JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 4 (1993) 448–459

Heparin-containing block copolymers

Part II *In vitro and ex vivo blood compatibility*

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Newly synthesized heparin-containing block copolymers, consisting of a hydrophobic block of polystyrene (PS), a hydrophilic spacer-block of poly(ethylene oxide) (PEO) and covalently bonded heparin (Hep) as bioactive block, were coated either onto glass, poly(dimethylsiloxane), polyurethane or PS substrates. Coated surfaces were characterized by determination of the surface-bound heparin activity, adsorption of AT III, plasma recalcification time assays, adhesion of platelets and by an *ex vivo* rabbit A–A shunt model.

It was demonstrated that heparin was available at the surface of all heparin-bound surfaces to interact with AT III and thrombin and to prevent the formation of clots. The maximum immobilized heparin activity was found to be 5.5×10^{-3} U cm^{-2}. Coated surfaces showed a significant prolongation of the plasma reclacification times as compared to control surfaces, due to surface-immobilized heparin. The platelet adhesion demonstrated that platelets reacted only minimally with the heparin-containing block copolymers in the test system and that the heparin-containing block copolymers seemed to passify the surface as compared to control surfaces. In the *ex vivo* A–A shunt experiments, which were carried out under low flow and low shear conditions, the heparin-containing block copolymers exhibited prolonged occlusion times, indicating the ability of the heparin-containing block copolymers to reduce thrombus formation at the surface.

1. Introduction

Several methods have been described to apply heparin to foreign surfaces [1–7]. These methods can generally be divided into chemical and physical [1] immobilization, and the former group can again be divided into surfaces with ionically bound heparin [2, 3] and surfaces with covalently bound heparin [4–7]. Surfaces with either physically or ionically bound heparin have the disadvantage of depletion of heparin into blood or plasma. This leakage is greatly reduced with covalently immobilized heparin. However, tedious methods are usually required to functionalize the surface.

An alternative route to the surface-immobilization of heparin is the use of heparin-containing triblock copolymers. A hydrophobic block will provide the interaction with the surface and insolubility in blood or plasma after application. The combination with a hydrophilic block and the active heparin block are used to create a phase-separated structure at the surface with the heparin moiety (partially) exposed. Okano *et al.* [4] have synthesized block copolymers of a poly(dimethylsiloxane) (PDMS) hydrophobic block, and poly(ethylene oxide) (PEO) to synthesize PDMS–PEO-heparin (PDMS–PEO–Hep) triblock copolymers.

Grainger *et al.* [8–10] investigated the properties of PDMS–PEO–Hep block copolymers. Using XPS [10] and Wilhelmy plate contact angles [9] it was

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shown that due to the very low surface energy of the PDMS block the PDMS phase is present on the outer surface of the coating after exposure to vacuum or air. However, after exposure to water or blood the surface rearranges and the hydrophilic phase (PEO and Hep) is evidenced at the surface. The block copolymers were shown to be effective in increasing blood compatibility of glass and Biomer® coated surfaces.

In this work the properties of a triblock copolymer with a PS hydrophobic block (PS–PEO–Hep) coated onto several substrates will be described. The first articles of this series [11] was directed to the surface characterization of the PS–PEO–Hep block copolymers by XPS and contact angle measurements. The aim of this study is the evaluation of the blood compatibility of heparin-containing block copolymers as a function of the copolymer composition. The relation of the polymer composition to the blood compatibility of the block copolymers coated either onto glass, poly(dimethylsiloxane) or polyurethane surfaces is described by: (a) an estimation of the surface-bound heparin activity; (b) the adsorption of AT III; (c) plasma recalcification time assays; (d) the adhesion of platelets; and (e) a low flow-rate *ex vivo* rabbit A–A shunt model [12].

2. Materials and methods

Acetic acid (E. Merck, Darmstadt, Germany), anhydrous sodium chromate (Na_2CrO_4, BDH (Laboratory Chemicals, Poole, UK), heparin sodium salt (Hep) from porcine mucosa with a specific activity of 165 U mg^{-1}, as indicated by the manufacturer (Diosynth B.V., Oss, Holland) and nitric acid 65% (HNO_3, E. Merck) were used as received. Aminotelechelic poly(ethylene oxide), with a molecular weight of 4000 (H_2N–PEO(4000)–NH_2), was a generous gift from Nippon Oil and Fats Company Ltd, Ibaraki, Japan and was characterized as described previously [13].

COATEST® Heparin (KabiVitrum, Stockholm, Sweden), consisting of 15 mg lyophilized chromogenic substrate S-2222 with added mannitol, 71 nkat lyophilized bovine F X_a, 10 IU lyophilized human AT III and sterile buffer containing 0.05 mol l^{-1} Tris and 7.5 mmol l^{-1} EDTA (pH 8.4) was used in the chromogenic antifactor X_a assay.

The thrombin-sensitive chromogenic substrate S-2238 was obtained from KabiVitrum. A lyophilized preparation of human thrombin and stabilizers (Sigma Chemie, Taufkirchen, Germany) with a specific activity of 3000 NIH U mg^{-1} was reconstituted with distilled water, resulting in a stock solution that contained 20 NIH U ml^{-1} thrombin in 150 mmol l^{-1} NaCl and 50 mmol l^{-1} sodium citrate. Human AT III was obtained from KabiVitrum and diluted to an activity of 1 IU ml^{-1}.

Lyophilized rabbit brain cephalin (Sigma Chemie) in 1 vial was diluted with 30 ml of an aqueous NaCl solution (0.85%) and frozen in 3 ml aliquots until used.

Polystyrene–poly(ethylene oxide) (PS–PEO) diblock copolymers were synthesized by a coupling reaction of aminosemitelechelic polystyrene with aminotelechelic poly(ethylene oxide), using toluene 2,4-diisocyanate as coupling agent, according to the procedures described previously [13–15].

