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New polyurethane compositions able to bond high amounts of both albumin and heparin

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In order to prepare polymers provided with better haemocompatibility with respect both to the coagulative cascade and to platelet aggregation and activation, we have synthesized new polyurethanes containing in the chain extender [di-(2-hydroxyethyl) hexa-decylamine (DHHA)] both a long chain alkyl group (able to bond albumin) and a tertiary ammonium group able, after suitable quaternization reaction, to bind ionically significant amounts of heparin. The amounts of heparin and albumin bonded to the polymer films were determined spectrophotometrically. A biological *in vitro* evaluation of the heparinized and albuminized films was also carried out, with respect to the blood coagulation factors (by APTT measurements) and to platelet adhesion. For the sake of comparison, polyurethanes containing as chain extenders two different monomers (containing, respectively, the long alkyl and the quaternary ammonium group), and polymer blends consisting of these copolymers, were also prepared and evaluated as above. It was seen that the type of the adsorption sequence for albumin and heparin, respectively, onto films of the various homopolymers, copolymers and blends, plays an important role in their biological properties.

1. Introduction

Segmented polyurethanes are widely used as biomaterials, due to their good physical and mechanical properties, joined with fairly good biocompatibility and antithrombogenicity characteristics. Among the numerous methodologies aimed at further improving their haemocompatibility, surface heparinization has been particularly investigated [1–4], for instance binding ionically heparin to polyurethanes containing, both in the main or in the side-chain, quaternary ammonium groups [5–7]. Moreover, since it had been observed that albumin-coated surfaces exhibited remarkable inhibition of the platelet adhesion and aggregation phenomena usually occurring when foreign material is contacted with blood, albumin was also (covalently) grafted to polymer surfaces. However, this covalently bonded albumin, after an *in vivo* permanence of a few months (or sometimes some weeks) undergoes deactivation (by denaturation, proteolysis, etc.). Since it had been observed that, *in vivo*, molecules containing linear long alkyl chains were able to bond albumin preferentially, polymers containing alkyl chains of 16 or 18 carbon atoms have been synthesized, in order to make possible, in the case of desorption or proteolysis of the adsorbed albumin, a continuous "*de novo*" adsorption of endogenous albumin from the blood onto the polymer films [8–10]. These surfaces, able to adsorb preferentially albumin, exhibited in *in vivo* tests, improved haemocompatibility.

In order to prepare polymer surfaces able to interfere both with the activation mechanisms of thrombin and with those responsible for the platelet aggregation, heparin was covalently bonded to albumin; polymer surfaces of different type, coated with these albumin–heparin conjugates, have shown, in *in vitro* tests, both decrease of platelet aggregation and prolongation of coagulation times [11, 12]. However, the employment of this technique is limited due to the probable denaturation of albumin by the reagents used for its coupling with heparin, and to the small resistance of immobilized albumin to proteolytic degradation under real *in vivo* conditions. In order to obviate these difficulties, we have synthesized polyurethanes able to bond both heparin (by ionic bonds, to quaternary ammonium groups), and albumin (adsorbed to long chain alkyl groups existing in the polymer molecule), being convinced that the advantages of a synergic type (not only due to the simple additive effect of the antithrombotic activity of heparin and the anti-platelet action exerted by the albuminization) could be obtained. In fact, according to several authors [13–15] polymer heparinization brings about increased platelet adhesion; this cannot be explained by electrostatic attractions among heparin molecules and platelet membranes (both negatively charged), but can be explained considering the fact that highly thrombogenic proteins, like fibrinogen, are preferentially adsorbed by heparinized polymer surface [16, 17]. Besides polyurethanes containing the functional groups responsible for the binding of albumin and heparin on the same atom, and since consequently this might bring about steric hindrance in the bonding of the two macromolecules, we

0957–4530

have also investigated polymers containing, in the repeating unit, spaced binding sites for the two molecules, in order to reduce possible hindrances.

2. Materials and methods

2.1. Polymer synthesis

Syntheses of DHHA and of polymers have previously been reported [18]. Polymerizations were carried out by a two-step procedure: in the first step a precursor was synthesized by reacting methylene-bis-phenyl isocyanate (MDI, Polyscience, Inc.) and poly(propyleneoxide) (PPO, Fluka) having a molecular weight of 1200 dalton, in a molar ratio 2:1, in a solvent consisting of a 1:1 mixture of anhydrous dimethylsulfoxide (DMSO, Fluka) and freshly distilled 4-methyl-2-pentanone (PENT, Fluka) under previously described reaction conditions [19]. The second reaction step, catalysed by dibutyl-tin-diacetate, was carried out for 24 h at room temperature, after addition of the DHHA (alone or in the mixture with butanediol at different ratios), in equimolar ratio with PPO. The different compositions of the obtained polymers are summarized in Table I.

