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THE CHEMISTRY OF MACROMONOMERS BASED ON PHYSIOLOGICALLY ACTIVE SUBSTANCES AND SOME PROBLEMS OF THE SYNTHESIS OF POLYMERS FOR BIOMEDICAL APPLICATIONS

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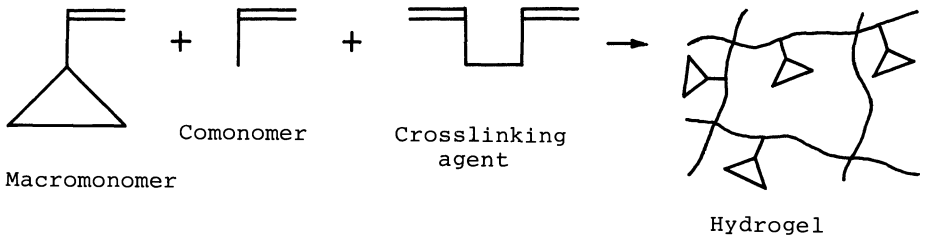
Abstract - In this paper an approach to the synthesis of macromonomers is described which permits to convert into reactive polymerisable species a wide circle of physiologically active substances without noticeable change of their activity. This approach which we developed and studied since 1975 at Moscow University gave us a chance to create a whole new range of macromonomers which were found to be capable of linear, three-dimensional and graft polymerisation. Products of these reactions are soluble physiologically active substances, hydrogels with immobilized physiologically active species, sorbents for biospecific chromatography, coatings which increase blood compatibility of polymeric prosthetic devices, etc.

Few years ago, mainly due to efforts of chemists studying ionic polymerisation processes, a new branch of monomer chemistry arose, namely, chemistry of macromonomers, i.e., organic species of rather high molecular mass (5000-10000) having the reactive group (double bond or cycle) at the end of the molecule. Most interesting results were obtained by E. Goethals in Belgium and J. Kennedy in USA (1,2). Polymerisation of such monomers results in graft copolymers and other types of block sequences in macromolecular chains. This new branch of "big" monomers is very attractive, in particular, by giving a chance to study the reactivity of the double bond (or cycle) which are under the influence of conformational rearrangements of the macromolecule itself.

First publications on the synthesis of physiologically active macromonomers in the middle of the 70-ies were related with immobilization of enzymes in polymeric hydrogels. The driving force for this research was the fact that, in the majority of methods, to carry out this process based on the interaction of physiologically active species (FAS) with a preactivated polymer matrix, there was no big chance to maintain the biological activity of immobilized FAS. The reason for that, as shown in Ref. 3, is the necessity to adapt FAS macromolecules to the structure of the polymer matrix which needed essential conformational changes of FAS. However, another approach could be developed when the polymer matrix is itself adopted to the macromolecules of FAS. This becomes possible if the formation of the matrix is carried out, for instance, simultaneously with the immobilization of FAS, i.e., the copolymerisation of an unsaturated derivative of FAS with corresponding monomers takes place:



How to make macromonomers? In order to introduce the double bond into the molecule of FAS, the most practical way is to use chloroanhydrides of acrylic or methacrylic acids, although acrolein (Ref. 4), acrylic esters (Ref. 5), maleic anhydride and 3,4-epoxybutene (Ref. 6) were also used.

The advantage of the use of chloroanhydrides is the easiness and efficiency of the acylation reaction which permits to minimize the alternative reaction - Michael reaction:

$$H_2C{=}CH{-}C\lesssim^{O}_{Cl} + HXR \begin{cases} \text{acylation} \longrightarrow H_2C{=}CH{-}C\lesssim^{O}_{XR} + HCl \\ \text{Michael reaction} \longrightarrow RXCH_2{-}CH_2{-}C\lesssim^{O}_{Cl} \end{cases}$$

It is known that, in principle, acyl chlorides can react with amino, hydroxy and sulfhydryl groups of FAS. That is why one of the first questions was to elucidate what groups are subjected to modification at first turn and how effectively this process proceeds as well as to know whether or not such activation influences the structure and biological properties of FAS. To answer this question is not so simple, because it is convenient to carry out acylation in water medium when most FAS are soluble and demonstrate their activity. At the same time, water reacts as a hydrolysing agent toward acyl chloride and, as a result, the real degree of acylation does not correspond to the initial ratio of raw materials (FAS/acyl chloride) (Refs 7-10).

A study of the acylation reaction of proteins (serum albumin, for example) with a model compound - ^{14}C-acetyl chloride - has shown that there is correction between the content of free amino groups and the number of acetyl groups (Fig. 1). It is shown that the number of acetyl groups introduced into serum albumin (SA) molecule is equal to that of "disappeared" free amino groups capable of a reaction with trinitrobenzenesulfonate.

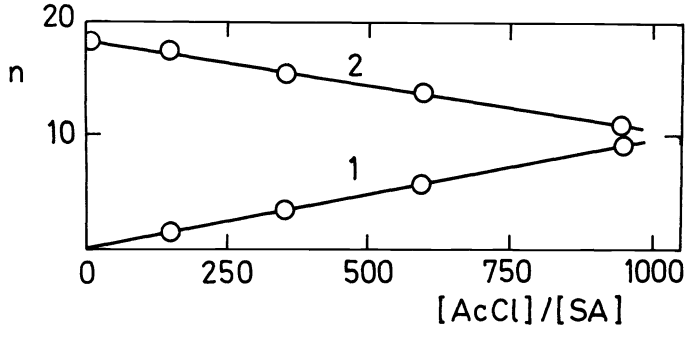


Fig. 1. The number of acetyl groups (1) and the number of the SA molecules after acylation of amino groups (2) versus molar ratio acryloyl chloride /SA in the initial mixture.

Using isoelectric electrofocussing technique, it was found that at the acyl chloride/protein ratio from 4:1 to 200:1 the isoelectric point of protein is shifted into the acidic region which shows a change in basicity of the SA molecule (Fig. 2). The isoelectric points of modified proteins depend on the initial ratio acylating agent/protein. However, at a ratio above 50:1 for acrylic acide chloroanhydride the isoelectric points are not changed any more. Moreover, at higher ratios partial denaturation of SA occurs (Ref. 11).

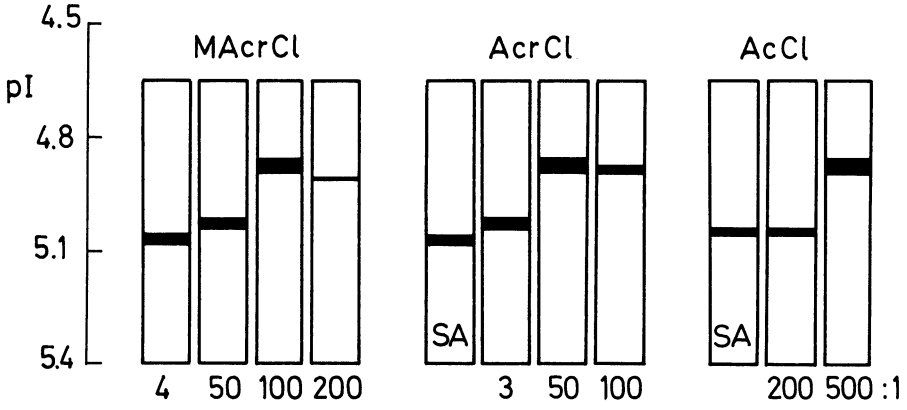


Fig. 2. Electrophoregrams of acylated SA at various acylating agent/protein ratios.