Polystyrene–poly(ethylene oxide)–heparin (PS–PEO–Hep) block copolymers were synthesized by: (a) coupling of PS–PEO–NH_2 with heparin performed in a DMF–H_2O (40:1 v/v) mixture, first by activating carboxylic acid groups of heparin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and subsequently reacting the activated carboxylic acid groups with amino groups of PS–PEO–NH_2, as described before [13]; and (b) coupling of PS–PEO–NH_2 with nitrous acid-degraded heparin performed in a DMF–H_2O (40:1 v/v) mixture, using cyanoborohydride as reducing agent, also described previously [14, 15]. An overview of the prepared block copolymers is shown in Table I.

2.1. Coating preparation

Glass beads (ø 250–300 μm, Tamson, Zoetermeer, Holland) were cleaned by treatment with 5% w/v

TABLE I Characterization of materials used[a]

	PS		PEO	Hep–NADHep	
Code	Synthesis	$\bar{M}_n$	$\bar{M}_n$	Coupling	$\bar{M}_n$
PS_0E_1	Radical	3300	500		
PS_1E_2	Anionic	8500	4000		
PS_2E_2	Radical	9300	4000		
PS_3E_2	Radical	11900	4000		
PS_4E_2	Radical	15700	4000		
PS_5E_2	Anionic	23000	4000		
$PS_0E_1H_1$	Radical	3300	500	EDC	11000
$PS_2E_2H_1$	Radical	9300	4000	EDC	11000
$PS_3E_2H_1$	Radical	11900	4000	EDC	11000
$PS_4E_2H_1$	Radical	23000	4000	EDC	11000
$PS_5E_2H_1$	Anionic	23000	4000	EDC	11400
$PS_1E_2H_2$	Anionic	8500	4000	$NaBH_3CN$	6000
$PS_5E_2H_2$	Anionic	23000	4000	$NaBH_3CN$	6000

[a] PS = aminosemitelechelic polystyrene, PEO = aminotelechelic poly(ethylene oxide), Hep = native heparin (H_1) and NADHep = nitrous acid-degraded heparin (H_2). For characterization methods see previous publications [13–15].

Na_2CrO_4 in HNO_3. After washing 3 times with double distilled water and 2 times with methanol, the glass beads were dried in an oven at 120 °C overnight. Clean, dry glass beads were then coated with 0.5% w/v solutions of block copolymers in DMF–H_2O (40:1 v/v). The coated beads were suction filtered and then dried at 37 °C under vacuum for 24 h.

Biomer® coated beads were prepared by using a 2% w/v solution of Solution Grade Biomer® (Ethicon, Somerville, NJ, USA) in *N,N*-dimethyl acetamide (DMAc). The beads were suction filtered and then dried at 60 °C under vacuum for 24 h.

Poly(ethylene oxide) (PEO) coated beads were prepared by using a triisocyanate (Colonate L®, Nippon Polyurethane Industrial Ltd, Tokyo, Japan) as a cross-linking agent (end group ratio 1:1) in a 2% w/v solution of H_2N–PEO (4000)–NH_2 in DMF. Curing overnight at 60 °C *in vacuo* produced a cross-linked, continuous film of PEO insoluble in water. After soaking in distilled water for 24 h, coated beads were vacuum dried at ambient temperature overnight and then mechanically sieved (US Standard #40) to remove aggregates.

Glass plates (dimensions 50×90 mm^2) were cleaned by treatment with 5% w/v Na_2CrO_4 in HNO_3. After washing 3 times with double distilled water and 2 times with methanol, the glass plates were dried in an oven at 120 °C overnight.

To obtain block copolymer coatings onto bare glass surfaces, clean glass plates were coated by slow, uniform dipping into 2% w/v solutions of block copolymers in DMF–H_2O (40:1 v/v). Then they were placed vertically in an oven at 37 °C. After 6 h, the coated plates were vacuum dried overnight.

Poly(dimethylsiloxane) coated plates were obtained by coating clean glass plates with a 2% w/v solution of Silastic RTV adhesive [poly(dimethylsiloxane), PDMS, General Electric, Waterford, NY, USA] in THF, forming homogeneous cross-linked PDMS films after a 24 h vacuum cure at ambient temperature. These PDMS coated plates were rinsed thoroughly with distilled water to remove acetic acid produced from curing. Then they were coated with block copolymers as described above.

Glass tubes (type RB $55 \times 11/12$, Fenes, Zeist, Holland) were cleaned with 5% w/v Na_2CrO_4 in HNO_3. After washing 3 times with double distilled water and 2 times with methanol the glass tubes were dried in an oven at 120 °C overnight. Tubes were then coated with a 2% w/v solution of Solution Grade Biomer® (Ethicon) in DMAc, forming homogeneous polyurethane films after a 24 h vacuum drying at 60 °C, or were coated with a 2% w/v solution of Silastic RTV Adhesive (General Electric) in THF, forming homogeneous cross-linked PDMS films after a 24 h vacuum cure at ambient temperature, or were coated with a 2% w/v solution of polystyrene (BASF KR 2521, $\bar{M}_n = 100\,000$, Ludwigshafen, Germany) in chloroform, forming homogeneous PS films after a 24 h vacuum drying at ambient temperature. The PDMS coated tubes were rinsed thoroughly with distilled water to remove acetic acid produced from curing.

The clean glass tubes, Biomer® coated, PDMS coated and PS coated tubes were filled with 2% w/v solutions of block copolymers in DMF–H_2O (40:1 v/v). After emptying the tubes, they were placed vertically in an oven at 37 °C. After 10 min and 1 h, any remaining solution was removed from the bottom using a pasteur pipette. After 6 h, the tubes were vacuum dried overnight.

Commercialized polyester–polyurethane tubing (2.0 mm OD × 1.5 mm ID, Miki Sangyo, Tokyo, Japan) was coated on its luminal surface with 1% w/v solutions of block copolymers in DMF–H_2O (40:1 v/v). The tubing was coated by pumping block copolymer solutions through the tubing with a peristaltic pump for 30 s, followed by 2 h drying under peristaltically-pumped air and 24 h vacuum drying at 40 °C.

All (block co)polymer solutions were filtered through 0.5 μm Teflon filters prior to the coating procedures. All (block co)polymer precoatings and coatings were clear, intact and continuous, as evidenced by optical microscopy and scanning electron microscopy (SEM).

