjugate species can be represented rather as a macromolecule of a segmented (block) copolymer in which the hydrophobic blocks, i.e. the sequences of copolymer and peptide unit pairs which have formed the covalent and salt bonds alternate with hydrophobic ones, i.e. the sequences of the copolymer chain not participating in the formation of double strand blocks. Such mechanism proposed, "frozen" of peptide molecules in the structure of conjugate at the unfolding state, which Trp environment are exposed to the solution.
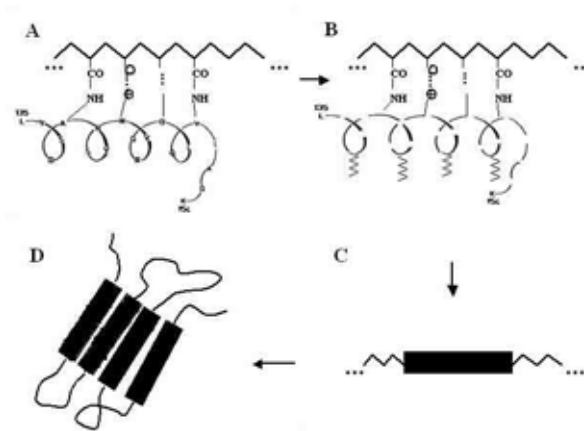
Figure 180. Schematic representation of polyelectrolyte-peptide conjugate species
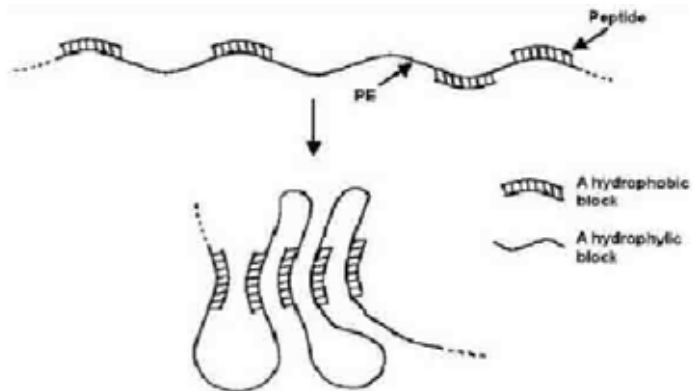
Figure 181. Schematic representation of polyelectrolyte-peptide conjugate species

Recently, we have shown that these peptide containing polymeric conjugates characterize the higher immunogenicity. This "intelligent" immunogens alike with another peptide containing Biopolymer systems were used in vaccinating guinea pigs for estimation of the potency against FMDV and dose dependent high protection was achieved. Such a modulated system is attractive for application as a novel immunogenic system in vaccine technology (see below).

Immunogenicity. For immunological experiments in ternary mixture [BSA.Pep]/[PE] = 2.0 ($C_{BSA-P}/C_{PE} = 2.0$) and metal/polymer ($n_{Cu}/n_{AA} = 0.25$) ratios were used.

The dynamics of antibody formation, induced by covalent bioconjugates of PE-FMDV peptides are presented in Figures 182 and 183.

It can be seen from these data, as determined by ELISA, that a single immunization of mice with FMDV polypeptide antigens solutions barely induced production of antibodies. The immunization of mice with solution of the bioconjugates PE-polypeptide led, in turn, to the development of a pronounced primary peptide-specific immune response. The mice, which were boosted 8 weeks later intravenously with the same concentration of free FMDV polypeptides and traced for the secondary immune response revealed no further increase in the antibody titers. In

contrast, the immunization of mice with conjugates evoked increased immune responses to polypeptides.
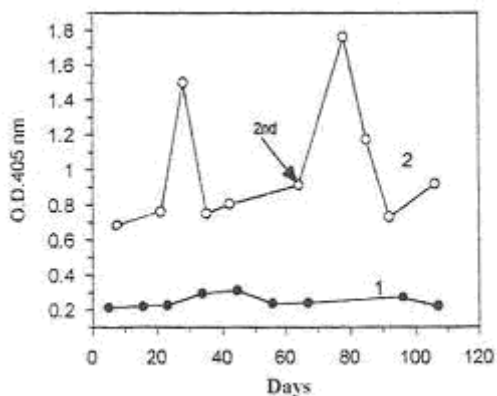
Figure 182. The dynamics of P2-specific (BSA-P2-specific) antibody formation [as assayed by ELISA (OD_{405})], induced by free P2(1) and CP2-P2(2) conjugates. 100 µg conjugate and P2 doses; intravenous injection
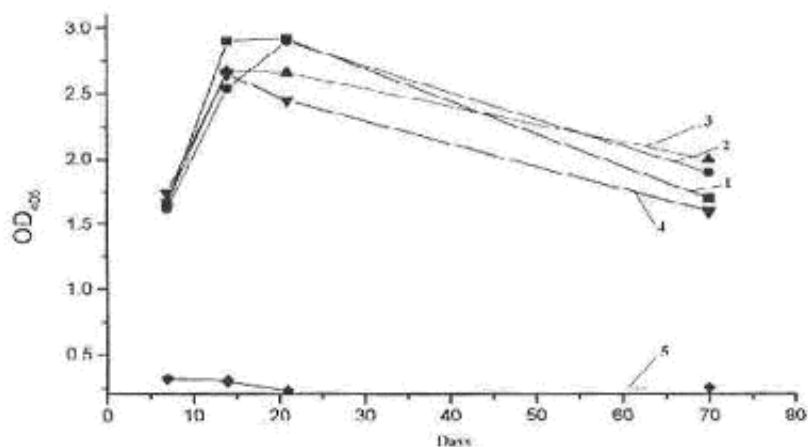
Figure 183. Peptide specific antibody formation dynamics in the mice immunized with biopolymer systems combined by two polypeptides (40-60 and 135-160 sequences) containing PE-peptide conjugates, electrostatic and icon coordination boods (Cu^{2+}) (1■) VACl, (2●) VAC2, (3▲) VAC3, (4▼) VAC4, (5♦) PI-P3

The dynamics of FMDV-specific antibody formation induced by electrostatic and Cu^{2+}-induced polycomplexes are presented in Figure. 7. It can be seen from the data that a single immunization of mice with polycomp1exes prepared with different methods, led to the development of a pronounced primary immune response. In mice, immunized with peptide-comprising Biopolymer Systems, the peptide-specific antibody activity increased in about 7 days and then maintained the very high level up to 70 days. The character of antibody formation is not dependent on the chemical structure of polymer carrier as well as on the method of the formation of Biopolymer Systems.

The physico-chemical mechanism(s) underlying the immunogenic activity of polymer-peptide conjugates may be related to an adjuvant effect of polymeric macromolecules. Free sites

of PE on bioconjugate may have the capacity to interact strongly with the membranes of immunocompetent cells. This may facilitate and stabilize the interaction of polymer bound antigen with specific cell receptors and hence enhance the immune response. The efficiency of such "forced" interactions and high epitope density (binding several peptide molecules to one polymer carrier) are high enough for the immune response.

In conclusion, a method is described for increasing the immune response to polypeptide antigens, which attacks immunological system and is of practical interest. It was found that PE-peptide as well as PE-BSAxPeptide conjugates conferred effective immunoprotection against Foot-and - Mouth Disease Virus.

Polymeric FMDV Vaccine.

Preparation of synthetic vaccine prototype by conjugation of synthetic polyelectrolytes and peptide antigens of 40-60 and 135-160 amino acid sequences of immunogenic VP1 capsid protein of "A" type FMDV which causes epidemics in Turkey was the aim of this project [356]. Thus, by the modification of the immunogenicity of the peptide antigens, development of the new FMD vaccines, diagnostic reagents, pharmaceuticals and biotechnological preparations was considered. In the author's knowledge, this work is the first synthetic peptide vaccine trial in Turkey.