The copolymers have been synthesized employing as chain extender: 1,4 butanediol (BD, Fluka), N, N-diethylaminoethyl-N-2,2-hydroxymethyl propionamide (DED) (20) and N-hexadecyl amide of 2,2-hydromethyl propionic acid (HDA) [21], at different ratios, as in Table II.

As far as the polymer blends are concerned, they have been obtained employing polymers previously synthesized and in particular PEUDED [20], PEUHDA [21] and PEU [19]. The compositions obtained are reported in Table III.

2.2. Characterization tests

The interaction of albumin and heparin with specific dyestuffs has been utilized for determining their concentration, through absorbance measurements, employing a double beam HITACHI-U-2000 spectrophotometer operating in the wavelength range 190–1100 nm.

The nuclear magnetic resonance spectra (^1H and ^{13}C NMR) have been recorded in solution in $CDCl_3$ (Merck) employing, for ^{13}C measurements, a BRUKER AC-200 instrument operating at 50.42 MHz, and for ^1H a VARIAN VXR 200 instrument. The chemical shifts are quoted in p.p.m. for tetramethylsilane (TMS)

TABLE I Different polymer compositions obtained

Polymer	Molar ratio	MDI:	PPO:	BD:	DHHA
PEUDHHA 100%		2	1	0	1
PEUDHHA 1/1		2	1	0.5	0.5
PEUDHHA 1/4		2	1	0.8	0.2
PEUDHHA 1/10		2	1	0.91	0.09
PEU		2	1	1	0

TABLE II Copolymer compositions

Polymer	Molar ratio	MDI:	PPO:	BD:	DED	HDA
Copolymer 1:1		2	1	0	0.5	0.5
Copolymer 4:1		2	1	0	0.8	0.2
Copolymer 3:3:4		2	1	0.4	0.3	0.3

TABLE III Composition of the prepared polymer blends

Polymer	Ratio (w/w)	Blend
PEUDEDQ 1/1 + PEUHDA 1/1	1/1	Blend 1:1
PEUDEDQ 100% + PEUHDA 100%	4/1	Blend 4:1
PEUDEDQ 100% + PEU + PEUHDA 100%	5/4/1	Blend 5:4:1

2.3. Heparin and albumin bonding reactions

In order to make the ionic bonding of heparin possible, the tertiary amine groups of the copolymers have been quaternized with CH_3I (Fluka). The reaction conditions and the amount of quaternization have previously been described [20]. The heparinization and albuminization were carried out on polymer surfaces supported on circular polyethylene films having an area of 40 cm^2 and a thickness of about 45 μm. The coated films obtained were reacted with 0.1% (w/w) water solution of heparin sodium salt (Fluka) at 60 °C for 24 h. The heparin adsorbed in a non-specific way was first removed by washing the films at room temperature for 48 h with distilled water and then with saline phosphate buffer (PBS 0.1 M) at pH = 7.4. The albumin coating was carried out on the same type of films, at room temperature for 24 h, using 0.15% (w/v) solutions of bovine delipidized albumin (BSA, Sigma) in 0.01 M Tris-(hydroxymethyl-aminomethane) buffer (TRIS, Fluka). This solution was also used for washing the films for 24 h. The amount of bonded albumin or heparin was determined measuring spectrophotometrically the difference between the amount of heparin or albumin in the solution before and after contact with the polymer films, adding to this latter term the heparin or albumin content of the washing solutions. The spectroscopic analytical methods utilize the reaction of albumin with the Comassie Brilliant Blue (CBB, Fluka) dyestuff [22], recording the absorbance of the albumin-CBB complex at 620 nm, and the reaction of heparin with toluidine blue, recording at 631 nm the absorbance of the dyestuff not complexed by heparin [23].