2.2. *In vitro* evaluation

Fresh platelet-rich plasma (PRP) was prepared by collecting blood from rabbits (New Zealand White, ± 2.5 kg) via a catheterized femoral artery into plastic syringes containing 3.8% sodium citrate solution (final dilution 9:1). NIH guidelines [16] for the care and use of laboratory animals have been observed. Blood was carefully transferred to Falcon tubes and centrifuged at 200 *g* for 10 min. PRP supernatant was collected and the residue was re-centrifuged at 1500 *g* to collect platelet-poor plasma (PPP). PRP was diluted with PPP to give PRP with a final platelet concentration of 3×10^8 per ml. After mixing, platelets were equilibrated at 20 °C for 60 min and used within 4 h.

2.3. Estimation of surface-bound heparin activity

2.3.1. APTT assay

The bioactivity of surface-immobilized heparin of heparin-containing block copolymers coated onto glass beads was analysed according to an APTT assay, modified to evaluate coated beads [17].

A standard curve was obtained as follows. Heparin standards in PPP were made in the range 0.1–0.5 U ml^{-1}. From these heparinized PPP standards 100 μl was incubated with 100 μl of Activated Thrombofax Reagent-Optimized (Ortho Diagnostic Systems Inc., Raritan, NJ, USA) for 2 min at 37 °C. Then 100 μl of 0.02 M $CaCl_2$ solution (Ortho Calcium Chloride Solution) was added and the time for a fibrin clot to form was recorded using a Fibrosystem Fibrometer (mechanical end point). A 6-point standard curve was constructed by averaging the APTT of 6 samples of each concentration ($n = 6$).

The bioactivity of surface-immobilized heparin of PS–PEO–Hep coated onto glass beads was determined by the following procedure. Four different

amounts of each type of bead (50, 100, 150 and 200 mg) were weighed into Fibrosystem Fibrocups in sets of 6. Each type of coated bead was then incubated with 100 µl PPP and 100 µl Activated Thrombofax Reagent-Optimized for 2 min at 37 °C. Finally, 100 µl of 0.02 M $CaCl_2$ solution was added and the clotting time was detected with the fibrometer. As a reference the bioactivity of PS–PEO coated onto glass beads was determined. The bioactivity of the surface-immobilized heparin was obtained by comparison of the APTT end points with the heparin standard curve.

2.3.2. Chromogenic antifactor X_a assay

The bioactivity of surface-immobilized heparin of heparin-containing block copolymers coated onto glass beads was analysed according to a chromogenic assay, modified to evaluate coated beads, as described by Teien *et al.* [18, 19].

A standard curve for measurement of the bioactivity of surface-immobilized heparin was obtained as follows. Heparin standards in Tris buffer (pH 8.4, 20 °C) were made in the range 0.1–0.6 U ml^{-1}. From these heparin standards 100 µl was diluted in polystyrene tubes (Greiner, Alphenaan der Rijn, Holland) with 100 µl of AT III (1 IU ml^{-1}) and 800 µl of Tris buffer. Aliquots of these solutions (200 µl) were incubated at 37 °C for 4 min and F X_a (100 µl, 7 nkat S-2222 ml^{-1}, 20 °C) was added and incubated for an additional 30 s. Then 200 µl of S-2222 (1 mmol l^{-1}, 37 °C) was added and incubated at 37 °C for 4 min. The reaction was terminated by adding 500 µl of 50% acetic acid and mixing. Samples were monitored spectrophotometrically at 405 nm using a Reader Microelisa® System (Organon Teknika, Boxtel, Holland) spectrophotometer against water blanks. Absorbances were measured 4-fold within 1 h after terminating the reaction with acetic acid. A 5-point standard curve was constructed by averaging the results of 5 samples of each concentration ($n = 20$).

The bioactivity of surface-immobilized heparin of PS–PEO–Hep coated onto glass beads was determined by the following procedure. Three different amounts of each type of bead (10, 20 and 40 mg) were weighed into polystyrene tubes (Greiner) in sets of 10. One half of each set ($n = 5$) was incubated for 4 min with 20 µl of AT III and 180 µl of Tris buffer at 37 °C with occasional shaking to promote wetting. The other half of each set ($n = 5$) was first equilibrated with 100 µl of Tris buffer for 30 h at 20 °C. Then 20 µl of AT III and 80 µl of Tris buffer were added and incubated for 4 min at 37 °C. F X_a, S-2222 and acetic acid were then added over the same time course and in identical quantities as for the heparin standards. As a reference the bioactivity of PS–PEO coated onto glass beads was determined in both the non-hydrated and hydrated states. Quantitation of the bioactivity of the surface-immobilized heparin was achieved by monitoring the absorbance at 405 nm (4-fold, $n = 20$) and comparing the results with the standard curve obtained for heparin.

2.3.3. Kinetic assay based on the inactivation of thrombin by AT III

The bioactivity of surface-immobilized heparin of heparin-containing block copolymers coated onto glass beads was determined by measuring the increase in the rate of thrombin inactivation by AT III due to the presence of heparin, as described by Chandler *et al.* [20].

Two stock reagents were prepared for the kinetic heparin assay: (a) substrate–blank reagent, consisting of 50 mmol l^{-1} Tris (pH 8.4, 20 °C), 150 mmol l^{-1} NaCl, 1 g l^{-1} polyethylene glycol 6000 (Carbowax® 6000, Fluka), 1 g l^{-1} bovine serum albumin (Sigma Chemie) and 0.2 mmol l^{-1} S-2238; and (b) substrate–AT III reagent, which was produced by adding AT III to a final concentration of 25 IU l^{-1} to the substrate–blank reagent. After mixing, both solutions were stored at 4 °C until used.

A standard curve for measurement of the bioactivity of surface-immobilized heparin was obtained as follows. Heparin standards in phosphate buffered saline (PBS, 0.9% NaCl, 10 mM Na_2HPO_4 adjusted to pH 7.4) were made in the range 0.5–4.0 U l^{-1}. From these heparin standards 50 µl was diluted in polystyrene tubes (Greiner) with 1.85 ml of substrate–AT III reagent. To start the reaction, 100 µl of thrombin (2 NIH U l^{-1}) was added. After 10 min, the reaction was terminated by adding 1 ml of 25% acetic acid and mixing. Samples were monitored spectrophotometerically at 405 nm using a Reader Microelisa® System (Organon Teknika) spectrophotometer. Absorbances were measured 8-fold within 15 min after terminating the reaction with acetic acid. A 9-point standard curve was constructed by averaging the results of 5 samples of each concentration ($n = 40$).

