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Use of the affinity chromatography principle in creating new thromboresistant materials

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Abstract

The principle of affinity chromatography was used for preparation of thromboresistant bilayer coatings. The outer biospecific layer containing ε-aminocaproic acid residues (from 2.2 up to 5.5 nmol/cm^2) was synthesized using a copolymer of maleic anhydride with *N*-vinylpyrrolidone and L-lysine dihydrochloride or *N*-ε-*tert*-BOC-L-lysine. This surface can selectively adsorb plasminogen (fibrinolytic zymogen) from blood. The biospecific layer (from 2.0 up to 3.6 μg/cm^2) was applied for covering chitosan (native or modified) or albumin interlayer. Such bilayer coatings (BCs) were stable and represented the insoluble polyelectrolyte complexes. BCs were proposed for bilayer modification of synthetic vascular grafts, polyethylene, and other materials contacting with blood. This technique allowed us to significantly reduce thrombogenic properties of polyethylene surfaces.

Keywords: Affinity chromatography principle; Bilayer coatings; Thromboresistant materials; Lysine; Plasminogen

1. Introduction

One of the main problems in creation of synthetic prostheses for implantation and other medical materials contacting with blood is to prevent thrombogenesis on their surfaces. Therefore, creation of materials possessing thromboresistant (antithrombogenic) properties (i.e., preventing blood coagulation on their surfaces) is topical.

The available antithrombogenic materials can be divided into the following groups—(a) materials releasing anticoagulant substances; (b) materials containing immobilized heparin or other natural anticoagulant bound covalently, or by chemosorbtion; (c) synthetic heparin-like polymer compounds [1]. Finally, polymeric materials with thromboresistant coatings which are able to resorption of anticoagulant substances in the process of exploitation were proposed. This method involves the use of biospecific chromatography. In particular, polymeric cholesterol-containing coatings capable of binding blood heparin without changing its activity were obtained by chemical modification of polyethylene [2]. These materials can bind up to 0.26 mg/cm^2 of heparin. However, this approach is rather labor-consuming because it involves preparation of monomer (cholesteryl ether of *N*-methacryloyl-β-alanine) followed by chemical modification of polyethylene by a mixure of the above-mentioned monomer and acrylamide.

Recently, the synthesis and application of a thromboresistant material based on polyurethane with a covalently photoimmobilizated polyacrylamide bearing lysine residues attached by α-amino group have been reported [3,4]. As a result, the lysine residues with free ε-amino groups were present on the surface. These residues represent ε-aminocaproic acid (ε-ACA), a specific ligand toward plasminogen (nonactive zymogen of fibrinolysis). A material based on dextran containing covalently bound dipeptide L-cysteinyl-L-lysine, so that the residues with ε-amino groups of L-lysine are accessible, was also reported [5].

Abbreviations: BCs, bilayer coatings; ε-ACA, ε-aminocaproic acid; HDPE, high density polyethylene; AP, affinity polymer; AP-A, affinity polymer synthesized by method A; AP-B, affinity polymer synthesized by method B; ε-IL, ε-isomer lysine (α-aminocoupled lysine); ACH, amphyphilic chitosan; VPMA, copolymer of maleic anhydride with *N*-vinylpyrrolidone; IA, binary mixture isopropyl alcohol:ammonia liquor; IAW, ternary mixture isopropyl alcohol:acetic acid:water; QTLC, quantitative thin-layer chromatography; FMOC, fluorenylmethoxycarbonyl; *t*-BOC, *tert*-butoxycarbonyl; DMF, *N*,*N*-dimethylformamide; TFA, trifluoroacetic acid; h, hour; ω (1/h), rate of desorption; τ (h), time of desorption

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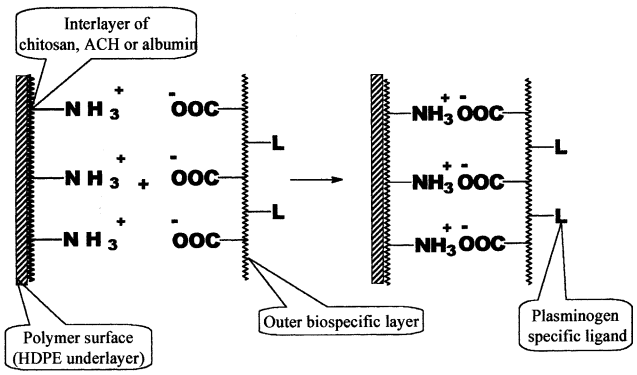

Fig. 1. Scheme of formation of bilayer coating on the polymer surface.