2.4. Biological tests

In order to evaluate the anticlotting effect of the heparin ionically bonded to the films, alone or in the presence of albumin, activated partial thromboplastin time (APTT) tests were carried out, by using an automatic coagulimeter (Koagulab MJ, Ortho Diagnostic System). The procedure recommended for the test by

TABLE IV Amounts of heparin and/or albumin bonded to quaternized polymer films with different content of DHHA

Polymer	Heparin (μg/cm²)	Albumin (μg/cm²)	Heparin after albumin (μg/cm²)	Albumin after heparin (μg/cm²)
PEUDHHA 100% Q	119 ± 15	72 ± 12	86 ± 7	59 ± 8
PEUDHHA 1/1 Q	56 ± 8	76 ± 12	69 ± 10	44 ± 13
PEUDHHA 1/4 Q	23 ± 3	49 ± 7	26 ± 4	47 ± 9
PEUDHHA 1/10 Q	5 ± 2	37 ± 4	5 ± 3	36 ± 1

the supplier of the reagents (Ortho Diagnostic System), was modified [24], in particular contacting directly during the tests the polymer surface with plasma. Furthermore, in order to realize an extended polymer surface, the polymer coating was carried out onto glass beads having an average diameter of 220 μm. Heparinization, albuminization and washing of the polymer-coated beads were carried out as previously described for the polymer films. For the sake of comparison, glass beads were coated with untreated polyurethane (PEU) in a similar way. In the case of PEU, DMF was used as solvent.

The determinations of platelet adhesion were carried out employing "bead columns" consisting of silicone tubes (diameter 5 mm) filled with polymer-coated glass spheres (diameter 0.53 mm). The procedures for the preparation of the bead columns and of the platelet rich plasma (PRP), and the test modalities have been described previously [21], and the platelet content of the plasma was determined by a Coulter counter.

3. Results and discussion

The composition of all the polymers synthesized was confirmed by ^{1}H and ^{13}C NMR. The amounts of heparin and albumin bonded to the PEUDHHA polymer films are reported in Table IV. A biological *in vitro* evaluation of the heparinized and albuminized films was also carried out, with respect to the blood coagulation factors, by APTT measurements, reported in Table V, and to platelet adhesion as in Table VI, and activation (by platelet count and SEM examination). It was seen that the type of the adsorption sequence, for albumin and heparin, respectively, onto the various homo- and copolymer films, plays an important role in their biological properties.

TABLE V APTT data for the heparinized and/or albuminized polymers

Polymer	APTT(s)		
	Heparin	Heparin after albumin	Albumin after heparin
PEUDHHA 100%Q	no clot	97 ± 8	31 ± 1
PEUDHHA 1/1 Q	79 ± 13	76 ± 2	30 ± 2
PEUDHHA 1/4 Q	37 ± 5	40 ± 1	30 ± 1
PEUDHHA 1/10 Q	37 ± 1	32 ± 1	31 ± 1

TABLE VI Platelet adhesion data for albuminized and/or heparinized polymers and for glass beads

Polymer	Platelet adhesion (%)[a]		
	Albuminized	Albuminized and heparinized	Heparinzed and albuminized
PEUDHHA 100% Q	7 ± 3	4 ± 2	39 ± 8
PEUDHHA 1/1 Q	11 ± 3	11 ± 3	34 ± 6
PEUDHHA 1/4 Q	26 ± 5	15 ± 1	33 ± 9
PEUDHHA 1/10 Q	28 ± 8	11 ± 4	39 ± 9
PEU	16 ± 6		

The platelet adhesion for glass beads was 51 ± 1%, and for different quaternized polymer compositions was in the range 35–40%
[a]Mean values obtained on at least five samples

In particular the albumin adsorption is mainly influenced by the hydrophylicity of the material, while the amount of bonded heparin increases with the concentration of quaternary ammonium sites in the polymer. It was observed that the contacting sequence of the two macromolecules with the polymer films (heparin + albumin or vice versa) very significantly influences the biological properties of the polymers, as far as their *in vitro* response to the anticoagulation factors and to platelet adhesion and aggregation is concerned.

It was hypothesized, also on the basis of data obtained from polymers previously synthesized in our laboratory and selectively treated with heparin, albumin or both, that the sites of bonding of albumin are not only the long alkyl chains present as a side-group in the polyurethane, but also its quaternary ammonium groups. Heparin too seems to form ionic bonds not only with the quaternary ammonium groups of the polymer, but also with basic groups of albumin, when this latter is pre-adsorbed onto the polymer film.

When the adsorption sequence onto the polymer is albumin + heparin, we have verified that both macromolecules maintain their biological activity, with respect to platelet adhesion and aggregation and coagulation factors, respectively, thus confirming our initial hypothesis that an albumin coating is able to oppose the negative action of heparin on platelets. Accordingly, we deem that these polymer compositions, able to capture continuously from the blood stream albumin even when it has been deactivated, deadsorbed or proteolytically degraded, can be particularly suitable for long-term *in vivo* applications.