From the results obtained it followed that in the examined conditions the acylation reaction involving only free amino groups takes place (ε-amino groups of lysine and end amino groups). Partially, acyl chloride is subjected to the hydrolysis. Schematically, these reactions could be written as:

$$R\text{-}COCl + H_2N\text{-}Protein \xrightarrow{k_1} R\text{-}CONH\text{-}Protein + HCl$$

$$R\text{-}COCl + H_2O \xrightarrow{k_2} R\text{-}COOH + HCl$$

The ratio between these two rate constants has an approximate value k_1^{ef}/k_2^{ef} = 85 ± 15 which exceeds the corresponding value for the reaction of acyl chloride with α-BOC-L-lysine, which is a low-molecular analog of the lysine residue of the SA molecule, the said value being 33 ± 4.

Figure 3 represents the results of comparative studies of acylation of some compounds by acetyl chloride (AcCl).

The following may be stipulated when protein molecules are being modified. It is seen that, on the one hand, acryloyl chloride is much more efficient acylating agent than acetyl chloride (k_1^{ef}/k_2^{ef} ratio for SA-AcrCl system equals 600±100) and, on the other hand, the efficiency of acylation by AcrCl is increased with the increase in molecular mass of the protein molecule. The effect can be explained by the solubilisation of acylating agent by macromolecules of the protein: the higher the hydrophobicity of the acylating agent, the higher the acylation efficiency. More hydrophobic molecules of AcrCl penetrate into hydrophobic portions of the protein globule, thus being

shielded from interaction with water. As a result, the amount of AcrCl being hydrolyzed is substantially diminished, while the amount of AcrCl for the acylation reaction is increased. The rise in acylation efficiency for proteins of higher molecular mass may probably be attributed to a certain extent to the concentration effect, i.e., the measure of effective concentration of amino groups within a single macromolecule. The higher activity of acyl chlorides towards amino groups of the proteins ensures the synthesis of unsaturated FAS derivatives via acylation of protein in aqueous solutions, in spite of the occurring hydrolysis of the acylating agent and even despite enormous molar excess of water.

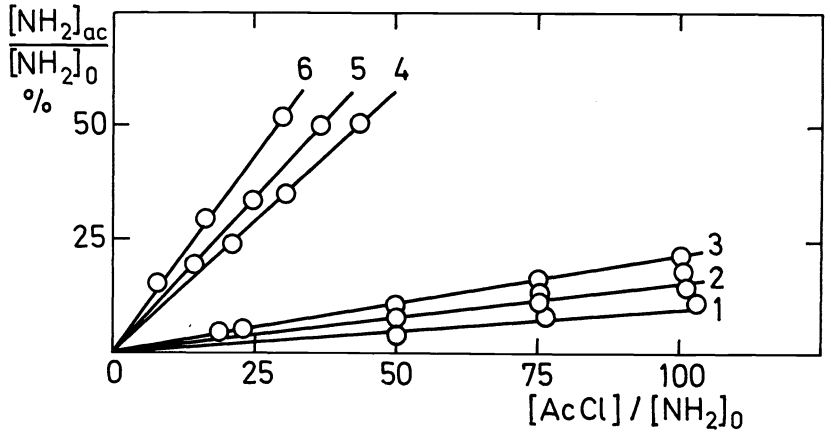


Fig. 3. The efficiency of acylation of α-BOC-L-lysine (1,2), SA (3), trypsin (4), SA (5) and fibrinogen (6) as a function of acyl chloride / number of NH_2-groups molar ratio. 1,3-acetyl chloride, 2,4,5,6-acryloyl chloride.

As regards the distinct point of attachment of the acyl radical to the protein molecule, it is determined by the accessibility and pK values of the amino groups entering the reaction. For instance, it seems likely that for SA the N-terminal amino group of asparagine is the first to become modified (pK = 9.82) and only then, by further increasing the amount of acyl chloride added, the remaining accessible amino groups of lysine (pK = 10.53) are modified. Indeed, even at ratios acyl chloride/protein as small as 2:1 there is no initial nonmodified SA left in the reaction mixture, while detection of the amino group in the N-terminal region of the protein coil according to Edman demonstrates that at low acylating agent/protein ratios (2:1 to 7:1) asparagine α-amino groups are modified primarily. The least practically feasible acyl chloride /SA ratio, that is 2:1 (which corresponds to the weight ratio of 1:1000) leads to the monoacylated, on the average, product.

The degree of acylation of FAS may be regulated by varying the acylating agent/FAS ratio, thus giving the means for the synthesis of "macromonomers" of distinct functionality.

One of the major aspects of this procedure is how acylation affects the physiological activity of the modified compounds. The possible conformational changes that might be the result of acylation in FAS molecules of protein nature were examined by the CD (circular dichroism) technique. The study re-

vealed that within a sufficiently broad range of the degrees of acylation the secondary structure of the albumin molecule remains actually unaltered (cf. Table 1).

It is also seen from the Table that one of the major properties of SA molecule in native conformation, that is the ability to interact strongly with organic dye molecule - bilirubin, also is fully retained.

A study of the catalytic activity of proteolytic enzymes and of the inhibitory activity of protease protein inhibitors, both modified by acylation procedure, permitted to establish the lack of noticeable effect of acylation even on such labile and "sensitive" macromolecules as enzymes (Table 2).

The fact that the activity of trypsin and fibrinolysin which are the serine proteases is retained during acylation indicates that acylation does not intrude in the catalytically active hydroxy functions of the serine residues but involves the amino groups of the protein.

TABLE 1. The relationship between helix content and binding constant or organic dye-bilirubin versus the number of acyl groups in the SA molecule.

The number of acyl groups in the SA-molecule	Acylating agent	The degree of helicity	$Kx10^8$, M^{-1}
0	-	0.55-0.65	0.3-0.5
2	AcCl,AcrCl	0.55±0.05	0.35±0.17
5	AcCl	0.55±0.05	-
7	AcCl	0.58±0.05	-
13	AcrCl	0.53±0.05	0.32±0.15

When modifying FAS that contain lysine residues at the active site, acylation must reduce the activity as is the case, for instance, in the acylation of pancreatic trypsin inhibitor (PTI), whose reactive site involves the lysine residue occupying the 15th position of the polypeptide chain (Table 3).