Such coatings should autoselectively bind the endogenic blood plasminogen, which, in turn can be converted into the active form, plasmin, by the endogenous plasminogen activators (tissue-type and urokinase-type). Plasmin can promote lysis of fibrinous clots.

In this work, we used the principle of affinity chromatography for obtaining thromboresistant coatings supported by synthetic vascular grafts and other blood contacting materials. To reach this end we prepared, without using organic solvents and coupling agents, non-covalent bilayer coatings (BCs) containing natural and synthetic polymers, which were applied successively to high density polyethylene (HDPE) films and tubes.

As an outer, "working," layer we used an affinity polymer (AP), which represents a non-toxic copolymer of maleic acid with *N*-vinylpyrrolidone bearing a plasminogen-specific ligand (ε-ACA residue). The biospecific layer was applied to the interlayer of chitosan, amphyphilic chitosan (ACH), or albumin deposited on the support (Fig. 1). For better adhesion of this interlayer to HDPE, we preliminarily hydrophilized HDPE or introduced hydrophobic groups into the chitosan structure (ACH). Some experiments were carried out involving both preliminary steps.

The cross-linked granulated form of this affinity polymer was successfully used for plasminogen isolation from blood plasma.

2. Experimental

2.1. Chemicals

L-Lysine dihydrochloride, FMOC chloride, *N*-ε-*t*-BOC-L-lysine, ninhydrin, ε-aminocaproic acid, Sephadex G-10 for gel filtration permeation chromatography and cellulose dialysis tubing were purchased from "Sigma–Aldrich" (St. Louis, MO, USA). Chitosan was purchased from "Bioprogress," Moscow, Russia) with a degree of deacetylation of 76% and a molecular mass of 450,000. Copolymer of maleic anhydride with *N*-vinylpyrrolidone (VPMA) was synthesized following the known procedure [6]. HDPE films and tubes were provided by "Plastik" (Moscow, Russia). The blood plasma was supplied by A.N. Bakulev Research Center of Cardiovascular Surgery Russian Academy of Medical Sciences (Moscow, Russia). Blood plasma Cohn fraction III was prepared by the Cohn technique [7]. Other chemicals were purchased from "Reakhim" (Moscow, Russia) and were of analytical reagent grade. Amphyphilic chitosan was kindly provided by Dr. V. E. Tikhonov (A.N. Nesmeyanov Institute of Organoelement Compounds).

2.2. Preparation of biospecific layer

2.2.1. Coupling of unprotected lysine to VPMA (method A, see Fig. 2)

VPMA (0.3 g, 1.4 mmol) was dissolved in DMF (0.6 ml). L-Lysine dihydrochloride (0.3 g, 1.4 mmol) was dissolved in 1 M $NaHCO_3$ (2 ml) and added to the VPMA solution and the reaction mixture was stirred for 1 h at room temperature to give AP-A (see Fig. 2), which was purified using gel permeation (Sephadex G-10). The purified product was concentrated and dried to constant mass in vacuo (20–30 mm Hg) over P_2O_5. The yield of AP-A was 0.29 g.