The bioactivity of surface-immobilized heparin of PS–PEO–Hep coated onto glass beads was determined by the following procedure. Batches (300 mg) of each type of beads were weighed into polystyrene tubes (Greiner) in sets of 10. One half of each set ($n = 5$) was first equilibrated with 300 µl of substrate–blank reagent for 30 h at 20 °C. Then, 1.60 ml of substrate–AT III reagent was added. The other half of each set ($n = 5$) was incubated with 1.90 ml of substrate–AT III reagent. To start the reaction, 100 µl of thrombin (2 NIH U ml^{-1}) was added. The absorbance of the solution at 405 nm was measured after the reaction was stopped (after 10 min) with 1 ml of 25% acetic acid. As a reference the bioactivity of PS–PEO coated onto glass beads was determined in both the non-hydrated and hydrated states. Quantitation of the bioactivity of the surface-immobilized heparin was achieved by monitoring the absorbance at 405 nm (8-fold, $n = 40$) and comparing the results with the standard curve obtained for heparin.

2.4. Adsorption of AT III onto heparin-containing surfaces

A two-step enzyme-linked immunosorbent assay (ELISA) was used to evaluate AT III interactions with PS–PEO–Hep and PS–PEO coated onto glass and

PDMS surfaces. These surfaces were obtained as described under coating preparation. The adsorption of AT III, either from PBS (0.9% NaCl, 10 mM Na_2HPO_4 adjusted to pH 7.4) solutions or from plasma (Bloodbank Twente and Achterhoek, Enschede, Holland, containing 0.15 g AT III l^{-1}, as determined spectrophotometrically at 280 nm using an extinction coefficient [21] $\varepsilon^{1\%}_{280\ nm} = 6.10$), for 1 h, onto the coated plates as well as onto hydrated coated plates was determined by means of specially constructed 24 well chamber in which each test surface area is 1 cm^2. Experimental details of the two-step ELISA have been given in the literature [22–24].

In order to detect AT III (human AT III, KabiVitrum) adsorbed onto the test surfaces, these surfaces were exposed to rabbit serum directed against human AT III (KH53P, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Holland). In the second step, peroxidase-conjugated sheep antibody directed against rabbit immunoglobulins (PK17E, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) was added. After this step, hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB, Fluka) were added. The reaction product that is formed by the action of the bound enzyme reacts with TMB and a dye is generated. The absorbance at 450 nm (A_{450}) of the dye is a measure of the amount of AT III that has been adsorbed onto the polymer surface.

2.5. Plasma recalcification time assay

Plasma recalcification times of plasma in contact with uncoated and with polymer coated glass tubes, Biomer® coated tubes and PDMS coated tubes were measured as follows.

(a) Fresh-frozen human CPD plasma (150 µl, Bloodbank Twente and Achterhoek) was pipetted into a tube. After an incubation time of 5 min at 37 °C, $CaCl_2$ (50 µl, 50 mM in H_2O) was added and the clotting time was detected with a Lode LC-6 Coagulatometer (optical method).

(b) The procedure involved incubation of 0.5 ml fresh-frozen human CPD plasma (Bloodbank Twente and Achterhoek) in the tubes at 37 °C for 1 min followed by addition of 50 µl rabbit brain cephalin solution and an additional incubation for 1 min. Finally, 50 µl of 0.2 M $CaCl_2$ solution was added and the solution monitored manually by dipping a stainless steel wire hook into the solution to detect fibrin threads. Clotting times were recorded at first signs of fibrin formation on the hook. Clots typically followed within a minute after fibrin was first detected.

2.6. Platelet adhesion onto polymer coated beads

Quantities of coated beads (140 mg) were carefully weighed into plastic disposable 3 ml syringes and equilibrated with 4 ml of PBS (pH 7.4, 0.15 M) overnight prior to adhesion studies. Buffer was squeezed out and 0.5 ml of PRP was introduced via another syringe and the syringes were then tapped to remove air bubbles. The syringes were sealed with parafilm and rotated through a water bath at 37 °C so that the beads were constantly exposed to PRP. Sets of syringes were arranged for adhesion time intervals of 15, 30, 40 and 60 min of PRP incubation. At each time point, the syringes were quickly removed from the rotating bath, emptied into Falcon tubes and the platelets left in the plasma counted immediately with a Coulter Counter® (Model ZBI, Coulter Electronics, Hialeah, FL, USA). A control sample of PRP incubated without beads was used as a reference for each time point. The number of adhering platelets was expressed as a percentage of control PRP at each time point.

2.7. *Ex vivo* A–A shunt model

Male rabbits (New Zealand White, ± 2.5 kg) were anaesthetized with ketamine–promethazine and atropine and the right carotid artery was carefully exposed surgically. Dried coated tubings were first equilibrated overnight with PBS (pH 7.4, 0.15 M) and cut into 30 cm lengths with the proximal and distal end cut at 45° angles. The coated tubing was rinsed and completely filled with fresh PBS with care taken to avoid introducing air bubbles. The carotid artery was clamped both distally and proximally and ligated at the center between clamps, and a small incision made on the proximal side of the ligation. The ligation was thoroughly washed with PBS to rinse away stagnating blood and one end of the shunt tubing was inserted into the artery and externally secured with suture with care taken to avoid pneumo-emboli. Another incision was made distally to the ligation, washed with PBS and the other end of the shunt inserted and secured with suture.

At time $t = 0$, the clamp was removed and the shunt flow was started. An ultrasonic flow meter (Model T201, Transonic Systems, Ithaca, NY, USA) was placed distal to the A–A shunt around the carotid artery and the flow rate was controlled to 2.5 ml min^{-1} (shear rate = 126 s^{-1}) using a suture tourniquet and a clamp proximal to the flow meter but distal to the shunt. Flow rate within the shunt was monitored continuously and the occlusion time was defined as the time for flow to decrease to zero.