TABLE 2. The activities of native and acylated (with AcrCl) proteins.

Protein	Activity	Native	Acylated
Trypsin	Esterase, μeq of substrate/mg min	41±2	45±2
Fibrinolysin	Esterase, μeq of substrate/mg min	34±2	31±2
Trypsin	Proteolytic, μmol	2.4±0.1	4.9±0.1
Soybean inhibitor of trypsin	Inhibitory, IU/mg[x]	1.16±0.04	1.11±0.04

[x] IU - inhibitory unit - the amount of inhibitor blocking the activity of 1 mg of trypsin.

Anti-tryptic activity of native PTI is 4.00±0.03 IU/mg. The molecular mass of PTI 6500 and trypsin 25000 having been taken into account, it implies that one PTI molecule "neutralizes" one trypsin molecule, i.e. it contains one reactive site. The reactive site may be protected, however, by acylating at pH 8.0 the trypsin-inhibitor complex and by isolating the modified inhibitor from the acylated trypsin after destruction of the complex at pH .0 (Ref. 12). Anti-tryptic activity of thus isolated PTI is the same as that of the native inhibitor, though 2 of its 7 amino groups are modified under these conditions (Table 3).

The effect of acylation on the pH optimum of FAS of protein nature appears essential. The fact that the pH optimum of such compounds is not shifted on acylation may be indirectly deduced from the finding that their activity is permanent at a certain pH value (optimal); still, the significance of the problem calls for direct proof of the conclusion. Figure 4 presents as an example the results of studies of the pH dependence of anti-tryptic activity for soybean trypsin inhibitor of Kunitz, both native and acylated.

TABLE 3. Anti-tryptic activity of PTI and the amount of free amino groups in a PTI molecule as a function of the PTI/AcrCl molar ratio.

Molar ratio PTI/AcrCl	The number of free NH_2-groups in PTI molecule	Anti-tryptic activity	
		IU/mg	% from initial activity
0	7.0±0.3	4.00±0.03	100
1:5	5.5±0.2	3.10±0.02	79
1:50	3.6±0.2	2.00±0.04	50
1:50[x]	5.0±0.2	4.00±0.03	100

The pH dependences of antitryptic activity are, as is seen, of the same mode and shape with maxima at the same pH 8.0 for both the native and acylated inhibitor, i.e. the pH optimum is indeed not shifted on acylation of the inhibitor.

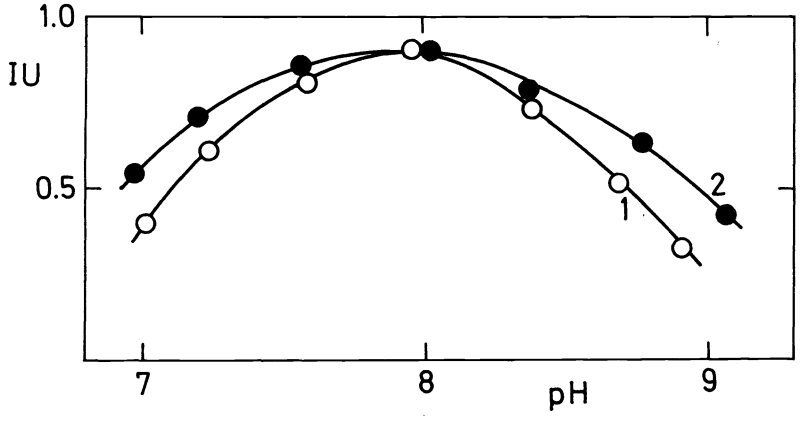


Fig. 4. Antitryptic activity of soybean trypsin inhibitor versus pH; 1 - native inhibitor, 2 - the inhibitor in which 4 or 5 NH_2-groups are substituted for the acrylic acid residue.

What is also worth attention is that the pH dependence of acylated inhibitor is definitely less pronounced, together with the increase in stability towards thermoinactivation on acylation for many proteins. It indicates that acylation is accompanied by additional stabilisation of the native conformation of biomolecule due to hydrophobic interactions which are enhanced when amino groups are modified with a more hydrophobic residues of unsaturated acids.

The macromonomers described are related to the class of reactive derivatives of acrylamide and may thus be anticipated to exhibit high reactivity of their double bonds in radical polymerisation processes. The huge substituent at the double bond, as is the macromolecule of FAS, may, however, inhibit the reaction. By investigating the homo- and copolymerisation of "acryloyl-trypsin" we have demonstrated that the said compound may enter the radical polymerisation reaction (Ref. 8). With sufficient ease macromonomers may just as well copolymerize with hydrophilic monomers, e.g., acrylamide, N-vinylpyrrolidone, hydroxyethyl methacrylate, etc. (Fig. 5).

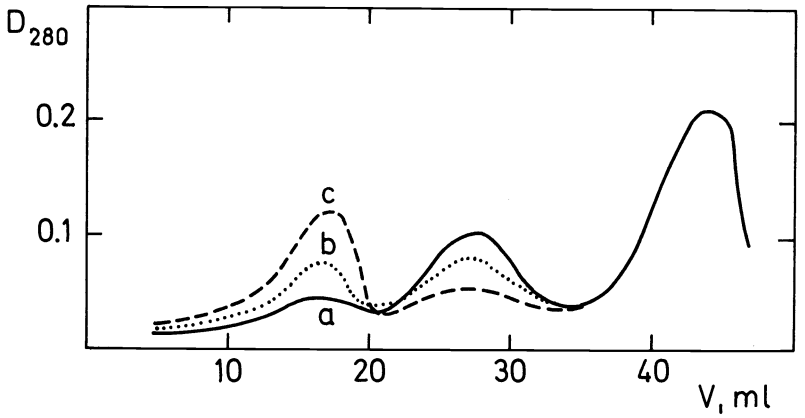


Fig. 5. Gel-chromatograms of copolymers of acylated trypsin with acrylamide. Eluent - tris-acetate buffer (pH=8.5). Polymerisation time, 10 (a), 15 (b) and 30 (c) minutes.

The Figure demonstrates the accumulation in the reaction mixture of the copolymer (peak II; peak I is acryloyl-trypsin) and the decrease of acrylamide (peak III) with time. The concentration of monomeric acryloyl-trypsin decreases proportionally to the increase in concentration of the copolymer.

A more detailed study has been recently performed by V.A. Postnikov and N.V. Dobrovol'skaya for an acylated SA-acrylamide system. Acryl-SA was obtained at the 2:1 AcrCl/SA ratio. Gel chromatographic analysis of the polymerisation mixture (initially composed of acyl-SA and acrylamide) demonstrates the presence of two peaks - one corresponds to the soluble product of copolymerization, the other - to the "unreacted" SA. Affinity chromatography of the first peak product on phenylsepharose confirmed that the copolymer contains SA. Its molecular mass was evaluated by ultracentrifugation to a value near 10^6, while the SA/AA ratio was found to be 1:3 by weight.