2.2.2. Coupling of N-ε-t-BOC-lysine to VPMA (method B, see Fig. 3)

Synthesis of sample I: VPMA (0.3 g, 1.4 mmol) was dissolved in a mixture of DMF (1 ml) and pyridine (0.2 ml). *N*-ε-*t*-BOC-L-Lysine (0.05 g, 0.2 mmol; VPMA:*N*-ε-*t*-BOC-L-lysine = 1:0.1 mol/mol) was added to the VPMA solution pH 7), and the reaction mixture was stirred for 1 h at 50 °C. Then distilled water (1 ml) was added and the reaction mixture was stirred for an additional 1 h at 50 °C. For the synthesis of samples II (VPMA:*N*-ε-*t*-BOC-L-lysine = 1:0.1 mol/mol; pH 9), III (VPMA:*N*-ε-*t*-BOC-L-lysine = 1:0.1 mol/mol; pH 10), IV (VPMA: *N*-ε-*t*-BOC-L-lysine = 1:0.5 mol/mol; pH 9), and V (VPMA:*N*-ε-*t*-BOC-L-lysine = 1:1 mol/mol; pH 9) the reaction mixture was adjusted to the required pH with 2 M NaOH. The synthesized products were precipitated with acetone, washed with methanol and acetone, dried, dissolved in distilled water, and the low-molecular-mass components were removed by dialysis through cellulose dialysis tubing first against 0.2 M NaCl and finally against distilled water. The dialysates were concentrated and dried. After drying, the products were *N*-ε-*t*-BOC-deprotected by TFA (2 ml) for 1 h and then concentrated. The final products (affinity polymer AP-B) were dried to constant mass in vacuo (20–30 mmHg) over P_2O_5. The yield of AP-B (sample I) was 0.26 g.

2.3. Lysine content determination

The lysine content in AP hydrolysates was determined by quantitative thin-layer chromatography (QTLC) using "Whatman" and "Merck" plates (Germany), a Hitachi-557 spectrophotometer (Japan) equipped with a scanning

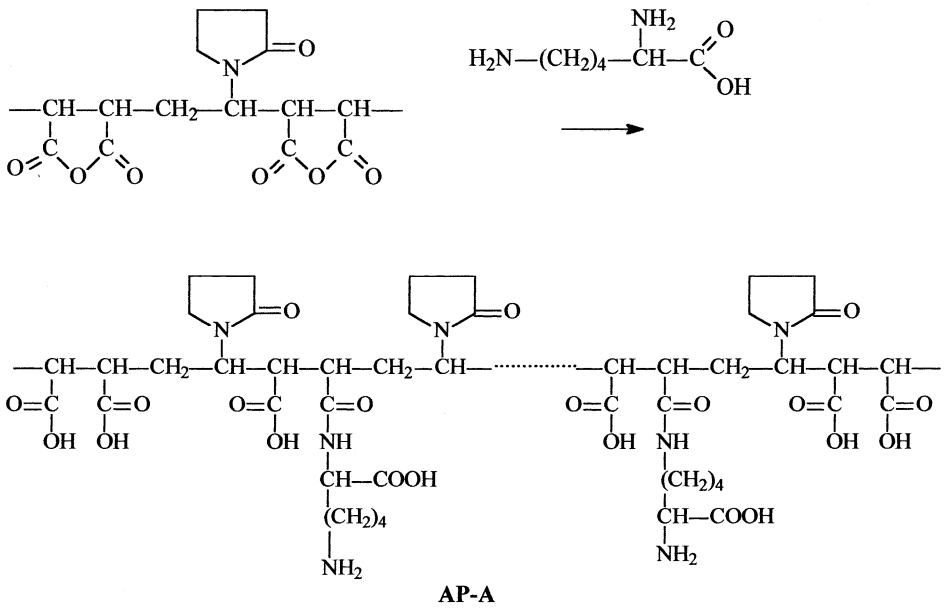

Fig. 2. Scheme of synthesis of affinity polymer AP-A with unprotected lysine (method A).

accessory, or a "Sorbfil" densitometer ("Mashinostroitel," Russia). The eluents used were a binary mixture isopropyl alcohol:ammonia liquor (25%) = 7:3 (v/v) (IA) and a ternary mixture isopropyl alcohol:acetic acid:water = 4:l:l (v/v/v) (IAW).

The AP-A samples were modified with FMOC chloride, the reaction product was hydrolyzed (see below), and then differential analysis of the content of the α- and ε-aminobound lysine residues (α/ε-isomers) was carried out using appropriate amino acid references.

The total lysine content in the AP-A and AP-B hydrolysates was determined after ninhydrin reaction (0.2% ninhydrin solution in acetone). All the AP samples were hydrolyzed with 6 M HCl in sealed ampules filled with N_2 at 155 °C for 1 h [8].