2.8. Determination of statistical significance

Statistical significance of differences in the amount of adsorbed AT III, the recalcification time ratios, the amount of adhered platelets and the A–A shunt occlusion times were determined using Student's t test.

3. Results

3.1. Estimation of surface-bound heparin activity

The estimation of the heparin activity on glass beads coated with heparin-containing block copolymers was performed using the following three methods: (a) APTT assay; (b) chromogenic antifactor X_a assay; and (c) kinetic assay based on the inactivation of

TABLE II *In vitro* quantitation of surface-immobilized heparin

Substrate	Heparin surface concentration			
	No hydration		30 h hydration	
	(10^{-3} U cm^{-2})	(10^{-2} μg cm^{-2})	(10^{-3} U cm^{-2})	(10^{-2} μg cm^{-2})
PS_4E_2[a]	0 ± 0.3	0		
$PS_0E_1H_1$[a]	1.1 ± 0.6	0.7 ± 0.4		
$PS_3E_2H_1$[a]	1.0 ± 0.3	0.6 ± 0.2		
$PS_4E_2H_1$[a]	0.8 ± 0.3	0.5 ± 0.2		
PS_5E_2[b]	0 ± 0.5	0	0 ± 0.5	0
$PS_5E_2H_1$[b]	4.1 ± 1.0	2.5 ± 0.6	5.5 ± 1.2	3.4 ± 0.7
$PS_5E_2H_2$[b]	3.3 ± 0.6	5.1 ± 0.9	4.8 ± 1.4	7.4 ± 2.2
PS_5E_2[b]	0 ± 0.1	0	0 ± 0.1	0
$PS_5E_2H_1$[c]	2.0 ± 0.3	1.2 ± 0.2	3.7 ± 0.5	2.3 ± 0.4
$PS_5E_2H_2$[c]	0.6 ± 0.1	0.9 ± 0.2	1.6 ± 0.2	2.5 ± 0.3

[a] Method (a) by APTT mean ± SD ($n = 24$).
[b] Method (b) described by Teien *et al.* [18, 19], mean ± SD ($n = 20$).
[c] Method (c) described by Chandler *et al.* [20], mean ± SD ($n = 40$).

thrombin by AT III. To investigate the effect of hydration on the availability of surface-immobilized heparin, glass beads coated with PS_5E_2, $PS_5E_2H_1$ and $PS_5E_2H_2$ were first equilibrated for 30 h with Tris buffer [method (b)] or with substrate–blank reagent [method (c)] before determining the activity of the surface-immobilized heparin. The amounts of heparin immobilized on the coated glass surfaces are summarized in Table II. For some of the triblock copolymer samples the heparin content was determined using a modified toluidine blue assay. The samples $PS_0E_1H_1$, $PS_1E_2H_1$ and $PS_3E_2H_1$ contained 29 ± 6, 19 ± 18 and 25 ± 12 wt % heparin, respectively (mean ± SD). This is less than can be calculated from the molecular weights of the blocks (74, 45 and 41 wt %, respectively).

In methods (a) and (b), the heparin standard curve demonstrated reliable linearity [for method (a): $r = 0.981$ and for method (b): $r = 0.993$]. The standard curve for method (c) showed a non-linear relationship between the heparin concentration and the absorbance at 405 nm. In method (a), the bioactivity of the surface-immobilized heparin of $PS_0E_1H_1$, $PS_3E_2H_1$ and $PS_4E_2H_1$ coated on glass beads was obtained by comparison of the observed APTT end points with the heparin standard curve. It was shown that for all three substrates 1.0×10^{-3} U heparin cm^{-2}, corresponding to ± 0.6×10^{-2} mg heparin (161.9 U mg^{-1} cm^{-2}, was available. As expected, PS_4E_2 coated glass beads demonstrated almost no heparin activity.

In methods (b) and (c), quantitation of the bioactivity of PS_5E_2, $PS_5E_2H_1$ and $PS_5E_2H_2$ coated on glass beads was achieved by observing the absorbance at 405 nm and comparing the results with the standard curve. The same was done for the substrates which were first hydrated for 30 h. From Table II it appears that the heparin activities determined with method (b) are consequently higher than those determined with method (c). Also, the heparin activities of the hydrated substrates are higher than those of the non-hydrated corresponding substrates. For the substrates tested, the maximum surface-bound heparin activity (5.5×10^{-3} U cm^{-2}) was found on hydrated $PS_5E_2H_1$ coated glass beads. As expected, PS_5E_2 coated glass beads showed almost no heparin activity.

3.2. Adsorption of AT III onto heparin-containing surfaces

Adsorptions of AT III from PBS solutions containing different concentrations of AT III (1 IU ml^{-1} and 0.067 IU ml^{-1}) to glass and PDMS coated with PS_5E_2 and $PS_5E_2H_1$ and to hydrated surfaces, after a contact time of 1 h, are shown in Fig. 1. The adsorption of AT III from PBS (1 IU ml^{-1}) to $PS_5E_2H_1$ coated on glass and PDMS respectively, was not significantly different as compared to the adsorption of AT III to PS_5E_2 coated on glass and PDMS, respectively. The hydrated surfaces showed the same amount of adsorbed AT III as compared to the amount adsorbed on the corresponding non-hydrated surfaces. The adsorption of AT III from 1:15 diluted PBS (0.067 IU ml^{-1}) to coated glass and PDMS, respectively, was the same on all surfaces. However, the amount of adsorbed AT III was significantly lower as compared to the amount adsorbed from undiluted PBS (1 IU ml^{-1}).