As it was mentioned above, two chemically synthesized peptides corresponding to VP1 protein region of FMDV were firstly conjugated to CP1, CP2 and BSA by using water-soluble carbodiimide. Two polyelectrolyte complexes were prepared by complex formation of BSA-peptide conjugates with cationic PECVP and Cu(II)-induced complex formation with anionic CP1 and CP2.

Table 42. Synthesized amino acid sequences of A Aydın98 FMDV strain [357]

P1	40-60 (21 mere)	Val-Lys-l1e-Asn-Asn-Thr-Ser-Pro-Thr-His-Val-I1e-Asp-Leu-Met-Gln-Thr-His-GIn-His-GIy
P3	135-160(26 mere)	Lys-Tyr-Ser-Ala-Thr-GIy-Glu-Arg-Thr-Arg-GIy-Asp-Leu-GIy-Ala-Leu-Ala-Ala-Arg-Val-Ala-Thr-GIn-Leu- Pro-Ala

Thus, 4 different vaccine compositions were prepared (VAC1, VAC2, VAC3 and VAC4).

Table 43. Composition of the synthetic peptide vaccines.

VAC1	(CP1-P1) + (CP1-P3)
VAC2	(CP2-P1) + (CP2-P3)
VAC3	(PECVP-BSA.PI) + (PECVP-BSA.P3)
VAC4	(CP-Cu^{2+}-BSA.PI) + (CP-Cu^{+2}-BSA.P3)

Side Effects in Guinea pigs: 4 animals for each vaccine were inoculated s.c. (2ml/ animal). The local and general reactions were detected and recorded during the 1 month of inspection period.
Side Effects in Cattle: VAC2, which passed both guinea pig tests was inoculated to 13 cattle. The local and systemic adverse reactions were inspected clinically for 7 days.
Vaccine Doses: Synthetic peptide quantities of vaccines, which used in immunisation and potency trials, are summarized in Table 44.

Table 44: Final synthetic peptide concentration of one vaccine dose for animals

Animal	P1 (μg/dose)	P3 (μg/dose)	P1 + P3 (μg/dose)	Vaccine dose (ml)
Mouse	50	50	100	0.2
Guinea-pig	500	500	1000	2
Cattle	1500	1500	3000	2

Mouse: 8 week old Balb/c mice were immunized intravenous (i.v.) with 4 vaccine candidates. Synthetic peptide combination (P1 + P3) was used as negative control. Animals were bled at weekly intervals and antibody titres were estimated with indirect ELlSA.

Cattle: 10 cattle (in a fattening farm near Ankara) were inoculated with VAC2 s.c. Animals were bled before vaccination (on the day of vaccination), 14th and 21st days pv.. Antibody response against both synthetic peptides and intact virus were tested with indirect ELlSA and LPB ELlSA respectively. Development of neutralizing antibodies against whole virus was also detected with NT with BHK cells.

Protection Test: Guinea pig protection test was applied as described by Barnett, CARABİN 2002 AND Wotzler et al 2002. [358,359]. Groups of four animals were immunized with two fold dilutions of the 4 vaccine candidates and an aluminium hydroxide-saponine adjuvant vaccine prepared with the inactivated A98 virus as control. Immunization was done subcutaneously 2ml vaccine/ guinea-pig where the final synthetic period concentration in each dilution was 1 mg, O.5 mg, 0.25 mg and 0.125 mg. 21 days post vaccination animals were challenged with 400 guinea-pig ID_{50} "A" 98 virus/ animal. A group of four unvaccinated guinea-pigs was also infected with the challenge virus as control.

Vaccine Site Reactions in Guinea-pigs*: Four vaccine formulations were tested in guinea pigs. There was abnormal reaction in the animals vaccinated with VAC1 and VAC3. A local hyperemia in the inoculation zone was detected for 1-2 days in the animals after vaccination with VAC2. Nevertheless, severe local and systemic reactions appeared just a few minutes after inoculation of VAC4. In coordinated pace, loss of appetite for 1 day, apses with large hyperemia and large swelling zone in the inoculation area. This severe side effects with VAC4 were attributed its CU^{+2} content of the polymer.

Vaccine Site Reactions in Cattle: All of the cattle were inspected for 1 week post vaccination. Mild reactions were detected such as Increase in body temperature of two animals (39.1-40.1) for 1 or 2 days and a small lump with 30-40 mm Φ insensitive to pressure lasting for 7 days maximum.

Immunogenicity:

Mice: The mice immunized intravenously with adjuvant-free polypeptide were not responded to the antigen. Whereas, primary peptide specific immune response increased in the first 7 days and the titers were steady up to 23rd day p.v. in the animals vaccinated intravenously with PE-polypeptide conjugates (Table 45). Since peptides are known as poor immunogens, unresponsiveness to the peptides without adjuvant was expected. The highest antibody level was detected in the sera of the mice inoculated with VAC2 14 days post vaccination and starting from the 21. day pv peptide specific antibody titre decreased gradually.

In fact, the characteristics of antibody development and the level of immune response was not dependant with the preparation method of the biopolymer systems and the structure of the polymer carriers.

Potency in Goinea-pigs: Guinea-pig potency test is stilI an acceptable and reliable method in determination of the poteney of the FMD vaceines. Starting from that point, 4 PE-polypeptide conjugates (VAC1, VAC2, VAC3, and VAC4) which developed the immunogenic activity in the mice were selected to be used in guinea-pig potency test. The conventional vaccine, with $AI(OH)_3$ adjuvant, inactivated virus which contains 8.9µg 146S antigen was protected all of the animals. Guinea-pigs vaccinated with VAC2 which contains 1 mg synthetic peptide/dose was also protected the entire animal. The protection ratio in the animals vaccinated with the same dose (1 mg) of peptide conjugated with different polymers (VAC3 and VAC4) was ¾. However, VAC1 developed weak protection. The importance of the adjuvant in the potency of the inactivated or subunit vaccines is a well known reality [360,361]. For that reason, recently most of the vaccine development studies are targeted to find out more effective adjuvant with minimum side effects. Also in the present study, guinea-pigs vaccinated with the same quantity of synthetic peptides conjugated with different PE's were protected against the same quantity of the virus in different

levels (Table 4).

VAC2 was selected as primary candidate for the further experiments because of its higher protective capacity and lower toxicity in Iab animals.

Vaccine Trial with Cattle: 15-18 months old 10 cattle were vaccinated subcutaneously with VAC2 which contain 3 mg synthetic peptide doses. Animals were bled 14 and 21 days post vaccination. Antibody response to homolog intact virus was evaluated with both LPB-ELISA and NT in BHK21 cell line. Although there was a mild increase in the titres of 8 animals out of 10, none of them passed over the acceptable levels. Since, NI 0.9-1.3 considered uncertain. Only one cattle (ear tag No.99) could reach up to that level with NI 1.2 in 14th day p.v. However, to be honest, this animal was probably primed before vaccination (NI 0.3 at day 0).

Table 45. Summary evaluation of the control tests applied to the synthetic peptide vaccines

TESTS			RESULTS	
Mice	Peptide specific Ab response (indirect ELISA)	VAC1	–	
		VAC2	+	
		VAC3	+	
		VAC4	ND	
Guinea-pigs	Side effects	VAC1	no	
		VAC2	negligible (1-2 days)	
		VAC3	no	
		VAC4	sever	
	Potency	V AC1	– (0/4)*	≤2ml (≤1mg)
		VAC2	+ (4/4)*	2ml (1mg)
		VAC3	+ (3/4)*	2ml (1mg)
		VAC4	+ (3/4)*	2ml (1mg)
Cattle	Side effects	VAC2	no	
	Virus specific Ab response (NT&ELISA)		±	
	Potency		ND	

*number of protected guinea-pigs /number of challenged
ND- not done

In the present study vaccination of the guinea-pigs with 1 mg of PE- conjugated synthetic peptides (40-60 and 135-160 amino acid. residues of VP1) developed complete protection. Antibody response in mice with $100\mu g$ of the same peptide vaccine conferred the effectiveness of preparation. Nevertheless, 2 ml vaccine with 2 mg synthetic peptide was not sufficient to develop antibody response against homolog virus.