2.3.1. Modification by FMOC chloride

AP-A (0.1 g) was dissolved in 1 ml 1 M $NaHCO_3$ at 0 °C. FMOC chloride (0.05 g, 0.2 mmol) was dissolved in 2 ml acetone and was added to AP-A solution and the reaction mixture was stirred at 0 °C for 1 h. Then the product was washed first with chloroform and finally with distilled water. Low-molecular-mass components were removed by dialysis against distilled water. Then the dialysate was concentrated and finally dried to constant mass in vacuo (20–30 mmHg) over P_2O_5. The yield of polymer was 0.09 g.

2.4. Surface modification of HDPE films and tubes by bilayer coatings (see Fig. 1)

HDPE films and tubes were first modified by—(I) chitosan; (II) amphyphilic chitosan (ACH); and (III) albumin [9] (were prepared at the A.N. Bakulev Research Center of Cardiovascular Surgery Russian Academy of Medical Sciences in the Laboratory of Chemistry and Technology of Materials for Cardio-Vascular Surgery). For variant I, the modification was carried out after preliminary treatment of HDPE by oxidants [10]; for variant II, the chitosan was modified by introducing hydrophobic (dodecylsuccinic) groups (from 5 up to 15 mol%).

For I and II variants the samples of HDPE were placed into the chitosan/CH_3COOH (0.2%) or ACH/CH_3COOH (0.2%) solutions for 30 min. Then the outer biospecific layer was applied after washing and drying films and tubes by inserting into the 0.1 M AP (with various pH) solution at room temperature for a certain period of time and then dried. The modified films and tubes were washed by distilled water and dried.

The kinetics of desorption of polymer layers was studied by batch method in bidistilled water (see Fig. 4) at 200 nm with use of Specord UV-Vis (Carl Zeiss Jena, Germany).

2.5. Extraction and affinity chromatography of plasminogen on cross-linked affinity AP-A sorbent (see Fig. 5)

Plasminogen was isolated from blood plasma Cohn fraction III by use of the cross-linked affinity AP-A sorbent (cross-link degree: 10 mol% of diaminodiphenyloxide).

Paste of blood plasma Cohn fraction III (75 g) was suspended in 75 ml of 0.01 M phosphate buffer (pH 7.4), containing 0.14 M NaCl and stirred for 20 h at room temperature. After centrifugation (10000 g, 45 min, 4 °C) the supernatant was collected and diluted (1:1 (v/v)) with 0.05 M phosphate buffer (pH 7.4). The obtained solution was applied onto a column (9.5 cm × 2.0 cm) packed with affinity AP-A sorbent (3 g) equilibrated with 0.05 M phosphate buffer (pH 7.4). Non-adsorbed proteins were washed out with 0.05 M phosphate buffer (pH 7.4) and non-specific bound proteins