Thus, the copolymer chains contain four SA molecules on the average interconnected with long polyacrylamide chain fragments of the molecular mass 200000.

At larger acyl chloride /SA ratios (i.e. more than 2:1) acyl-SA was produced that leads, when brought to copolymerize with acrylamide, to the formation of insoluble crosslinked products.

We may now resume that acylation of protein with chlorides of unsaturated acids produces macromonomers which, while fully retaining the biological activity of initial unmodified FAS, are capable of radical polymerization.

The distinctive feature of the macromonomers synthesized, when compared to the low-molecular compounds, is that not all of the double bonds of a single macromonomer molecule are involed in the polymerisation. Some of the double bonds "attached" to the FAS molecule are unaccessible to the radicals of the growing chain, probably due to steric retardations. Figure 6 illustrates the fact that the amount of immobilized protein increases initially in proportion to the "amount" of double bonds on the protein molecule to reach a constant value at 1.2 - 1.4 double bonds per protein molecule. It implies that only one of the 1.2 - 1.4 double bonds is involved in copolymerization. The greater number of double bonds per protein molecule results only in an increase in the number of points of attachment of the protein molecule to the polymeric matrix, which is indicated by an increase in thermostability of the immobilized protein.

The actually indefinite conversion of the macromonomer double bonds as well as the great difference in the molecular masses of the macromonomer and the low-molecular monomers (by 2-3 orders of magnitude) do not allow the determination of true value of the copolymerization constants which could have been an estimate of the true activity of macromonomers. We may only state that r_2 (copolymerization constant of the macromonomer) is close to zero, practically only dimers and trimers can usually be obtained in the homopolymerization process.

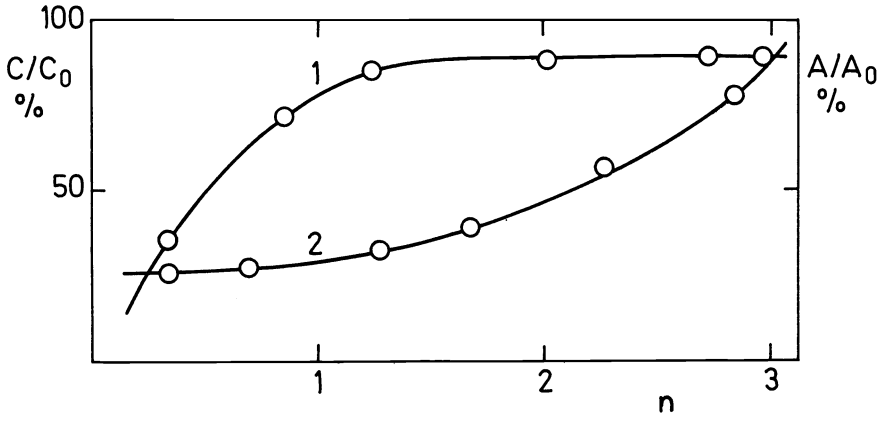


Fig. 6. The degree of incorporation of ovomucoid from the whites of duck eggs (C/C_0) (curve 1) into the polyacrylamide gel and the antichymotryptic activity (A/A_0) remaining after heating for 30 minutes at 100 °C (curve 2) versus the number of double bonds on the ovomucoid molecule.

The physiological activity of homo- and copolymerization products depends both on the nature of FAS and on the nature of substrate. For instance, the catalytic activity of enzymes, i.e. trypsin or fibrinolysin towards low-molecular substrate (α-N-benzoyl-L-arginine ethyl ester), is not actually changed as they are converted to polymeric derivatives (42±2 and 34±2 meq /mg min for native enzymes vs. 44±3 and 32±2 for their polymeric derivatives). The activity of the enzymes towards high-molecular substrates is decreased as the molecular mass of the polymeric form increases. The activity of trypsin itself and of acyl-trypsin (elution volume is 26 ml) towards casein, for example, is 2.38 and 4.92 μmol/mg min, respectively, while the activity of poly-acyltrypsin amounts to 2.42 (elution volume is 20 ml) and 1.18 (elution volume is 16 ml) μmol/mg min. The reason for this effect is the decrease in accessibility of the enzyme active sites to molecules of the high-molecular substrate in the case of polymeric enzyme derivatives - the effect examined already in the literature.

The results presented here gave hope that the described reactions might be of use for chemical modification of polymeric materials with FAS; this called for such conditions of the process that would ensure sufficient accessibility of the active sites of immobilized FAS, and thus permitting to excape the decrease in activity observed in homo- and copolymerization.

The formation of polymeric matrix taking place simultaneously with covalent immobilization of FAS, when the polymeric microenvironment is adjusted to the FAS macromolecule, results in a structure of the gel that is quite beneficial to displaying the functions of FAS.

The resultant hydrogel was noticeably different from gels synthesized in the absence of macromonomers. When compared to the latter, gels containing immobilized FAS are characterized by a narrower macropore size distribution, the cell structure is better developed, and the dividing walls are thinner. For instance, the polyacrylamide gel at a given certain degree of concentration is characterized by the swelling of 1.4 g of water per g of dry polymer and the mean pore size (diameters) 4.6 μm at 2.9 μm mean square deviation, while analogous gels containing 0.03 and 0.3 mass-% of immobilized SA swell to imbibe 4.0 and 11.0 g water per g of dry gel and have the mean pore size of 8.5 ± 1.5 μm and 5.8 ± 1.0 μm, respectively.

What are the effects in regard to the physiological activity of immobilized FAS which accompany the described structural changes? By coupling the results of investigations with FAS of various chemical nature, two major effects may be followed (Table 4, Fig. 7).

The first effect is that immobilization by itself, i.e. combination of the protein molecule with the polymer, is not accompanied by noticeable changes in the activity of the immobilized molecule (the binding constants for immobilized FAS, when its concentration in the polymeric matrix is low, do not actually differ from the respective constants for native FAS) (Table 4).

TABLE 4. The relationship between the relative binding constant of organic dye-bilirubin versus the concentration of immobilized SA.

The concentration of immobilized SA, mg/g of the gel	4.0	4.7	8.0	17.9	21.9	36.5	55.5
$K=K_{imm}/K_{sol}$ +10 %	1.0	1.0	1.0	1.0	0.8	0.6	0.5

K_{imm} - binding constant of bilirubin with immobilized SA,
K_{sol} - binding constant of bilirubin with SA in solution.

The second effect is that the specific physiological activity measured falls down as the concentration of immobilized protein increases. The reason for the first effect is quite apparent: no noticeable change of the conformational state of the protein macromolecule occurs during the formation of the gel, while the gel itself is permeable to the substrate. The decrease in activity of the immobilized protein observed at its higher contents in the polymeric matrix is related to the fact that some parts of the immobilized molecule, more strictly its active sites, become inaccessible to the substrate molecules when they (the immobilized macromolecules) are densely packed at the macropore surface.