Adsorptions of AT III from plasma containing 1 IU AT III ml^{-1} to glass and PDMS coated with PS_5E_2 and $PS_5E_2H_1$ and to hydrated surfaces, after a contact time of 1 h, are shown in Fig. 2. When coated glass surfaces were incubated for 1 h with plasma containing 1 IU AT III ml^{-1}, the adsorption of AT III on $PS_5E_2H_1$ coated glass was significantly higher than on PS_5E_2 coated glass ($p \leq 0.0005$). The adsorption on hydrated coated glass surfaces was not significantly different as compared to the adsorption on the corresponding non-hydrated surfaces. When similar experiments were carried out with coated PDMS surfaces as substrates for the adsorption of AT III, the adsorption on $PS_5E_2H_1$ coated PDMS was significantly higher than on PS_5E_2 coated PDMS ($p \leq 0.005$). For the hydrated coated PDMS surfaces the adsorption was significantly higher ($p \leq 0.005$) than the adsorption on the corresponding non-hydrated surfaces.

It has been shown [11] that the coatings on PDMS expressed a higher content of the hydrophobic block

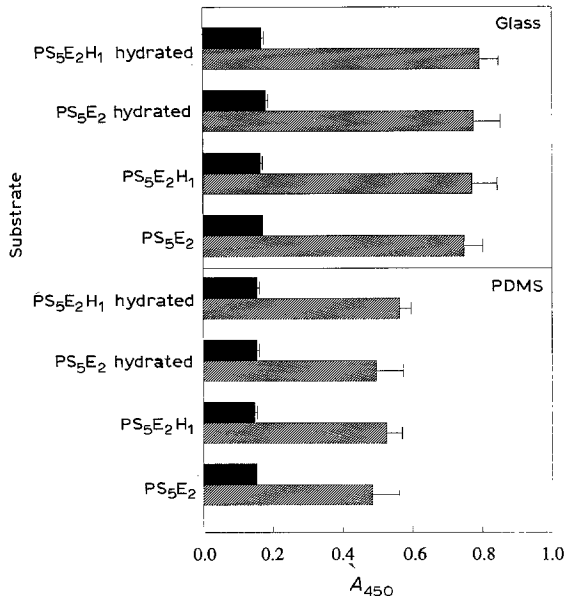

Figure 1 Adsorption of AT III from buffer containing different concentrations of AT III (light bars = 1 IU ml^{-1} = 0.15 gl^{-1} and dark bars = 0.067 IU ml^{-1} = 0.01, l^{-1}) onto glass or PDMS coated with PS_5E_2 and $PS_5E_2H_1$. Mean value ± SD (n = 10).

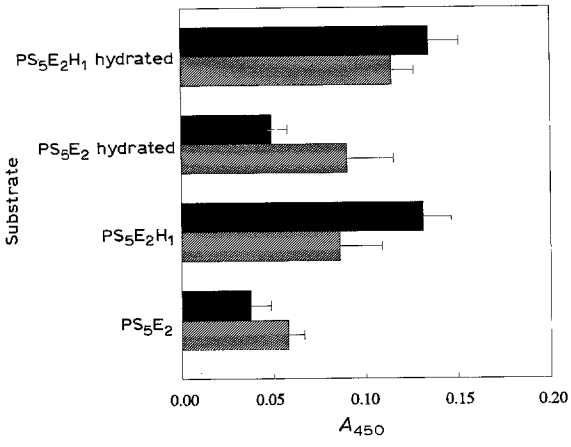

Figure 2 Adsorption of AT III from plasma onto glass (light bars) and PDMS (dark bars) coated with PS_5E_2 or $PS_5E_2H_1$. Mean value ± SD (n = 10).

on the surface than the coatings on glass. Thus, the heparin fragment seems to be buried under the PS fragment of the block copolymers when coated on PDMS.

3.3. Plasma recalcification times

Recalcification times of plasma exposed to uncoated glass and glass tubes coated with PS, PS–PEO–NH_2 and PS–PEO–Hep were determined by method (a). The recalcification time of plasma in contact with glass was measured for each experiment and used as a reference. The observed recalcification times for plasma exposed to the different substrates were divided by the value observed for glass, yielding recalcification time ratios given in Fig. 3. Average ratios were obtained by repeating the experiments several times (n = 7). From Fig. 3 it appears that the recalcification times were significantly prolonged when the glass

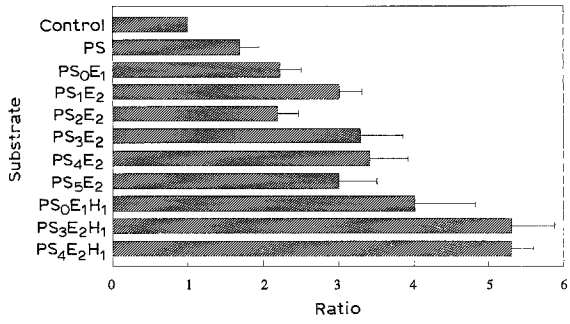

Figure 3 Recalcification times of plasma exposed to uncoated and polymer coated glass tubes, determined by method (a). Normalized data, mean value ± SD (n = 7).

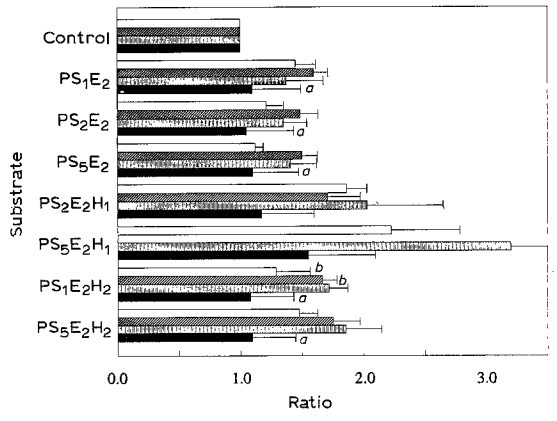

Figure 4 Recalcification times of plasma exposed to glass (white bars), Biomer® coated (light grey bars), PDMS coated (dark grey bars) and PS coated (black bars) tubes coated with heparin-containing block copolymers, determined by method (b). Normalized data, mean value ± SD (n = 7). *a* $0.1 < p \leq 0.375$ and *b* $0.05 < p \leq 0.1$, when compared to the corresponding control/prepolymer value; in all other cases $p \leq 0.025$.

tubes were coated with heparin containing block copolymers ($p \leq 0.0005$).