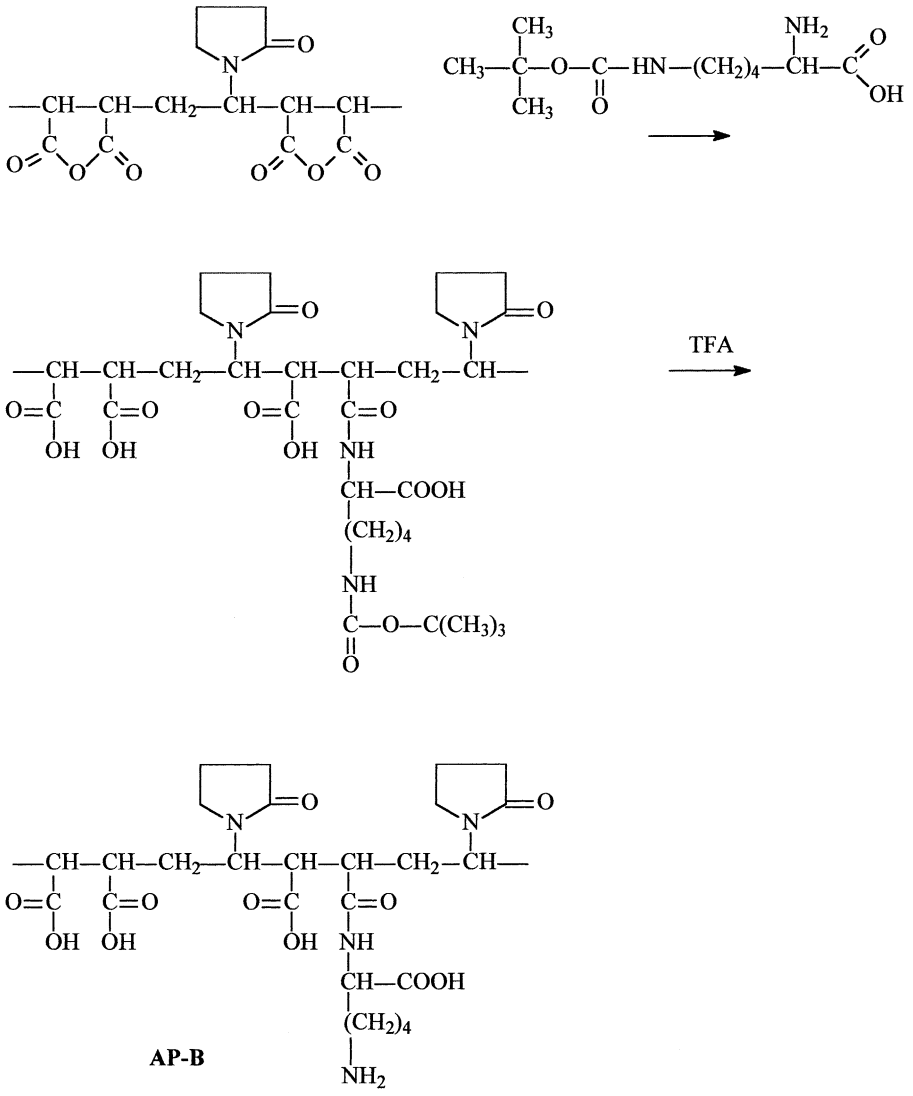

Fig. 3. Scheme of synthesis of affinity polymer AP-B with *N*-ε-*t*-BOC-lysine (method B).

were eluted with 0.5 M NaCl in 0.05 M phosphate buffer (pH 7.4) until the absorbance at 280 nm reached the baseline. Plasminogen was eluted with 0.2 M ε-aminocaproic acid solution in 0.05 M phosphate buffer (pH 7.4). Then, plasminogen was separated from ε-aminocaproic acid by precipitation with ammonium sulphate (0.4 g/ml) at 4 °C and centrifuged (10000 × *g* for 30 min). The precipitate was dissolved in 0.1 M sodium acetate buffer (pH 5.0), dialyzed against the same buffer and against distilled water, lyophilized. The yield of homogeneous plasminogen was 0.039 g.

3. Results

The AP-A (see Fig. 2) was prepared in water solutions without using of toxic reagents in optimum conditions. Thus lysine is bound preferentially (but not exclusively) by its α-aminogroups with maleic anhydride residues of VPMA and thus ε-ACA residues on polymer surface were exposed. As the result, it was prepared the AP-A bearing the ε-ACA (the affinity ligand toward plasminogen) and α-ACA (the non-specific ligand) residues.

For the obtaining of AP bearing only required ε-ACA plasminogen specific residues we elaborated the second method of synthesis (B) (see Fig. 3) with *N*-ε-*t*-BOC-L-lysine. The synthesis was carried out in two stages—at the first stage, we have obtained copolymer *N*-vinylpyrrolidon-[*N*-1-(carboxy)-5-(*tert*-butoxycarbonylamino)-pentyl]-monoamide of maleic acid, then; at the second stage, removed the *N*-ε-*t*-BOC-protection by acid hydrolysis and finally have obtained the copolymer *N*-vinylpyrrolidon-[*N*-1-(carboxy)-5-(carbonylamino)-pentyl]-monoamide of maleic acid (AP-B). The AP-B contained only ε-ACA residues.