In Table VII the amounts of heparin and/or albumin bonded to copolymer films are reported. In Tables VIII and IX APTT data and platelet adhesion, respectively, of these variously heparinized and albuminized copolymer films are given. A similar influence of the heparinization/albuminization sequence was observed, with complete loss of heparin activity, when the albumin-coating is carried out after the film heparinization.

In order to study the effect of possible microphase segregations, polymer blends of similar composition have been investigated. In Table X the amounts of heparin and albumin bonded to polymer blends are reported, while in Table XI the corresponding APTT values are shown. In this case it was seen that the anticoagulation activity of the heparinized blends still remains after albuminization.

4. Conclusions

New polyurethane compositions able to bond molecules provided with good anticoagulant properties, like heparin and albumin, have been investigated. It was observed that the high amount of albumin bonded to polymer films can be explained supposing that its adsorption be not caused only by hydrophobic interactions with long alkyl chains, but mainly by ionic interactions, employing its acidic residues, with quaternary ammonium groups, while the amount of bonded heparin is proportional to the quaternary ammonium sites. Furthermore, heparin seems to form ionic bonds not only with the quaternary ammonium groups of the polymer, but also with basic groups of albumin, when this latter is pre-adsorbed onto the polymer films.

The biological data show that the adsorption sequence of the two macromolecules with the homo-, copolymer and polymer blends films affects their *in vitro* behaviour. In fact, when the adsorption sequence onto the polymer is albumin + heparin, we have verified that both macromolecules maintain their biological activity, whereas when the sequence is heparin + albumin all polymers, except for polymer blends, lose their anticoagulant properties after albuminization.

In the case of films from polymer blends, because of the phase segregation occurring on their surface, we can suppose the existence of heparinized zones sufficiently large to be not hindered, as far as their biological activity is concerned, by interactions with surrounding albumin molecules.

TABLE VII Amounts of heparin and/or albumin bonded to quaternized copolymer films

Polymer	Heparin ($\mu g/cm^2$)	Albumin ($\mu g/cm^2$)	Heparin after albumin ($\mu g/cm^2$)	Albumin after heparin ($\mu g/cm^2$)
Copolymer 1:1	96 ± 13	144 ± 19	105 ± 16	43 ± 7
Copolymer 4:1	107 ± 7	196 ± 21	85 ± 12	23 ± 4
Copolymer 3:3:4	84 ± 4	102 ± 10	87 ± 14	10 ± 2

TABLE VIII APTT data for the heparinized and/or albuminized copolymers

Polymer	APTT(s)		
	Heparin	Heparin after albumin	Albumin after heparin
Copolymer 1:1	69 ± 7	57 ± 5	33 ± 2
Copolymer 4:1	86 ± 5	69 ± 5	33 ± 2
Copolymer 3:3:4	65 ± 5	56 ± 2	31 ± 1

TABLE IX Platelet adhesion data for the heparinized and/or albuminized copolymers and for glass beads

Polymer	Platelet adhesion (%)[a]		
	Albuminized	Heparinized after albumined	Albuminized after heparinized
Copolymer 1:1	21 ± 3	5 ± 2	19 ± 6
Copolymer 4:1	12 ± 4	2 ± 2	19 ± 2
Copolymer 3:3:4	14 ± 2	22 ± 8	26 ± 1

The platelet adhesion for glass beads was 51 ± 1%, and for different quaternized copolymer composition was in the range 39–50%
[a]Mean values obtained on at least five samples

TABLE X Amounts of heparin and/or albumin bonded to polymer blends

Polymer blend	Heparin ($\mu g/cm^2$)	Albumin ($\mu g/cm^2$)	Heparin after albumin ($\mu g/cm^2$)	Albumin after heparin ($\mu g/cm^2$)
Blend 1:1	28 ± 5	85 ± 3	10 ± 5	53 ± 11
Blend 4:1	70 ± 2	126 ± 3	39 ± 2	34 ± 2
Blend 5:4:1	38 ± 1	90 ± 4	21 ± 2	27 ± 3

TABLE XI APPT data for the heparinized and/or albuminized polymer blends

Polymer blend	APTT(s)		
	Heparin	Heparin after albumin	Albumin after heparin
Blend 1:1	102 ± 1	79 ± 1	32 ± 1
Blend 4:1	159 ± 7	93 ± 5	52 ± 2
Blend 5:4:1	117 ± 4	85 ± 4	40 ± 4

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