What are the distinctive features of the described immobilization procedure? These are, primarily, the possibility to vary the number of conjugation points of the macromolecule to the matrix and the chemical nature of the polymeric microenvironment of the macromolecule. This, in turn, allows to enhance the stability of immobilized FAS towards thermal inactivation and permits to regulate the effective pH optimum of immobilized FAS, which is necessary when the FAS modified polymer is to act in a medium the pH of which is different from the pH optimum of the FAS. By introducing ionogenic monomers to the initial monomer mixture, the polymeric matrix will assume electric charge which affects the local concentration of hydrogen ions in direct vicinity of the immobilized protein, thus providing means to adjust it to the optimal value dependent on the pH of the medium (Fig. 8) (Ref. 13).

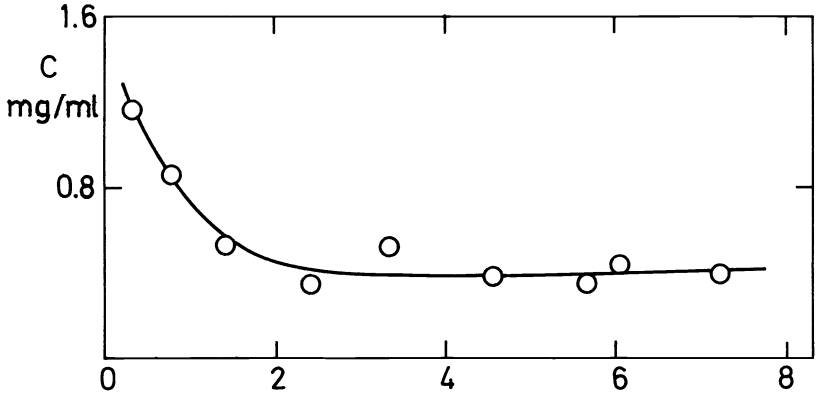


Fig. 7. The capacity of soybean inhibitor containing adsorbents as a function of the content of immobilized inhibitor.

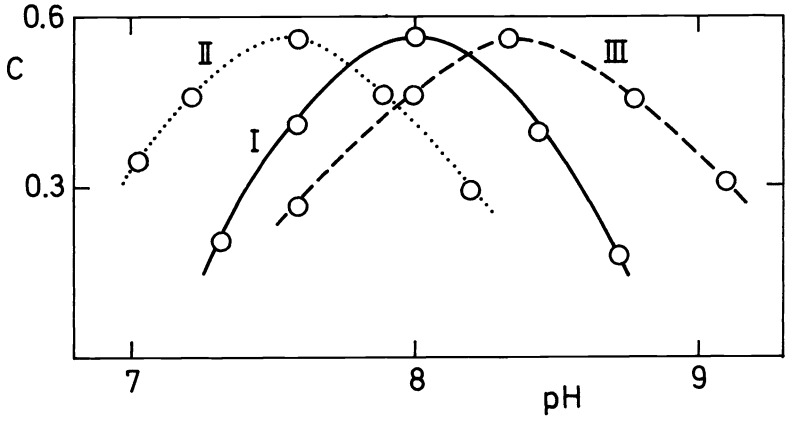


Fig. 8. The capacity of soybean inhibitor containing polyacrylamide gel versus pH: I - polyacrylamide gel; II - copolymer of acrylamide (50 %) and dimethylaminoethyl methacrylate (50 %), quaternized with ethyl bromide; III - copolymer of acrylamide (50 %) with acrylic acid (50 %).

The interaction of soybean inhibitor, immobilized in the polymeric acrylamide gel, with trypsin is, as seen in the Figure, at its highest at pH 8.0. The addition of quaternized with ethyl bromide dimethylaminoethyl methacrylate

$$H_2C=C(CH_3)-CO-O-(CH_2)_2-\overset{\oplus}{N}(CH_3)_2(C_2H_5)Br^{\ominus}$$

to the initial monomer mixture allows us to vary the positive charge of the polymeric matrix, thus shifting the effective pH optimum to acidic pH. For instance, the addition of 50 % by weight (from the concentration of acrylamide) of a positively charged monomer to the initial acrylamide-crosslinking agent mixture shifts the pH optimum of immobilized inhibitor to pH = 7.4, which is a physiological one. The amount of trypsin associated is higher than that obtained from the non-charged gel at pH 8.0 (Fig. 8, curve II). Such hydrogel demonstrates maximal sorption capacity, for example, when trypsin is removed from blood.

If the negatively charged monomer, e.g. acrylic acid, is introduced, the effective pH optimum is shifted to the basic pH (Fig. 8, curve III).

The increase in thermostability of the immobilized protein is easily attained by the greater number of conjugation sites of the protein macromolecule to the polymeric matrix, i.e. by increasing the number of double bonds attached to the macromolecule (Fig. 6 and Fig. 9). Figure 9 presents the results of studies on the thermostability of trypsin immobilized via copolymerization of acyltrypsin with acrylamide and a crosslinking agent (Ref. 14). As is seen, the rate constant for a reversible thermoinactivation of the enzyme drastically falls down to a certain limiting value, as the number of bonds between the enzyme and the polymer increases, which is due to a significant retardation of conformational changes leading to irreversible thermoinactivation.

Relying on the described features of the copolymerization procedure for physiological active macromonomers, we may now give the basic criteria for the

positive effect to be attained if the macromonomers are used for chemical surface modification of polymeric materials. The FAS immobilized at the polymer surface must have a hydrophilic polymer environment and long spacer, and it must be accessible to the substrate. Consequently, the chemical modification of polymeric materials must involve graft copolymerization of a mixture of a low-molecular monomer, to form a surface layer of a hydrophilic gel, and of a macromonomer. The macromonomer is in this case attached to chains of the grafted hydrophilic polymer, but not directly to the hydrophobic surface of the polymer. This provides for the remoteness of FAS from the polymeric surface via a long spacer and thus provides for the increase in its activity (Table 5) (Ref. 5).

TABLE 5. Proteolytic activity of the modified polypropylene as a function of the composition of initial monomer mixture.Irradiation dose is 0.3 Mrad, substrate is casein.