The content of α/ε-aminocoupled lysine and covalent (total) lysine in AP were detected by QTLC after acid hydrolysis. This method has allowed us to combine

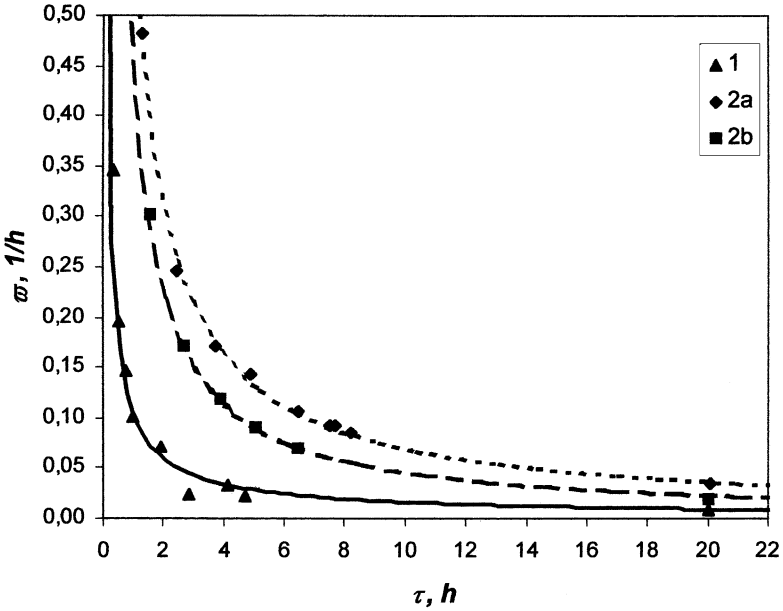

Fig. 4. The kinetics of chitosan desorption from HDPE (1), and affinity polymer (AP) desorption from BC at pH 2.5 (2a), and pH 7.5 (2b), of polymer solution applied on interlayer.

in one experiment the separation of high-molecular and low-molecular mass components of the obtained products, and also to estimate quantitatively their composition. The use of the method A the content of α-aminocoupled lysine (ε-isomer lysine (ε-IL), $R_f = 0.69$ in IA solutions system) in polymer AP-A was found to be equal to 1.0 ± 0.2 mmol/g of AP (21.1 ± 4.9 mol%) and it exceeded the content of non-active ε-aminocoupled lysine (α-isomer lysine (α-IL), $R_f = 0.51$ in IA solutions system) 5.1 ± 1.6-fold.

As shown in Table 1 (for method B), the content of specific ligand ε-IL ($R_f = 0.51$ in IAW solutions system) in AP-B (I–V) was varied from 0.21 up to 0.53 mmol/g (from 4.6 up to 11.6 mol%) and depended on synthesis conditions. The largest degree of substitution (0.53 ± 0.06 mmol/g; 11.6 ± 1.3 mol%) was achieved at pH 9 and equemolar ratio of initial reagents. It is note worthy, that the amount of lysine in samples of AP-B was smaller than in AP-A. It can be explained by steric impediments when *N*-ε-*t*-BOC-protection group of lysine was used for AP-B preparation. Besides, it has appeared that variant B was much more expensive and more labor-consuming, although it has allowed us to obtain the required ligand – ε-IL without any admixture of the side product, the non-active isomer of lysine, α-IL. Therefore, we have used the AP-A for the subsequent studies.

It is necessary to note, that biospecific properties of AP-A were investigated for its cross-linked granulated form.