Antoni et al (1988) showed that the cattle with high antibody titer at 21st day p.v. against synthetic peptide vaccine developed generalized lesions after challenge with 10.000 ID_{50} of the homolog virus [362]. Contrary, in some experiments some of the cattle with insufficient antibody titer could be protected after challenge [363] it is clear that there is some other factors play important role in the protection mechanism of the animals. This can be cellular immunity or other type immunological responses [358].

For this study increasing the quantity of the synthetic peptide per dose could also be a solution to the problem. Tam et al (1989) showed that sufficient protection in cattle could be achieved with 5 mg synthetic peptide [338]. Another alternative is preparation of new vaccine combinations with some additional amino acid residues. Volpina et.al. (1999) declared that besides mice, guinea-pigs and rabbits also sheep and cattle responded to the vaccination with synthetic peptide vaccines contain 170-188 amino acid residues of VP1 protein of A type FMDV [364]. The last solution but not the least is to make some modifications in synthetic polymer composition and coupling mechanisms.

6. BIOPOLYMER SYSTEMS IN RADIOBIOLOGY

Theories of radiation protection can be considered at both the molecular and biochemical-physiological levels. Four molecular level protection hypothesis, radical scavenging, hydrogen

transfer reactions, the mixed disulfide hypothesis and the endogenous nonprotein sulphydryl hypothesis, probably describe different aspects of the actual protection mechanism, although each has inconsistencies [365]. At the biochemical-physiological level, hypothermia induction, hypoxia induction and biochemical shock may be involved in protection of the organism against radiation induced damage and death. It is most likely that no single mechanism can account for the protection offered by a radioprotective drug.

Water-soluble synthetic polyelectrolytes and their various polyelectrolyte complexes (or conjugates) with functionally molecules have potential possibility of radioprotective activity. One of the mechanism of the action of polyelectrolyte in biological systems is the cooperative interaction of polyelectrolyte with the biomacromolecular components of organism. This idea have been based on the results of experiments in polyelectrolyte-protein and polyelectrolyte-cell systems [372]. It is remarkable that the higher immunologically active polyelectrolytes also have radioprotective properties.

As it was mentioned above a relatively new technique involves the use of transition metal (Cu (II)) compounds as a means of activating the polymer carrier and allowing direct coupling of proteins without prior derivatization of the activated polymer, through formation of chelates (Mustafaev and Kabanov, 1981; Mustafaev et al., 1990, 1996). It is known that synthesis and fabrication of polymeric material for biomedical application can be done by radiation techniques such as polymerization, grafting, crosslinking and etching (Swallow, 1973; Spinks and Woods, 1990) [366,367]. Thus, bioreactor, biosensor, artificial organ and drug delivery systems have been studied and developed. Recently, the signal-responsive chemical delivery systems which are a combination of sensor and biofunctional system, prepared by irradiation technology have been studied (Yoshida et al., 1989; Okuda et al., 1999) [368,369].

It is known that superoxide dismutase (SOD), which is present in cytosal of eukaryotic cells is copper-zinc enzyme which catalyses the dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen. Superoxide dismutase is a beta barrel protein with 152 amino acids and consists of two subunits of identical molecular weight joined by a disulfide bond containing two Cu (II) and two Zn (II) atoms per molecule. Studies of enzyme by pulse radiolysis have indicated reduction and reoxidation of the Cu2+ during the catalytic cycle (Mc Cord and Fridovich, 1969; Keele et al., 1971); Forman and Fridovich, 1973). Radio-protective effects on mice of superoxide dismutase have also been reported (Bannister et al., 1971; Akita et al., 1984; Feher et al., 1990). It appears that metal ions in both systems: (PE-Cu2+-BSA) and SOD show similar protective effect against radiation damage.

Recently, the effects of Cu on stability and composition of water-soluble ternary polyelectrolyte-Cu-protein complexes against radiation damage was studied before evaluating their possible usage as a radioprotector [121,370,371].

Fraction of polyacrylic acid (240 kDa), BSA and superoxide dismutase (SOD) [378-382] were used as a components for preparation of polycomplexes.

To produce polymer-protein mixtures, BSA and SOD solutions (l g/l) were added to PAA (l g/l), dissolved in phosphate buffer, pH = 7.2. The ternary (PAA-Cu^{2+}-protein) mixtures were, in turn, prepared by different methods: by adding protein solutions to the polymer-metal complex (PMC), by adding polymer solutions to the protein-Cu^{2+} complexes and by adding Cu^{2+} ions ($CuS0_4.5H_2O$, pH = 4.0) to the polymer-protein mixtures. The pH values were adjusted with 1 M NaOH to the desired pH. BSA/PAA ratios (n_{BSA}/n_{PAA}) were calculated using the equation $n = cN_A/M$ where n is the number of molecules in 1 ml; M is the molecular weight of components; N_A is the Avagadro number; c is the concentration in g/l. The heterogenecity of polymers and proteins and the fraction compositions of the mixtures were estimated by using two HPLC systems.

γ-radiolysis of the aqueous solutions of PAA, BSA, SOD, PAA-BSA, PAA-Cu^{2+}-BSA and PAA-SOD mixtures, open to air, was performed by using a ^{60}Co γ-source (Picker 9 V). 5 ml solutions of samples were put in bottles. The samples were irradiated at a position of 10 cm from

the source. The dose rate was measured to be 54.5 Gy/h as determined by Fricke dosimetry. A Shimadzu UV-2401 PC spectrophotometer was used for spectroscopic analyses.

      The spectrophotometric results of the irradiation experiments are presented in Table 46. As it can be seen from comparisons of the results of optical densities of irradiated and unirradiated solutions in aerated conditions, unlike the solutions of free components (PAA, BSA, PAA-Cu) for the ternary mixture PAA-Cu-BSA the values of %OD in the dose up to 1.2 kGy are changed insignificantly. A significant decrease in the radiation change of the values of %OD of these mixtures was observed for solutions containing N_2. A percentage change of optical densities in N_2O saturated solutions of BSA was higher than aerated and N_2 saturated solutions (Table 46).

Table 46. Percentage changes in optical density values {%OD = [(ΔOD/OD)*100]} in γ-radiolisis (medium: aerated)

Dose(Gy)	PAA	PAA-Cu^{2+}	BSA	PAA-Cu^{2+}-BSA
33.34	0.04	0.65	2.28	0.02
100.03	2.12	1.92	5.71	0.09
133.37	5.16	4.09	8.24	0.12
471.94	11.69	6.51	11.41	0.53
655.12	15.37	9.01	26.35	8.50
675.28	16.49	10.2	38.01	8.81
1036.66	17.08	-	49.43	9.74
1044.91	17.19	-	51.40	10.35
*655.12	10.25	3.31	18.41	2.23
**655.12	16.40	9.12	56.76	6.59

*Medium in N_2 ** Medium in N_2O

      Although the radiation –chemical changes were measured by UV-Vis spectrophotometry, more detailed information was obtained by the method of HPLC. The HPLC results of the irradiated and unirradiated PAA, BSA, PAA-Cu^{2+}, PAA-BSA and PAA-Cu^{2+}-BSA solutions in O_2 atmosphere are shown in Figure 184. No change was observed in BSA solutions irradiated at low doses (up to 0.655 kGy). However, the values of retention time (RT) and form of these peaks (heterogenecity) of BSA solutions irradiated at 1.044 kGy significantly differ from unirradiated protein solutions. For the PAA and PAA-Cu^{2+} solutions deformation of the peaks was observed at the higher irradiation dose (1.2 kGy).
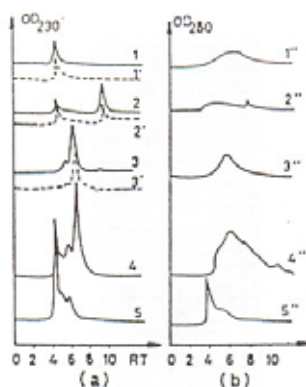
Figure 184. HPLC results of unirradiated and irradiated solutions of PAA (1), PAA-Cu^{2+} (2), BSA (3), PAA-BSA (4), PAA-Cu^{2+}-BSA (5) in the presence of O_2; 1, 2, 3, 4, 5 (Unirradiated samples); 1′, 2′, (1.044 kGy); 3′ (0.675 kGy) 1″, 2″, 3″, 4″, 5″ (1.2 kGy) [Cu^{2+}] = 1.388 x 10^{-3} M, $C_{PAA} = C_{BSA} = 0.1$ g/dl

On the bases of these results, as well as other from earlier investigations [372,373], it can be proposed that BSA and PAA undergoes degradation and crosslinking at this dose. Similarly, denaturation and aggregation have been obtained with irradiation of proteins such as bovine and human serum albumines, egg albumin, casein and β -lactoglobulin [373].