Weight ratio acrylamid/acylenzyme in initial mixture	Activity, μg tyrosine/min cm^2	
	Fibrinolysin	Protease from Act. Sphaeroides
0	0.05±0.01	0.07±0.01
200	0.47±0.03	0.96±0.03
400	2.00±0.10	2.78±0.12
800	2.16±0.11	2.70±0.12

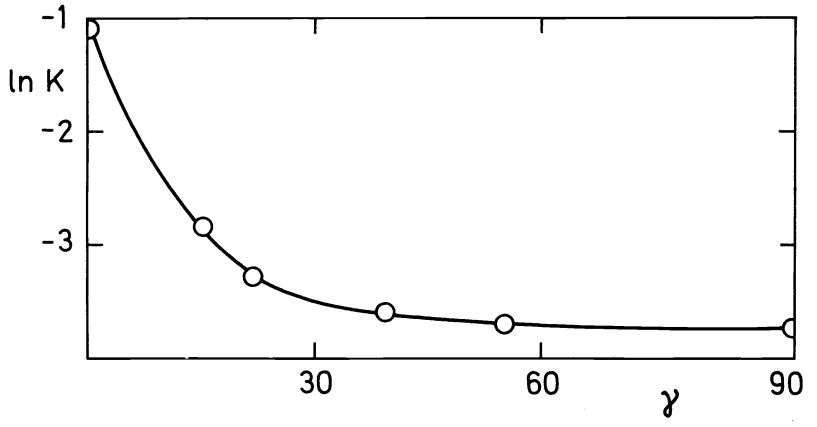


Fig. 9. The rate constants for irreversible thermoinactivation of immobilized trypsin as a function of the acylation degree of trypsin (γ).

The approach just outlined is, quite naturally, also applicable to the immobilization in polymeric hydrogels of low-molecular compounds. We have already described synthetic adsorbents of albumin, containing hydrocarbon fragments of 12-22 carbon atoms covalently conjugated with the polymeric matrix (Refs 15-16). The adsorbents were synthesized by copolymerization of the unsaturated derivatives containing hydrocarbon fragments with a hydrophilic monomer and a crosslinking agent (Fig. 10). The paraffinic fragments display

strong affinity for the second class of binding sites of the SA molecule. Figure 11 shows the disc-electrophoregrams of plasma treated with the described adsorbent; the treatment results in the removal of SA from plasma. It should be noted that the affinity of SA to the 16 carbon atom ligand in solution and in the immobilized state is not very different (Fig. 12).

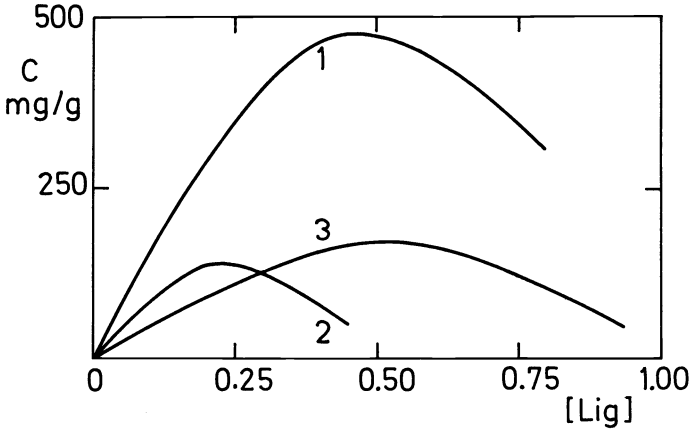
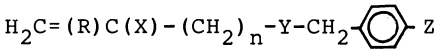


Fig. 10. The adsorbent capacity versus ligand content: I - the ligand of 16 carbon atoms, II - of 22 carbon atoms, III - of 12 carbon atoms.

The efficiency of biospecific adsorbance of SA is dependent on the distribution of the ligand within the chains of the hydrophilic polymer, which within the framework of the described approach may be regulated by varying the composition of the monomer mixture. Figure 13 illustrates the fact that the maximal adsorbent capacity is observed when the ligand units are situated in an isolated manner along the polymer chain. When the probability to find diads and triads of ligands is increased, the adsorption capacity goes down.

Biospecific adsorbents derived from unsaturated structural analogs of concurrent inhibitors of serine proteases (Ref. 17) of the general structure



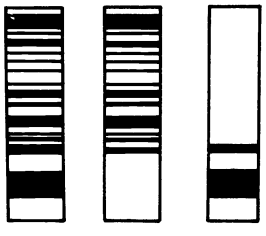


Fig. 11. Electrophoregrams of human blood plasma (1), of plasma treated with the adsorbent (2) and of protein adsorbed by gel (3).

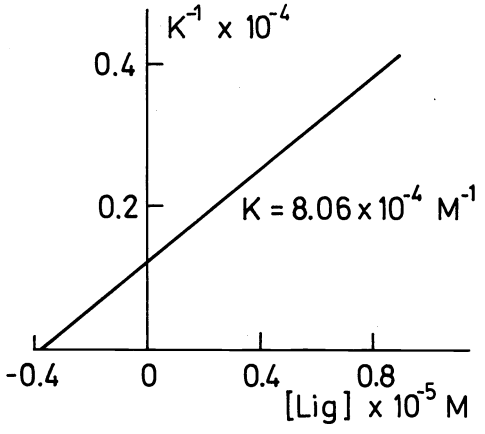


Fig. 12. The graphic determination of the binding constant of albumin with immobilized ligands.

(where R = -H, $-CH_3$, n = 0-11, Z = -Cl, H; Y = -C-C-, -CONH-, -COO-) are yet another illustrations of such approach. By using in the new monomers those benzylamine and benzamidine fragments which act as thrombin inhibitors, immobilization via flexible spacers of different length (Fig. 13) was achieved.

Figure 14 illustrates some of the properties of the adsorbents synthesized in this study. The adsorbent capacity as a function of ligand concentration passes through a maximum which as in the previous case is the result of association of hydrophobic ligands on swelling of the adsorbents in aqueous solutions (Fig. 14). The activity of thus purified thrombin is 2500 NIH units.

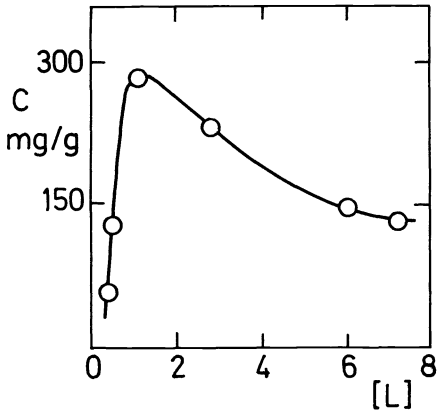


Fig. 13. The adsorption capacity and the length of ligand associates versus ligand/monomer molar ratio.

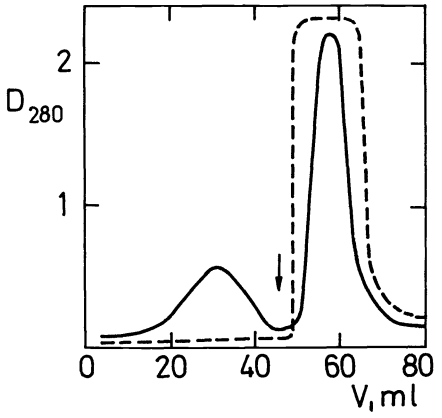
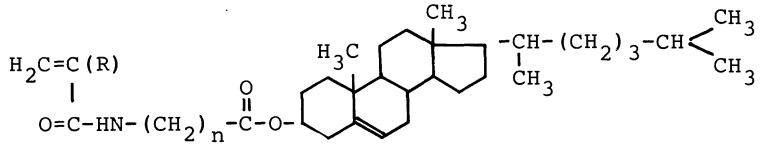


Fig. 14. Gel-chromatograms of thrombin samples.