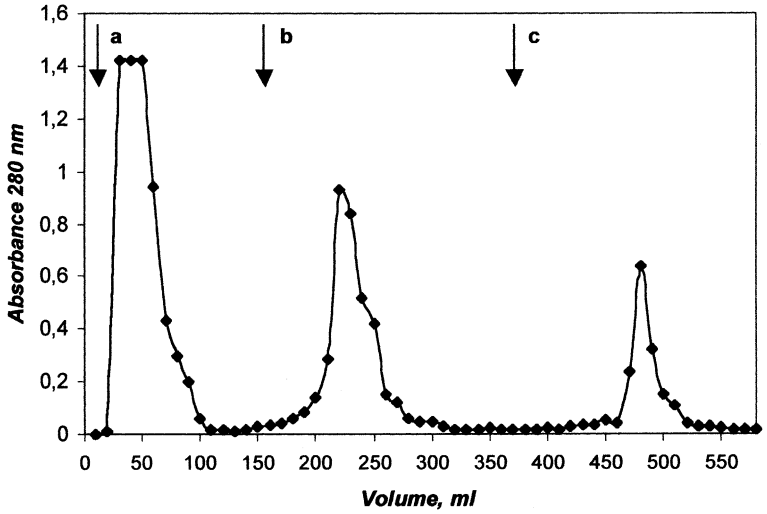

Fig. 5. Affinity chromatography of plasminogen on cross-linked affinity AP-A sorbent (a) 0.05 M phosphate buffer (PH 7.4), (b) 0.5 M NaCl in 0.05 M phosphate buffer (pH 7.4), (c) 0.2 M ε-aminocaproic acid in 0.05 M phosphate buffer (pH 7.4)).

Table 1
The content of specific ligand in affinity polymer synthesized by method B

Variant of synthesis	pH of reaction mixture	VPMA:*N*-ε-*t*-BOC-L-lysine (in the reaction mixture) (mol/mol)	Content of ε-IL (specific ligand) (mmol/g) (mol%)
I	7	1:0.1	0.21 ± 0.01 (4.6 ± 0.3)
II	9	1:0.1	0.31 ± 0.01 (6.9 ± 0.1)
III	10	1:0.1	0.32 ± 0.16 (7.3 ± 3.5)
IV	9	1:0.5	0.33 ± 0.26 (7.3 ± 5.7)
V	9	1:1	0.53 ± 0.06 (11.6 ± 1.3)

Table 2
Content of interlayer and outerlayer on the surface of polyethylene devices

Sample	Interlayer		Outerlayer	
	Interlayer type	Adsorbed interlayer on surface (μg/cm^2)	Adsorbed outerlayer bearing plasminogen specific ligand (μg/cm^2)	Specific ligand (ε-ACA) density (nmol/cm^2)
I	Chitosan	4.1 ± 0.5	3.6 ± 0.8	5.5 ± 1.2
II	ACH[a]	4.9 ± 0.5	3.0 ± 0.8	4.1 ± 1.1
III	Albumin	1.0 ± 0.5[b]	2.0 ± 0.5	2.2 ± 0.6

[a] 5 mol% of hydrophobic groups.
[b] Data of A.N. Bakulev Research Center of Cardiovascular Surgery Russian Academy of Medical Sciences, the Laboratory of Chemistry and Technology of Materials for Cardio-Vascular Surgery.

Such affinity sorbent contained covalently coupled lysine residues from 0.6 up to 1.2 mmol/g (thus ε-IL from 0.3 up to 0.7 mmol/g). When this sorbent was used, the homogeneous plasminogen was isolated from blood plasma (see Section 2, item 2.5.) with yield 13 mg/g of sorbent (specific activity of obtained plasminogen was 14 casein units/mg).

The prepared AP-A was used as an outer biospecific layer in BC, which were proposed for modification polymer materials contacting with blood (for example for polyethylene). To improve the adhesion of hydrophilic polymer coatings with hydrophobic polymer underlayer, we have undertaken preliminary hydrophilization of HDPE films and tubes (variant I, see Section 2) (or), vice versa, introduced hydrophobic groups into chitosan structure (ACH-variant II, see Section 2). HDPE was also modificated by albumin (variant III, see Section 2). The AP layer was applied on an interlayer of chitosan (native or ACH) or albumin, which were deposited on the ground material, reduced the negative charges of maleic acid residues of AP, and exposed the specific ligand on the surface (see Fig. 1). Earlier, we have shown that the polymers used as BC formed the unsoluble polyelectrolyte complexes that were stable in wide range of pH values and ionic strengths [11].

The investigation of polymer desorption from a surface of modified HDPE films and tubes at neutral pH water solutions (the representative curves of desorption see Fig. 4), has shown that at batch conditions after 5–6 h of desorption only strongly attached chitosan (Fig. 4(1)) or complex of chitosan with AP (Fig. 4(2a and 2b)) remained on the surface.

The data of the quantitative analyses of polymer content in interlayer and biospecific layers are presented (Table 2). The obtained materials contained 4.1 ± 0.5 μg/cm^2 of chitosan and 4.9 ± 0.5 μg/cm^2 of ACH. The amount of chitosan (or ACH) on the surface may be varied by alteration of chitosan concentration in starting solution. The larger chitosan concentration leaded to more thick layers.