Fig. 4 shows the recalcification time ratios of plasma exposed to uncoated glass tubes, Biomer® coated glass tubes, PDMS coated glass tubes and PS coated glass tubes coated with PS, PS–PEO–NH_2 and PS–PEO–Hep, respectively, determined by method (b). It appears that the recalcification times were significantly prolonged when the glass tubes (except for $PS_1E_2H_2$; $0.05 < p \leq 0.1$), the Biomer® coated glass tubes and the PDMS coated glass tubes (except for $PS_1E_2H_2$; $0.05 < p \leq 0.1$) were coated with heparin-containing block copolymers ($p \leq 0.025$). No prolongation in plasma recalcification times was observed when PS coated glass tubes were coated with $PS_1E_2H_2$ and $PS_5E_2H_2$ block copolymers ($0.1 < p \leq 0.375$). In contrast, when $PS_2E_2H_1$ and $PS_5E_2H_1$, respectively, were used as the coating, the observed recalcification times were significantly prolonged as compared to the recalcification times of plasma in contact with PS_2E_2 and PS_5E_2 coated PS coated glass tubes, respectively ($p \leq 0.025$).

Also of importance is the observation that plasma removed from tubes coated with heparin-containing block copolymers (after an incubation time of 15 min) showed recalcification times comparable to those of plasma exposed to uncoated tubes.

3.4. *In vitro* platelet studies

Fig. 5 shows the adhesion of platelets on different substrates, over a 60 min time course, determined by quantifying the platelet depletion from PRP at the various time points. As can be seen, platelet adhesion to Biomer® and PS coated glass beads seems to reach a plateau value after 60 min, while the platelet adhesion to PS–PEO and PS–PEO–Hep coated glass beads reaches a plateau value after only 30 min. Considering the 30 min interval (see also Fig. 6), a statistically significant ($p \leq 0.005$) reduction in percentage of adhered platelets was measured when control (Biomer® and PS coated) beads and block copolymer coated beads were compared. However, the platelet adhesion on PS–PEO coated glass beads was not significantly different from adhesion on PS–PEO–Hep coated glass beads. Although, it seems from Fig. 5 that PS–PEO–Hep coated glass beads show a slightly higher degree of platelet adhesion, over the 60 min time course, than PS–PEO coated glass beads ($PS_5E_2H_1$–PS_5E_2: $0.01 < p \leq 0.025$ and $PS_5E_2H_2$–PS_5E_2: $0.05 < p \leq 0.1$).

In addition, although no quantitative analysis was obtained, platelet aggregates in PRP were qualitatively noted from Coulter Counter® distributions to increase over time in contact with Biomer®, PEO and PS coated glass beads and remain small in contact with PS–PEO and PS–PEO–Hep coated glass beads.

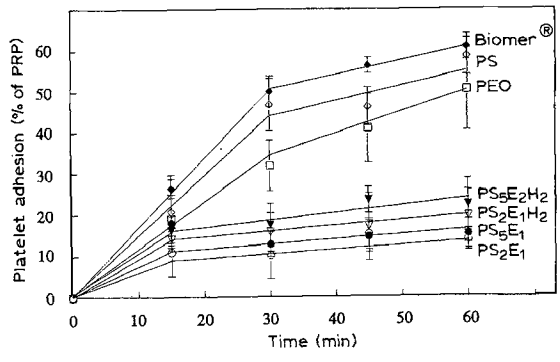

Figure 5 Platelet adhesion to Biomer® (◆), PS (◇), PEO (□), PS_2E_2 (○), PS_5E_2 (●), $PS_5E_2H_1$ (▽) and $PS_5E_2H_2$ coated (▼) glass beads. Mean value ± SD ($n = 4$).

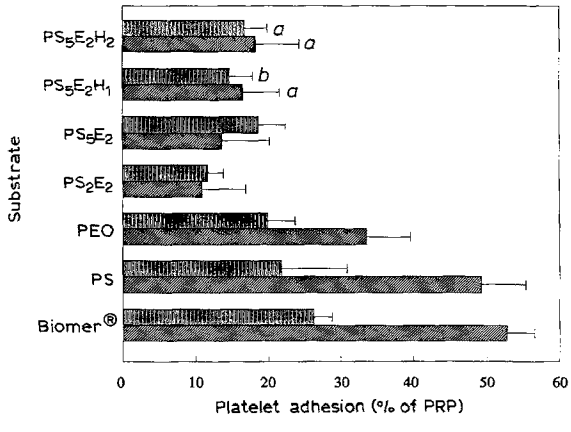

Figure 6 Platelet adhesion to polymer coated beads, for 15 (light bars) and 30 (dark bars) min. Mean value ± SD ($n = 4$). *a* $0.1 < p \leq 0.375$ and *b* $0.025 < p \leq 0.05$, when compared to the corresponding prepolymer value; in all other cases $p \leq 0.005$, when compared to the corresponding control/prepolymer value.

3.5. *Ex vivo* A–A shunts

The occlusion times of whole blood in contact with polyester–polyurethane tubings coated with heparin-containing block copolymers are shown in Fig. 7. The three control surfaces (uncoated and coated with PEO and PS_5) performed poorly in the shunt experiments, all demonstrating occlusion times less than 45 min. The tubings coated with PS–PEO–Hep remained patent, even under these extreme flow conditions, for approximately 180 min. As shown in Fig. 7, blood in contact with tubings coated with PS–PEO showed significantly longer occlusion times than blood in contact with the control surfaces ($p \leq 0.005$). Compared to PS–PEO–Hep coated surfaces, these occlusion times were significantly lower ($p \leq 0.01$).

4. Discussion

4.1. Estimation of surface-bound heparin activity

With the use of the following three methods: (a) APTT assay; (b) chromogenic antifactor X_a assay; and (c) kin etic assay based on the inactivation of thrombin by AT III; it was possible to estimate the availability of heparin on glass beads coated with heparin-containing block copolymers. The effect of hydration on the availability of surface-immobilized heparin was evaluated using only methods (b) and (c). As expected, in all cases PS–PEO coated glass beads showed almost no heparin activity.