The last example is related to the biospecific adsorbents of heparin, a natural blood anticoagulant. The well-known interaction of cholesterol with immobilized heparin was brought to our minds to result in the creation of reverse system.

The adsorbents were synthesized by copolymerization of acrylamide and a cross-linking agent with the unsaturated derivative of cholesterol of the general formula



where R = -H, $-CH_3$, n = 2-11 (Ref. 18).

Some of the properties of the synthesized hydrogels are compiled in Table 6. The gels are capable of adsorbing heparin from the plasma solution. The maximal amount of adsorbed heparin is increased with the increase in mobility and accessibility of the cholesterol grouping for heparin, i.e. when the length of the methylene spacer is increased. The distinct values are 0.7, 0.9 and 1.3 mg/mg of imm. chol. for cholesterol esters of N-methacryloyl-β-alanin (n=2), N-methacryloyl-ω-aminoenantic acid (n=6) and N-methacryloyl-ω-aminolauric acid (n=11), respectively.

TABLE 6. The properties of cholesterol-containing hydrogels.

Ligand and its concentration in the gel, mg/g of gel	The amount of adsorbed heparin, mg/g of gel ±20 %	Blood clotting time [x] on contact with gel ±10 %			
		I	II	III	IV
Cholesterol ester of N-methacryloyl-β-alanin n=2 9 ± 2	2.0	14	9	13	16
	6.0 [xx]	27	-	30	28
Cholesterol ester of N-methacryloyl-ω-amino-enantic acid, n=6 13 ± 1	0.5	11	-	20	18
	4.0	25	-	25	27
	12.0	30	8	30	30
Cholesterol ester of N-methacryloyl-ω-amino-lauric acid, n=11 11 ± 2	1.0	11	-	10	11
	4.0	27	9	30	25
	14.0 [xx]	30	8	30	30

I - the gel prepared with a 1 % of heparin in plasma solution; II - the gel with adsorbed heparin, subsequently washed with NaCl solution; III - the washed gel treated with heparin; IV - the gel subjected to washing and heparin adsorption procedures 5 times.

The adsorbed heparin fully retains its anticoagulant activity. The blood clotting time is proportional to the amount of adsorbed heparin and exceeds reasonably the clotting time of blood after the contact with heparin-free gel. The data presented demonstrate that repeated readsorption after the gel has been washed from the adsorbent is possible.

The principally analogous results were obtained with polyethylene films, modified by simultaneous graft-copolymerization of acrylamide and unsaturated cholesterol derivative. The activity of such "surface bonded" cholesterol was almost at the same level as of cholesterol immobilized in a polyacrylamide gel: the adsorption capacity of graft-copolymers of cholesterol esters of N--methacryloyl-β-alanin, containing 0.3 mg of cholesterol/cm^2, was 0.26 mg heparin/cm^2 or approximately 0.9 mg heparin/mg cholesterol. The blood clotting time at the modified polyethylene surface has increased from 6.0 to 30 minutes after the adsorption of heparin and remained actually the same after six cycles: heparin absorption, washing, repeated adsorption.

Thus, we have demonstrated a procedure for the formation of polymeric materials capable of repeated heparin adsorption while retaining its anticoagulant activity.

SOME ASPECTS OF BIOMEDICAL APPLICATION OF MODIFIED POLYMERS

The synthetic biospecific adsorbents seem to become a powerful tool in preparative biochemistry as means for the isolation of highly purified substances. The prospects for the isolation of highly purified serum albumin and thrombin by making use of low-molecular ligand have been discussed above. Here is an example of how high-molecular ligands can be utilized in adsorbents based on

proteases and their protein inhibitors (Table 7).

TABLE 7. Properties of biospecific adsorbents

Ligand and its concentration, mg/ml adsorbent	The adsorbed substance and its activity	The amount of adsorbed substance and the activity of the eluted substance
Trypsin, 5-10	Inhibitor of trypsin (soybean), 0.2 IU/mg	1.25-1.90 mg, 0.9±0.1 IU/mg
Trypsin, 5-10	Pancreatic inhibitor of trypsin, 0.8 mg IU/mg	0.53-0.65 mg, 4.00±0.03 IU/mg
Trypsin, 5-10	Ovomucoid from chicken egg, 0.3 IU/mg	1.5-2.0 mg, 1.0±0.1 IU/mg
Ovomucoid from whites of duck eggs, 0.2-7.1	Trypsin, 10 μeq/mg min	0.2-5.7 mg, 41±1 μeq/mg min
Pancreatic inhibitor of trypsin, 0.05-0.06	Trypsin, 10 μeq/mg min	0.2-0.3 mg, 39±2 μeq/mg min

The data from this Table demonstrate that isolated proteins exhibit a high biological activity that is actually the same as that of pure substances.

As regards medical application, surface modification of polymeric materials with FAS enhancing the hemocompatibility of these materials gave interesting results.

One of the FAS used for the purpose is heparin which is the anticoagulant of direct action. Heparin, as shown above, having been adsorbed on cholesterol-containing polymers retards the blood clotting process, i.e. enhances the hemocompatibility of the polymer. We have recently observed (Refs 19, 20) that heparin covalently bonded at the surface of synthetic polymer by some another chemical procedure improves hemocompactibility of the polymer as well. In this case the effect does not consider the leaking of heparin into the environment (Table 8).

It is seen from Table 8 that heparinization of polymers, though leading to an increase in platelet adhesion, results in an enhancement of the hemocompatibility of the polymers.

The changes occurring in blood after contact with heparin-containing polymers (HCP) may be detected by thromboelastography. The typical ones are shown in Fig. 15. It is seen that when blood contacts HCP, a noticeable hypocoagulation effect is observed together with essential changes of the parameters of the clotting system. This is,primarily, related to the endurance of the clotting invisible phase (R-) and of the time of onset of the fibrin clot formation (K) to the decrease in the fibrinogen concentration, the number and functional activity of platelets and of the fibrin-stabilizing factor (M_a), etc. The mode of the observed changes of the parameters of blood clotting system is confirmed by examination of blood coagulograms (Table 9).

TABLE 8. Hemocompatibility of some heparin-containing polymers

Polymer	Concentration of imm. heparin, mg/cm^2	RBCT[x]	RIPA[xx]
Polyurethane	0	1.7±0.2	1.0±0.2
Polyurethane	1.7	12.0±1.0	1.0±0.2
Polyurethane	2.9	12	7.0±1.5
Polyurethane	1.1	12.0±1.0	3.0±0.6
Polyurethane	1.4	12	4.0±0.8
Biomer^R	0	1.7±0.2	0.5±0.1
Biomer^R	1.5	10.0±1.0	6.0±1.2
Polyethylene	0	1.2±0.1	6.0±1.2
Polyethylene	0.3	1.8±0.2	3.9±0.5
Polyethylene	1.5	10.0±1.0	6.0±1.2

[x] - RBCT - relative blood clotting time - ratio of blood clotting time at the surface of a polymer to that at a standard surface (glass). [xx] RIPA - relative index of platelet adhesion - ratio of the number of platelets adhered to these surface.