The amount of AP (Table 2) applied on underlayer modified by chitosan/ACH or albumin depended on pH of AP solution and type of interlayer. For example, for a material with chitosan interlayer the amount of outer biospecific layer after desorption was 1.3 ± 0.2 μg/cm^2 at pH 2.5 of AP solution applied on interlayer and 3.6 ± 0.8 μg/cm^2 at pH 7.5 of AP solution, thus the amount of a specific ligand on a surface consisted from 2.2 ± 0.6 up to 5.5 ± 1.2 nmol/cm^2. The better results were obtained with BC when chitosan interlayer was used.

The content of the ligand in BC approximately corresponded to content of the specific ligand in modified polyurethane [4] −0.8 to 6.4 nmol/cm^2 and in this range a specific binding of plasminogen was possible.

The biological investigations of degree of BC thrombogenicity were carried out at the A.N. Bakulev Research Center of Cardiovascular Surgery Russian Academy of Medical Sciences in the Laboratory of Chemistry and Technology of Materials for Cardio-Vascular Surgery. It was shown that the amount of thrombotic mass adsorbed on the BC materials was 2- to 3-folds less than on the unmodified materials [12].

4. Conclusions

In the present study, the new affinity bilayer coatings for synthetic vascular grafts and other materials contacting blood are proposed. Such BC are based on the stable unsol-

uble polyelectrolyte complexes of non-toxic biocompatible polymers. The outerlayer of BC contained a ligand specific to fibrinolysis zymogen (plasminogen). The preliminary study of thromboresistance properties of the proposed BC confirmed a legitimacy of this approach for obtaining of antythrombogenic materials. Further, in case of endurance of the obtained biological effect, it is possible to cover the synthetic vascular grafts and other materials coming in contact with blood by BC, which will allow us to reduce significantly their thrombogenic properties.

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References

[1] M. Jozefowicz, J. Zorefonvicz, Pure Appl. Chem. 56 (10) (1984) 1335.
[2] N.A. Plate, L.I. Valuev, Zhurnal Vsesoyuznogo khimicheskogo obshestva imeni Mendeleeva, Mendeleev J. All-Union Chem. Soc. 30 (4) (1985) 402.
[3] W.G. McClung, D.L. Clapper, S.-P. Hu, J.L. Brash, J. Biomed. Mater. Res. 49 (2000) 409.
[4] W.G. McClung, D.L. Clapper, S.-P. Hu, J.L. Brash, Biomaterials 22 (2001) 1919.
[5] P.H. Warkentin, K. Johansen, J.L. Brash, I. Lundstrom, J. Colloid Interface Sci. 199 (1998) 131.
[6] A. Conix, G. Smets, J. Polym. Sci. 15 (79) (1995) 221.
[7] E.J. Cohn, L.E. Strong, W.L. Hughes, D.J. Mulford, J.N. Ashworth, M. Melin, H.L. Taylor, J. Am. Chem. Soc. 68 (1946) 459.
[8] G.L. Lookhart, B.L. Jones, D.B. Cooper, S.B. Hall, J. Biochem. Biophys. Methods 7 (1982) 15.
[9] H. Mobus, S.P. Novikova, N.B. Dobrova, in: Proceeding of the 26th Microsymposium on macromolecules—Polymers in Medicine and Biology, Prague, Czechoslovakia, 1984, p. P59–1.
[10] M. Shalkauskas, A. Vashkyalys, Khimicheskaya metallizatsiya plastmass (Chemical Metallization of Plastic Materials), Khimiya, Leningrad, 1985.
[11] N.A. Samoilova, M.A. Krayukhina, I.A. Yamskov, Russ. J. Phys. Chem. 76 (9) (2002) 1502.
[12] N.A. Samoilova, M.A. Krayukhina, S.P.Novikova, N.V.Vasiliev, I.A.Yamskov, in: Proceedings of the First International Congress on Biotechnology—State of the Art and Prospects of Development, Moscow, Russia, 2002, p. 76.