Figure 184(b) compares HPLC results of the irradiated and unirradiated PAA, PAA-Cu^{2+} and BSA solutions with those of PAA-BSA and PAA-Cu^{2+}-BSA mixtures at the high irradiation dose (1.2 kGy). The chromatograms obtained for the irradiated solutions of PAA-BSA mixture clearly demonstrate the formation of a new form of the protein and macromolecules at this dose. The behaviour of BSA upon irradiation in the presence of PAA macromolecules do not change essentially. The denaturation and aggregation by the crosslinking way of macromolecules in mixture takes place as in the case of individual components. At the same time, as can be seen from the results (Figure 184(b)), the behavior of ternary PAA-Cu^{2+}-BSA mixture after irradiation was not significantly different from unirradiated solutions. Although the shapes of the peaks and RT values remained the same, the peak areas decreased upon irradiation.

Addition of Cu^{2+} ions to PAA-BSA mixture reduces the extent of radiation–induced change of the protein and PAA macromolecules in the particles of ternary polycomplexes. This phenomenon can be considered to "protect" (or stabilization) of the macromolecules against radiation damage. Preservation of native structure of BSA in ternary PAA-Cu^{2+}-BSA complexes upon irradiation was observed and this was confirmed by the immunological methods recently. Injection of irradiated ternary PAA-Cu^{2+}-BSA complexes to animals resulted on the production of BSA-specific antibodies.

Studies of the fraction composition of polymer-protein mixtures at different irradiation doses by HPLC permit to elucidate some important features characterizing the obtained "protection" phenomenon.

6.1. PAA-BSA Systems:

The HPLC results of the unirradiated and irradiate PAA-BSA mixtures at different irradiation dose are shown in Figure 185.
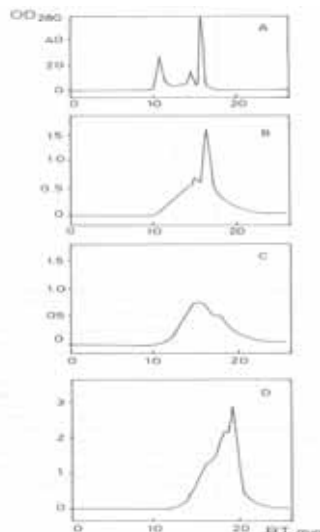
Figure 185. HPLC results of the unirradiated (A) and Irradiated (B-D) PAA-BSA mixture at different irradiation doses (Gy): 100 (B), 300 (C), 1200 (D), $C_{BSA} = C_{PAA} = 0.01$ g/l

As it is seen from this figure, the unirradiated PAA-BSA mixture is characterized by a multimodal distribution of components. The comparison of the values of retention times (RT) corresponding to the peaks of the mixture and of the individual components (RT of the protein and PAA under the similar conditions are equal to 15.77min (monomer), 14.63min (dimer), l3.8min (trimer) and 10.8 min (PAA), respectively) shows that the interaction between BSA and PAA at the pH 7.0 was weak, if not negligible. The preexisting electrostatic repulsive forces between similarly (negatively) charged PAA and BSA prevent the formations of polycomplexes. The results are consistent with the results obtained by sedimentation and HPLC analysis of PAA-BSA systems (Kabanov et al., 1978; Mustafev et al., 1996, 1998). Stable bioconjugation of PAA with BSA took place, however, upon irradiation of the mixture PAA-BSA (B, C, D). The peak with the RT of pure PAA is absent in chromatograms and the value s of RT, the distribution of compounds and the shape of the peaks (heterogenecity) of irradiated PAA-BSA solutions significantly differ from unirradiated polymer-protein mixtures.

The increase of RT values and narrowing of the heterogenecity of reaction products were observed at the higher irradiation dose. On the basis of the results, it can be proposed that in the mixture of PAA-BSA, the macromolecules undergo degradation and crosslinking under these conditions. Degradation and crosslinking reactions are reported for the γ-radiolysis of powdered PAA and polymethacrylic acid (Afanas'ev et al., 1985; Hill et al., 1990) (dose fare of approximately 10 kGy) [374,375]. Denaturation and aggregation have been obtained with irradiation of proteins such as bovine and human serum albumins, egg albumin, casein and β-lactoglobulin (Urbain, 1977).

Figure 186 compares HPLC results of the irradiated and unirradiated PAA-Cu^{2+}-BSA mixtures at different irradiation doses. As suggested by the change in chromatograms (Figures 187A and 186B (1)) stable complexation of PAA with BSA via Cu^{2+} took place upon addition of copper ions into PAA-BSA mixture. It is remarkable that the character of the distribution of compounds in ternary mixture in contrast to PAA-BSA mixtures practically does not change during irradiation up to 1.2 kGy. At the high irradiation dose (2.5 kGy) the areas of the peak with law RT decreased and the distribution of compounds and the heterogenecity of solutions significantly differ from solutions irradiated at $\leq$ 1.2 kGy. This may cause the radiation-induced covalent crosslinking of particles. Therefore, the addition of Cu^{2+} ions to PAA-BSA mixture protects the PAA and BSA components of ternary PAA-Cu^{2+}-BSA complexes against radiation damage. The mechanism underlying the protection effect might be related to the conversion of superoxide anion (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) via Cu^{2+} ions (Mustafev et al., 1996).

One can as time that in the case of the PAA-BSA mixtures containing Cu^{2+}, the reaction of radiation-induced crosslinking starts after the complete oxidation of Cu^{2+} ions in composition of polycomplex particles with superoxide anions (O_2^-).
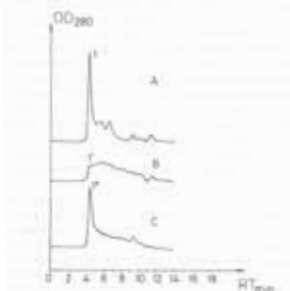
Figure 186b compares the results of HPLC analysis of unirradiated and irradiated solutions of ternary PAA-Cu^{2+}-BSA mixtures prepared at different initial n_{BSA}/ n_{PAA} ratios and more high concentration of Cu^{2+}. When PAA-Cu^{2+} solution is titrated with protein solution ($n_{BSA}/ n_{PAA} < 0.1$), BSA is complexed with the polyion via copper ions (A-D). The particles of ternary polycomplex moved in chromatograms as a pure PAA (peaks 1) and some free BSA molecules (or BSA-Cu^{2+} complexes) remain in solution (peaks 2). The pattern changes significantly on further increase of the ratio, $n_{BSA}/ n_{PAA} \geq 1$ (E). Under this condition, a further increase in BSA content ($n_{BSA}/ n_{PAA} = 2.0$) led than to the decrease of peak 1, the intensity of peak 2 corresponding to free (or BSA-Cu^{2+}) BSA increased. Notice that the intensity of peak with RT between peaks 1 and 2 corresponding to the dimer form of BSA content increased considerably. Thus, it can be proposed that a further increase in BSA content to breakdown some of the polycomplex as in mechanism (1) by the formation of BSA-Cu^{2+}-BSA and BSA-Cu^{2+} complexes and free PAA-Cu^{2+} or (PAA): (Mustafev et al., 1996):