TABLE 9. Results of coagulogram examination of blood

Volumetric ratio HCP/blood	Duration of contact, s	Plasma tolerance for heparin, s		Recalcification time, s		Activity of fibrin-stabilizing factor %		Concentration of fibrinogen, mg %	
		I	II	I	II	I	II	I	II
1:4	30	135	353	117	170	80		425	300
1:2	30	40	125	2	160	--		440	250
1:1	30	60	495	60	150	57		365	210
1:1	60	73	1800	87	290	41		510	250

I - before contact of HCP with blood, II - after contact.

Having examined the adsorption by HCP of SA, fibrinogen, fibrinolysin and thrombin, we have found that the adsorption of fibrinogen and thrombin is the highest. These are the very proteins whose absorption was detected by thromboelastography and coagulation studies of blood. The binding constants of these proteins with immobilized heparin are $(1.4\pm0.5)\times10^5 M^{-1}$ and $(8\pm2)\times10^5 M^{-1}$ for fibrinogen and thrombin, respectively. The interaction of fibrinolysin with imm. heparin is of much lower extent while SA does not actually interact with it at all. As imm. heparin interacts with the same proteins as heparin in solution, it was quite natural to suggest that physiological properties of the resultant complexes are the same. The first of these properties is the ability of the complexes to dissolve the unstabilized fibrin clot. It was found that complexes of imm. heparin with fibrinogen, thrombin and, to a lesser extent, with fibrinolysin demonstrate the lytic action towards unstabilized fibrin (Fig. 16). This fact allows to understand why the

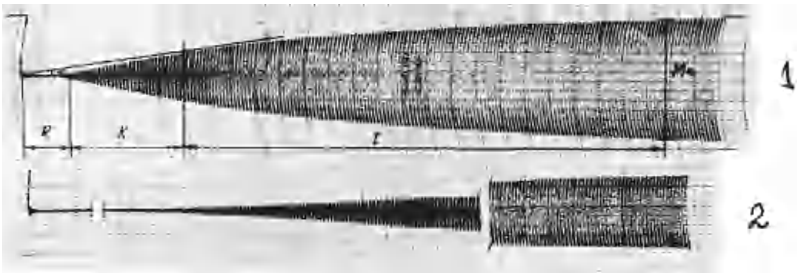


Fig. 15. Thromboelastograms of canine blood before (1) and after (2) contact with heparin containing polymer.

enrichment of the HCP surface with the most thrombogenic proteins, which are thrombin and fibrinogen, does not result in thrombin formation. The explanation is that these proteins having been associated with heparin are not only removed from the reaction of thrombin formation but also acquire ability to dissolve the fibrin clots.

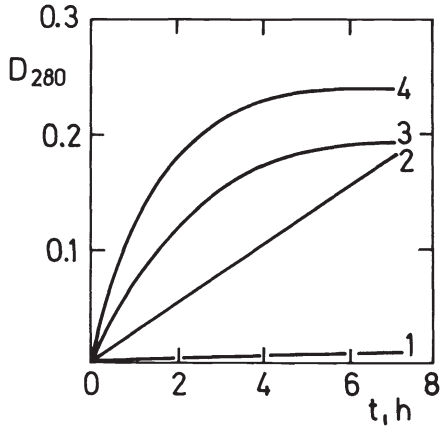


Fig. 16. The kinetics of unstabilized fibrin hydrolysis with complexes of imm. heparin with fibrinogen (1), thrombin (2), fibrinolysin (3) and albumin (4).

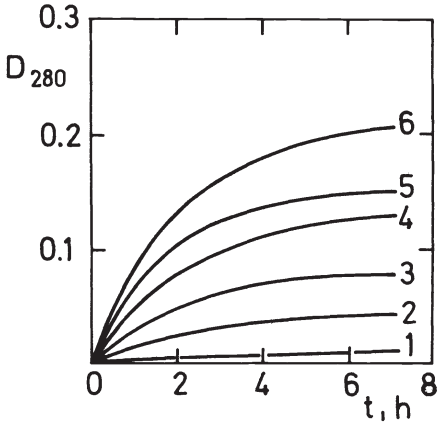


Fig. 17. The kinetics of stabilized fibrin lysis by gels containing (mg/g) 2.35 heparin (1), 0.46 trypsin (2), 1.43 heparin+0.29 trypsin (3), 3.15 heparin+0.46 trypsin (4), 1.29 trypsin (5) and 1.29 trypsin+1.5 heparin (6)

Altogether, such stages as platelet adhesion and dissolution of nonstabilized fibrin clot are still out of the scope of action of imm. heparin. As the increased adhesion of platelets at the HCP surface is due to the higher surface concentration of fibrinogen, which is reactive towards some enzymes of the platelet membrane, the cp-immobilization of heparin with the fibrinogen hydrolysing enzyme (protease) may be expected to reduce the platelet adhesion.

Tables 10 and 11 compile the results of studies of hemocompatibility of polymers modified with heparin and trypsin. It is seen that co-immobilization of heparin and trypsin on the polyethylene surface leads to the increase in blood clotting time and tends to decrease the platelet adhesion, while the changes of the blood clotting system parameters are of the same character as that accompanying the interaction of blood with HCP but essentially exceeding the latter. Consequently, the described binary systems, being brought into contact with blood plasma, act as active anticoagulants, and moreover display fibrinolytic action (Fig. 17).

TABLE 10. Hemocompatibility of polyethylene modified with binary systems of heparin and trypsin

Imm. heparin concentration, mg/g of polyethylene	Imm. trypsin concentration, mg/g of polyethylene	RBCT ±10%	RIPA ±15 %
0	0	1.2	1.8
0.3	0	1.8	3.9
1.5	0	10.0	6.0
0	0.14	1.2	1.3
0.31	0.16	2.1	2.3
0.31	0.26	3.2	1.6

TABLE 11. The results of investigation by coagulograms

The concentration of FAS in polymer, mg/g	Plasma tolerance for heparin, s		Recalcification time, s		Activity of fibrin-stabilizing factor, % from initial	Concentration of fibrinogen, mg %	
	I	II	I	II		I	II
Heparin 1.9	73	80	44	69	56	250	200
Heparin 3.2+ Trypsin 0.5	73	93	44	91	11	250	110
Heparin 1.5+ Trypsin 1.6	73	x	44	x	x	250	40

x - the clot does not form

Thus, the synthesis of hydrogel polymeric systems with immobilized physiological active substanc es, in particular via the described pathways involving macromonomers, is one of the possible approaches towards preparation of modified materials for biological and medical applications.

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