$$[PAA - Cu^{2+} - BSA] \xrightarrow{BSA} [(BSA)_2 - Cu^{2+}] + PAA(Cu^{2+})$$ (1)
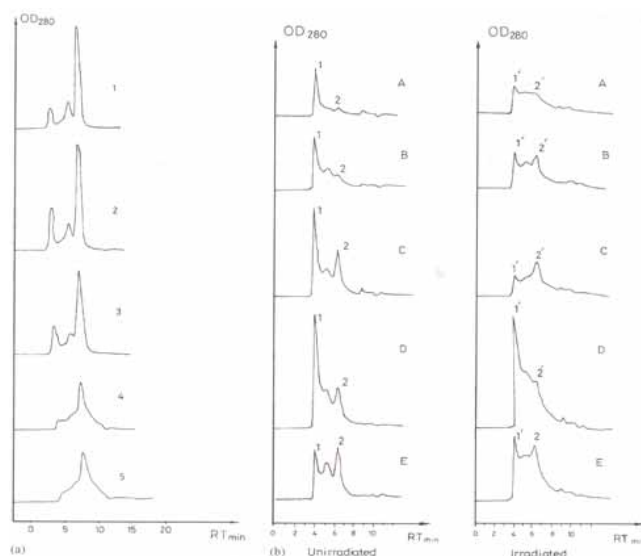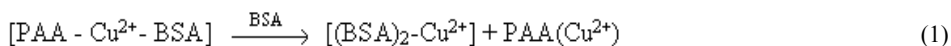
Figure 186. (a) HPLC results of the unirradiated (1) and irradiated (2-5) PAA-Cu^{2+}-BSA mixtures at different irradiation dose (Gy): 300 (2), 1200 (3), 2500 (4), 3000 (5); $C_{BSA} = C_{PAA} =$ 0.01 g/l. [Cu^{2+}] = 1.4 x 10^{-4} g mol/l. (b) HPLC results of the PAA-Cu^{2+}-BSA mixtures, prepared at different initial n_{BSA}/n_{PAA} : 0.1 (A); 0.2 (B); 0.5 (C); 1.0 (D); 3.0 (E); [Cu^{2+}] = 1.4 x 10^{-4} g mol/l; irradiation dose: 1200 Gy

The higher capacity of BSA in complex formation with Cu^{2+} than PAA (Lau and Sarkar, 1971; Dixon and Sarkar, 1974) is consistent with this proposal.

An analysis of the irradiated ternary PAA-Cu^{2+}-BSA mixtures at different initial n_{BSA}/n_{PAA} ratios of components deserves some consideration. On the whole, the addition of Cu^{2+} ions protects the components of PAA-BSA mixture, prepared at different n_{BSA}/n_{PAA} ratios, against radiation damage, although some difference on the heterogenecity of the solution after irradiation took place. Taking into account the fact of the radiostability of fraction (2′), under these can one may consider that fraction 2 in the mixture (Figure 186b unirradiated solutions) contain BSA-Cu^{2+} complexes.

Figure 187. HPLC results of the unirradiated PAA-Cu^{2+}-BSA mixtures prepared in water (A) and in 0.15gmol/l NaCl solution (B); (C) After irradiation of mixture A and adding 0.15gmol/l NaCl; $C_{BSA}/C_{PAA} = 0.01$ g/l; [Cu^{2+}] = 1.388 x 10^{-3} g.mol/l

The suggested mechanism $Cu^{2+} \rightarrow Cu^{1+}$ by O_2^- may led to the weakening of ternary polycomplexes, that exert influence on the redistribution of solution components after irradiation. Figure 187 compares the results of HPLC analysis of unirradiated solutions of ternary PAA-Cu^{2+} mixtures prepared in water and in 0.154 g mol/l NaCl solution. As it can be seen, the heterogenecity of the mixture prepared in the presence of law molecular salts, significantly differs from those, which do not contain a specially added NaCl. Besides, the OD_{280} values in salt-containing mixture are essentially lower than in water system. On the basis of these results, it can be proposed that a soluble ternary polycomplex is not stable under physiological conditions (pH = 7.0; 0.154 g mol/l NaCl) and the interaction becomes a complicated character. The charge neutralization of particles (free section PAA, etc.) in mixture by NaCl leads to the decrease the size of particles, (broad peaks in chromatograms by high-diffusion coefficient of compact particles). Figure 187c corresponds to the HPLC results of the PAA-Cu^{2+}-BSA mixtures preparing in the presence of 0.154 g mol/l NaCl after irradiation at 2.5 kGy. Addition of NaCl to this irradiated ternary mixture does not lead to essential change in the character of the chromatograms of the solution. This phenomenon can be explained due to radiation-induced covalent crosslinking of protein and polymer macromolecules in ternary polycomplex after irradiation at 2.5 kGy, which lead to the additional stabilized polycomplex particles against NaCl.
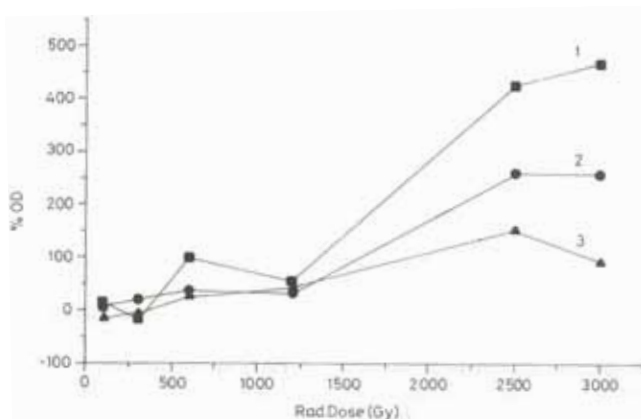
Figure 188. Percentage changes in optical density values %OD [%OD = (ΔOD / OD) x 100] %OD versus radiation dose (Gy). 1 – PAA; 2 - (PAA - SOD); 3 - SOD; $C_{BSA} = C_{PAA} = 0.01$ g/l

The preparation order of irradiated and unirradiated mixtures does not affect composition. The results have shown that the character of the formation of ternary complexes is same. Initially, addition of BSA into (PAA-Cu^{+2}) the mixture or addition of PAA into (Cu^{+2}-BSA) is practically same. One can assume that process of Cu^{2+} -induced complex formation between PAA and protein molecules is the equilibrium reaction.

6.2. Poly(NIPAAm)-BSA Systems

Poly(N-isopropylacrylamide) homopolymer do not contain the corresponding functional groups for protein covalent binding and complex formation in neutral water solution. The covalent binding of poly (NIPAAm) with BSA was carry out by irradiation method recently [371]. The solutions of poly (NIPAAm) and BSA were irradiated at different doses with a ^{60}Co γ-source. The change of the Tripthophan fluorescence intensity of polymer-protein mixture with increasing irradiation dose and temperature is shown in Figure 189.
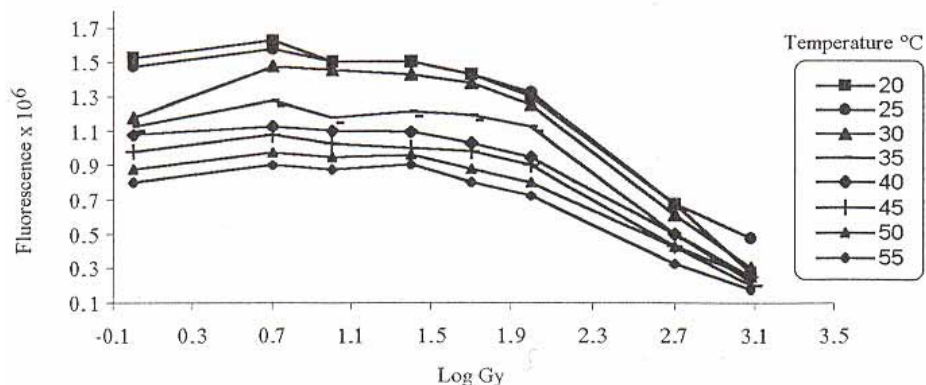
Figure 189. Fluorescence intensity of poly(NIPAAm)-BSA conjugates at different radiation
dose and temperature

It was observed that the fluorescence intensity of polymer-protein mixture decreased
with increasing irradiation dose and temperature. At the same time it was observed that the
fluorescence intensity showed a very little change for absorbed dose of up to 0.1 kGy, but it
decreased considerably for doses higher than 0.1 kGy. It was suggested that covalent conjugates
occurs under this condition and this phenomenon was caused with the structural alteration of
protein molecules in the composition of covalent conjugates. Formation of radiation-induced
covalent conjugates in poly(NIPAAm)-BSA mixtures was confirmed by the HPLC and
immunological methods recently. Injection of irradiated poly(NIPAAm)-BSA mixtures to animals
resulted on the production of BSA-specific antibodies (Mustafaev et al. unpublished results).

6.3. PAA-SOD Systems

Figure 188 illustrates the dependence of the percentage changes in the optical density values
%OD of the solutions of free PAA, SOD and equimolar mixture, PAA-SOD on the irradiation
dose. As can be seen from comparison of these results, a significant increase in the values of
%OD was observed for solutions of free PAA. Percentage change of optical density in solutions
of PAA-SOD was lower in solutions of free PAA and higher in solutions of free SOD. The OD
results obtained after irradiation indicate that all systems undergo some change in chemical
structure (degradation -COOH groups, hydrogen abstraction from the polymer chain and
crosslinking reactions (Hill et al., 1992) [375].
Figure 190a shows the results of HPLC analysis of unirradiated and irradiated solutions
of free SOD, PAA and PAA-SOD mixtures at different irradiation doses. The irradiated solutions
of PAA were characterized in chromatograms only by one peak and the retention time
corresponding to the peaks practically does not change and remains equal to that of unirradiated
PAA. The values of areas did not change considerably over a wide range of irradiation dose (up to
2.0 kGy). However, there exists a critical irradiation dose in the system (> 2.0 kGr) at which the
areas of these peaks increased and reached a maximum although the values of RT did not change.
(We cannot analyze the degradation and crosslinking of polymer chains after irradiation by HPLC
methods because PAA macromolecules before and after irradiation is the polyelectrolytes with
unfold structure and do not separate on molecular weight in HPLC). On the basis of these results,
as well as from earlier investigations (Urbain, 1977; Pietrzak, 1995) [373,376], it can be proposed
that the increase of the optical density with the dose is probably due to an unidentified radiolytic
product which absorbs at the same wavelength. This might be due to organic peroxide formation.

Other results obtained for the irradiation of SOD solutions are shown in Figure 190a (B). It can be seen from the results that the SOD solutions in chromatograms were characterized by one peak (up to 0.6 kGy) at the low irradiation dose, but for the dose > 0.6 kGy, a new peak appears in chromatograms; the new peak appears with increasing values of RT and the irradiated SOD solutions were characterized by bimodal distribution. The bimodal distribution of components was obtained up to 3.0 kGy irradiation dose and change in the areas of these peaks was weak, if not negligible, with increasing irradiation dose.

Bovine erythrocyte SOD was subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulfate ± β-mercaptoethanol (Keele et al., 1971) [377]. It was shown that sodium dodecylsulfate was able to cause the dissociation of the enzyme only in the presence of β-mercaptoethanol. On the basis of these results Keele et al., concluded that SOD is composed of two subunits of equal size, the association of two subunits does involve at least one disulfide bridge. With radiation changes in the primary structure of proteins involved, oxidation of SH groups, partial deamination, decarboxylation and oxidation of phenol radicals and radicals of the heterocyclic amino acids. The principal reaction of oxygenated aqueous solutions of proteins is degradation and crosslinking by the peroxides (Swallow, 1973) [367]. We may conclude that the peroxide was able to destroy the disulfide bonds which is the most weak covalent bonds in protein structure.

HPLC results of PAA-SOD mixtures at different irradiation doses are given in Figure 190a (C). The mixture of PAA-SOD up to 0.6 kGy irradiation dose was characterized in chromatograms by two peaks corresponding to PAA (RT= 10.58-10.96 min) and SOD (RT= 16.96-17.24 min). The area of these peaks in the mixtures analogous to free polymer and enzyme systems did not change with increasing irradiation dose. Moreover, at irradiation doses $\geq$ 0.6 kGy, the peak corresponding to the SOD fraction doubled and behaved as in the free SOD solutions by the increasing irradiation dose. This result demonstrates that SOD is a scavenger of superoxide radicals and prevents the covalent conjugate formation. The effect of transition from unimodal to bimodal distribution of SOD fraction by irradiation is probably conditioned by destruction of the disulfide bond and separation of the two subunits of the enzyme. The superoxide anion (O_2^-) which is formed by the univalent reduction of O_2 by ionizing radiation will be captured with transient metal ions of the SOD molecule. Both copper and zinc ions might take part in catalysis of the reaction $O_2^- + O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2$ and protect the macromolecules against radiation damage.

6.4. Mechanism of the radiation-induced conjugation

Our results indicate that water-soluble PAA-BSA bioconjugates are formed at natural pH upon irradiation. The preexisting electrostatic repulsive forces between PAA and BSA (pI = 4.9) do not prevent the formation of covalent conjugates in the radiolysis of PAA-BSA mixtures.

The unirradiated mixture of PAA-SOD was characterized by two peaks corresponding to free PAA and SOD fractions. At neutral pH, PAA and SOD (the isoelectric point of the enzyme is 4.95) have negative charges and are incapable of binding to each other.

This protein consists of two subunits of identical molecular weight joined by a disulfide bond and contains two Cu(II) and two Zn (II) atoms per molecule. Zn^{++} plays a structural role and lends it enhanced stability whereas Cu^{++} is directly involved in the catalytic activity and binds two histidine residues. It can be proposed that the higher capacity of SOD in complex formation with copper-zinc ions than PAA prevents the ternary PAA-metal-enzyme complex formation.
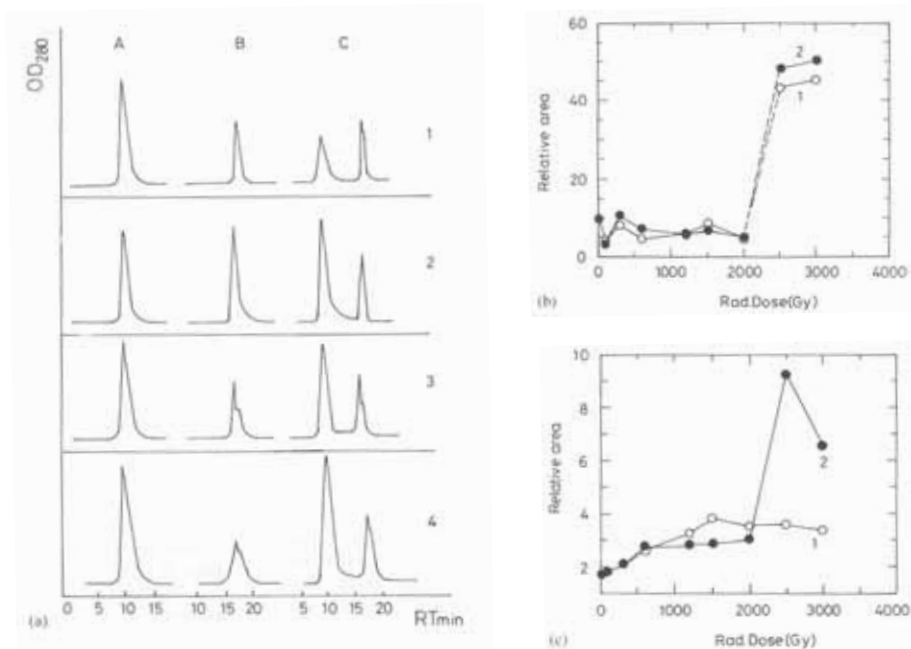
Figure 190. (a) HPLC chromatograms of unirradiated and irradiated solutions of PAA (A), SOD (B), PAA + SOD (C); 1 - unirradiated solutions; irradiated solutions at different irradiation dose (Gy): 600 (2); 2500 (3); 3000 (4); $C_{BSA} = C_{PAA} = 0.01$ g/l (b) Relative area of the peaks, corresponding free PAA (1) and PAA in mixture PAA-SOD versus radiation dose (2). (c) Relative area of the peaks, corresponding free SOD (1) and SOD in mixture PAA-SOD versus radiation dose (2)

Mechanism of protection

The effect of high energy radiations on water [367] may be summarized as:

$$H_2O \rightarrow 2.7\ (e^-_{aq}) + 2.7\ (OH\bullet) + 0.55\ (H\bullet) + 0.45\ (H_2) + 0.71\ (H_2O_2)$$
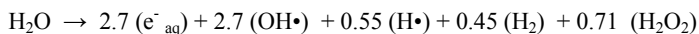
where the numbers before chemical symbols represents G-values. Larger yields of HO_2 and O_2^- are formed in aerated solutions by reaction of e^-_{aq} and H with oxygen [366]
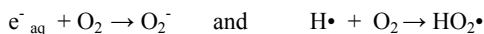
$$e^-_{aq} + O_2 \rightarrow O_2^- \qquad \text{and} \qquad H\bullet + O_2 \rightarrow HO_2\bullet$$

With radiation, changes in the primary structure of proteins involved oxidation of SH groups, partial deamination, decarboxylation and oxidation of phenol radicals of the heterocyclic amino acids. The principal reaction of oxygenated aqueous solutions of protein is:

$$RCONH\text{-}CHR_2 + O_2 + H_2O \rightarrow RCONH_2 + RCOR + H_2O_2$$
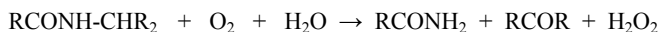
Irradiation of polymers in the presence of oxygen can give rise to peroxides, which may subsequently lead to degradation and crosslinking of the polymer chain. The higher change in optical density of BSA solutions saturated with N_2O could be due to the increased yield of OH radicals in N_2O saturated solutions, where e^-_{aq} is converted to an equivalent amount of OH radical:

$$e^-_{aq} + N_2O \xrightarrow{\ +H_2O\ } OH\bullet + OH^- + N_2$$
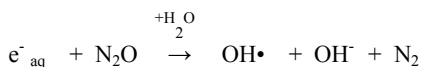
According to Mustafaev [121], the mechanism underlying the protection effect in the

mixture of PAA-Cu^{2+}-BSA might be related to the complexation of copper in polyelectrolyte complex (PEC) with superoxide anion (O_2^-) and the formation of the following equilibrium:

$$PEC.Cu^{1+} + O_2$$
$$\rightleftharpoons (PEC.Cu^{2+} * O_2^-) \rightleftharpoons PEC.Cu^{1+} * O_2$$
$$PEC.Cu^{2+} + O_2^-$$
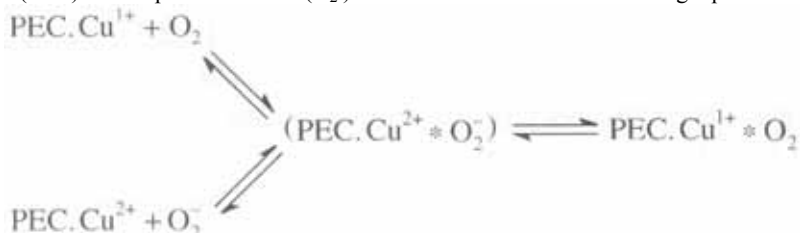
Measurement of Cu(I) ions in irradiated solutions of 0.1% PAA-Cu(II) and 0.2% PAA-Cu(II) by neocuproine showed that the concentration of Cu(I) increases with increasing radiation dose.(Figure 191)

Figure 191. Effect of radiation dose on formation of Cu (I) in PAA-Cu(II) system; $[Cu^{2+}] = 1.388$ x 10^{-3} M, $[PAA] = 0.1$ g/dl and 0.2 g/dl, $n_{Cu}^{2+}/n_{PAA} = 0.05$

Copper ions may also react with OH and HO_2:

$OH\bullet + Cu^{2+} \rightarrow Cu^{3+} + OH^-$ and $HO_2\bullet + Cu^{2+} \rightarrow Cu^{1+} + O_2 + H^+$

Besides, one can notice that when polyanion macromolecules reacts with the protein globules to mask a group that is practically radiation sensitive and may act as a "sacrificial" protective agent. Both physical (charge and energy transfer) and chemical (reacting the amino acids with carb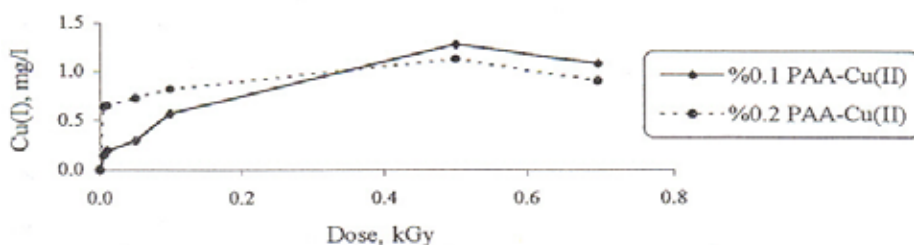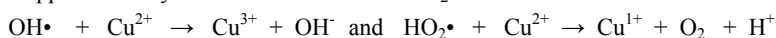onyl compounds and Cu, recombination of forming radicals, "repair effect", etc.) protection can occur in this system. Stabilization of PAA chains in ternary polycomplexes was partially realized by Cu^{2+}. As shown earlier, the degradation of aqueous solutions of high molecular weight polymetacrylic acid caused by HO_2 radicals formed during irradiation is also inhibited by the protective SH compounds, amines, alcohols, cyanides, etc.

We can propose that in the mixture of PAA-SOD, Cu-Zn-superoxide dismutase is the scavenger of the superoxide anion by the following equlibrium:

$Me^{2+} - SOD + 2 O_2^- + 2 H^+ \rightleftharpoons Me^{1+} - SOD + H_2O_2 + O_2$

Metal ions act as protective agents of the protein globules against radiation damage and prevent the radiation-induced covalent conjugation of PAA macromolecules with SOD molecules. In conclusion, the presented results show the preparation of water-soluble covalent conjugates in the mixture of polyanions with similarly (negatively) charged proteins by radiation-induced method. Injection of irradiated PAA-BSA mixtures to animals resulted on the production of BSA-specific antibodies (Mustafaev et al., unpublished results), which open the new possibilities for the creation of immunogenic biopolymer systems by these methods. A considerable interest exists for the establishment of the influence of transient metal ions on these processes. Comparison of these results with the radioprotective activity of polymeric compounds will be of interest for the elucidation of the mechanism of the action of PE in the organism. In addition, such reactions may

simulate, for instance biomolecule reactions in the presence of transient metal ions.

7. CONCLUSION

The data presented in the monography provide factual evidence concerning the mechanism of binding of linear polyelectroleytes to proteins in aqueous solutions and the structure of soluble polycomylexes formed thereby. Some peculiarities and regularities of complex formation are discussed, and structural models of the synthetic compounds are proposed. An attempt has been made to classify the specific effects underlying the interactions between linear and globular macromolecules. It has been demonstrated that the use of fractions with predetermined molecular mass and a narrow range of molecular mass distribution as complex forming polymers males it possible to elaborate adequate approaches to the study of complex formation reactions in such systems and to apply a wide variety of present day physico-chemical methods for their investigation.

The experimental results testify to the fact that various proteins, regardless of their functional activity and physico-chemical properties, form soluble complexes with polyelectrolytes according to a common mechanism. Depending on the chemical nature of the polymeric carrier and environmental conditions, two types of soluble polymer-protein complexes may be constructed.

Complexes of the first type are formed in those systems which display a marked cooperativity of binding and a tendency towards self-organization. One characteristic feature of these complexes is the uneven distribution of protein molecules between the adsorbing polyions, i.e., the alternation of maximally loaded with protein polyelectrolytes and unloaded macromolecules. The components of such complexes have rod-like structure: the core of the rod is made up by protein globules that are closely linked together; the linear macromolecule is twisted around the protein globule, the length of the rod, i.e., the number of protein globules on it, being directly proportional to the degree of polymerization of the linear macromolecule. Under conditions when the compactization of polyions occurs as a result of nonpolar interactions of lateral hydrophobic radicals, the structural organization of the polymeric complexes is characterized by complex interactions between the polyion and the protein. The compact polyelectrolyte macromolecule forms the particle core on which protein molecules are situated, in many sites polymerized regions come to the surface.

Complexes of the second type are formed, as results of the uniform loading of protein globules between the polyelectrolyte chains, i.e., the protein molecules are randomly distributed between the adsorbing polyions. In this case the structure of soluble complexes being formed retains the conformation of a statistical coil of the polyelectrolyte carrier. By changing the chemical composition of the complex-forming polymer and environmental conditions (ionic strength, pH) one may induce an intermediate transition from the distribution of the "all or nothing" type to the uniform distribution. Possible causes of the existence of two types of soluble polycomplexes differing in their structural organization have been described. It has been supposed that such aggregates may be regarded as models of specific natural biocomplexes (nucleoprotein and polysaccharide-protein complexes), many of which may also be formed from proteins and naturally occurring polyelectrolytes as a result of self-organization.

The self-assembly reactions described herein are accouip1ished via relatively simple and nonspecific physico-chemical interactions between linear and globular macromo1ecules. The general structural and thermodynamic principle of self-assembly of highly ordered aggregates from chemica1ly complementary linear and globu1ar macromolecules has been proposed. Quite probably, the 'minimum' requirements concerning the nature and dynamics of macromolecular interactions are sufficient for such a self-assembly to be implemented in systems of different chemical nature, including those containing no biopolymers. One may assume that these results provide compelling evidence of the simplicity and uniformity of fundamental physico-chemical

principles underlying the occurrence and functioning of living systems.

The approaches and methodology used in the study of two-component polymer-protein systems play a very important role during the analysis of interactions occurring within multicomponent systems. The results cited herein suggest that the mode of distribution of protein fractions between the complex-forming polymer (both in artificial serum protein mixtures and in whole sera) is, in a greater degree, regulated by the possibility of 'positive' interactions between the identical subunits (globules) of the complex components which allows the synthesis of both individual and mixed (chimeric) polycomplexes. It has been shown that in solution self-organization, macromolecular exchange and substitution are also inherent in complex mixtures. Under these conditions the reaction equilibrium is reached rather quickly, almost at a rate of the reagent mixing. Specifically, the modes of formation, structure and composition (and, as a matter of consequence, the reactivity of the complex components) depend on the ionic strength and pH of the medium.

These data testify to the high practical utility of synthetic polymer-protein complexes in immunological, chemical and enzymological studies as well as m clinical medicine.

Very encouraging result were obtained in immunologic studies employing the use of polyelectrolyte complexes of natural antigens for the construction of polymer-subunit immunogens; these complexes served as a basis for creating effective vaccinating compounds aimed at the protection of animals from various infections. Thus, it has been shown that conjugation of model (BSA, BGG, OVA, etc), microbial and viral antigens (B. tuberculosis antigen, influenza virus, H-antigen of Salmonella, α-fetoprotein, etc.) with polyelectrolytes of different origin results in stable complexes endowed with an exceedingly high immunological activity. Joint investigations conducted in collaboration with our Indian colleagues at the National Institute of Immunology in New Delhi have culminated in the synthesis of highly immunogenic polyelectrolyte complexes of gonadotropic hormones whose application will open promising perspectives for the construction of anti fertile vaccines designed for birth control in animal and man. Noteworthy, these polycomplexes elicit thymus-independent immune responses, stimulate immunological memory (secondary immune responses) and, as was revealed by animal studies, afford rather an effective immunological protection.

One of the most perspective trends in modern-day immunology is the search for ways and means of lowering the immunogenecity of protein preparations (hormones, plasma components, etc.) injected to man curative purposes. For instance, multiple injections of insulin to patients with diabetes mellitus induce sensitization which, in turn, diminishes the biological activity of the drugs and evokes side reactions, e.g., allergy. Therefore the lowering of immunogenicity of protein preparations seems to be a very perspective approach. The working models of polymer-antigen complexes proposed herewith demonstrate that depending on the "architecture" of the antigen-polyelectrolyte particles the same polymeric carriers can be used for the construction of both highly immunogenic and weakly immunogenic preparations.

Hence a natural question arises as to what is the further fate of the polymeric carrier entering the organism. Marked progress has been attained in the synthesis of polymeric carriers capable of being split off and exported from the organism. As far as the choice of effective carbo-chained polymeric carriers is concerned, it does not present any serious problem. Thus, the construction of highly immunogenic preparations through the binding of nontoxic polyelectrolytes with natural antigens via transient metal ions (ternary polyelectrolyte-metal-antigen complexes) provides an illustrative example. This method is universal and allows the synthesis of polyelectrolyte complexes on the basis of a vast variety of polymers irrespective of their molecular mass (involving those, with universally accepted values of molecular masses) and composition, thus significantly expanding the range of polymers of immunological and clinical purpose.

Studies of complex formation between synthetic polyelectrolytes and biopolymers have culminated in the synthesis of highly immunogenic artificial antigens capable of affording

effective immunological protection from various infections as well as of radiopritector preparations displaying a high specific activity and stability. Analysis of physico-chemical capabilities of such polycomplexes in vitro and under conditions of the whole organism makes them a helpful tool in theoretical immunology and radiobiology studies as well as in other areas of the biological science. Thus, a direct correlation has been found between the ability of unnatural polyelectrolytes and their polycomplexes to enter multipoint interactions with proteinaceous and cellular components of the blood in model systems, on the one hand, and their physiological activity in vivo, on the other. This finding sheds additional light on the mechanisms underlying various effects of polymeric compounds in vivo. These results, however, do not indicate that the mechanisms underlying the physiological effects of such complexes should necessarily be based on polyelectrolyte interactions with blood serum proteins, e.g., serum albumin or other biosystem components selected as model compounds. No doubt, the observed correlation between the results obtained in vitro and in vivo implicates a similarity of mechanisms underlying the multipoint interactions inherent in polyelectrolytes as biphilic cooperative systems. These results may also be interpreted as being due to the lowered toxicity of polyelectrolytes without any alterations in their physiological activity. The latter circumstance is of key importance for practical medicine.

Further continuation of in depth studies in this field will inevitably lead to the solution of a vitally important task, namely, the synthesis of polyelectrolyte complexes of low and high molecular weight natural compounds having a predetermined structure and composition. These studies will develop along several lines namely further elaboration of theoretical concepts of polyreactions biomodelling and construction of ,radioprotectors, artificial polydeterminant antigens, drugs and vaccines of the future.

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