The determination of the anticoagulant activity of the surface-immobilized heparin of $PS_0E_1H_1$, $PS_3E_2H_1$ and $PS_4E_2H_1$ coated on glass beads using method (a) showed, for all three substrates, 1.0×10^{-3} U heparin cm^{-2}. Although there are differences in molecular weights of the PS and PEO blocks in the heparin-containing block copolymers, the weight percentages of heparin are approximately the same (for $PS_4E_2H_1$ not determined). Modification of heparin carboxylic acid groups leads to a decreased anticoagulant activity, as pointed out by several authors [25–27], so the anticoagulant activity of heparin in heparin-containing block copolymers is different from that of native heparin in solution. Furthermore,

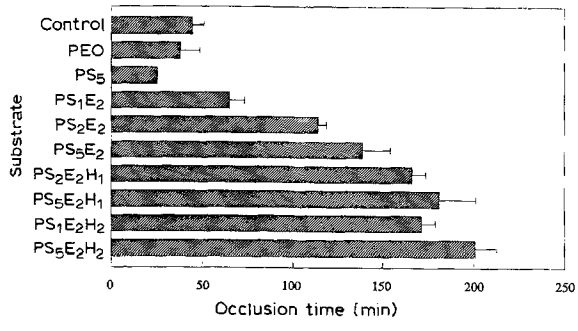

Figure 7 Ex vivo A–A shunt occlusion times of whole blood in contact with polyester–polyurethane tubings coated with heparin-containing block copolymers. Mean value ± SD ($n = 3$).

$PS_0E_1H_1$ has 4 PS–PEO chains coupled per heparin molecule, while $PS_3E_2H_1$ bears 2 PS–PEO chains per heparin molecule. Despite the possible differences in anticoagulant activities and the number of PS–PEO chains coupled, the tested coatings of heparin-containing block copolymers showed the same amount of heparin available at the surface, as determined by the ability to prevent the formation of clots.

Estimation of the antifactor X_a activity of PS_5E_2, $PS_5E_2H_1$ and $PS_5E_2H_2$ coated on glass beads was performed with a chromogenic antifactor X_a assay, analogous to that used for the determination of the antifactor X_a activity of PDMS–PEO–Hep coated on glass beads [4] [method (b)]. F X_a inactivation requires the binding of heparin with AT III [28, 29], thus this assay detects only heparin on the surface which is bioactive in forming complexes with both AT III and F X_a. The results of this *in vitro* assay are summarized in Table II. The calculated amount of bioactive immobilized heparin is 4.1 ($\pm$ 1.0) $\times 10^{-3}$ U cm^{-2} for $PS_5E_2H_1$ coated on glass beads and 3.3 ($\pm$ 0.6) $\times 10^{-3}$ U cm^{-2} for $PS_5E_2H_2$ coated on glass beads. These values are lower than those found by Okano *et al.* [4], who reported a bioactivity of 2.66×10^{-2} U cm^{-2} for PDMS–PEO–Hep coated on glass beads. These investigators also found antifactor X_a activity (2.65×10^{-3} U cm^{-2}) on glass beads coated with PDMS–PEO, possibly due to some nonspecific adsorption of F X_a to this coating. From our results there is no indication for nonspecific adsorption of F X_a to the PS–PEO coating.

The differences in antifactor X_a activities for glass beads coated with $PS_5E_2H_1$ and $PS_5E_2H_2$, can be explained as follows. In the synthesis of $PS_5E_2H_1$, native heparin (165 U mg^{-1}) was coupled to PS_5E_2 using EDC, while $PS_5E_2H_2$ was synthesized using the less bioactive nitrous acid-degraded heparin (65 U mg^{-1}) in combination with reductive amination [15]. As expected, the $PS_5E_2H_1$ block copolymer showed a higher bioactivity as compared to that of the $PS_5E_2H_2$ block copolymer. The same differences in antifactor X_a activities are found when the coated glass beads were first hydrated for 30 h, although the calculated amounts of surface-immobilized heparin are higher. Two possible explanations for the later observation can be given. (a) Water is penetrating the coating surface, allowing relaxation and mobilization of heparin molecules near the surface. (b) The PEO domains are swelling in contact with water to compose a larger area of the coating surface exposed to water. Both explanations give rise to a greater availability of surface-immobilized heparin to play an active role in the inactivation of F X_a.

For samples PS_5E_2, $PS_5E_2H_1$ and $PS_5E_2H_2$, the amount of active heparin available at the surface of coated glass beads was also estimated using method (c). To neutralize thrombin activity, heparin molecules must contain binding sites for both AT III and thrombin. From Table II it can be seen that for both $PS_5E_2H_1$ and $PS_5E_2H_2$ coated glass beads the same trend is observed as is observed for those obtained by method (b). Although, all heparin activities estimated with the former method (b) are consequently higher than those estimated with method (c). Heparin is heterogeneous and contains molecules which bind both AT III and thrombin [30]. Molecules which only bind either AT III or thrombin do not contribute to the thrombin neutralization activity. Thus, this kinetic assay [method (c)] estimates only those heparin molecules with binding sites for both AT III and thrombin, whereas the antifactor X_a assay [method (b)] estimates heparin molecules with binding sites for AT III.

In conclusion, all heparin-immobilized surfaces studied showed that heparin was available at the coating surface to interact with AT III and thrombin and to prolong the formation of clots. The calculated amounts of bioactive heparin molecules available at the surface of the coating are a factor of ten lower than found by other investigators [4, 5, 20]. This is possibly due to the fact that the coating technique traps or buries part of the heparin molecules within the coating matrix, allowing only the detection of heparin at the surface that is actually bioactive. This was also found in the surface analysis of these materials using XPS and contact angle measurements [11].

4.2. Adsorption of AT III onto heparin-containing surfaces

The adsorption of AT III onto glass and PDMS coated with PS_5E_2 and $PS_5E_2H_1$ was studied using ELISA. From Fig. 1 it appears that there are no significant differences in the adsorption of AT III from PBS solutions containing different concentrations of AT III (1 IU ml^{-1} and 0.067 IU ml^{-1}) onto glass and PDMS coated with PS_5E_2 and $PS_5E_2H_1$ and hydrated surfaces, after 1 h contact time at ambient temperature. Studies by Hennink *et al.* [23] demonstrated that immobilized heparin was able to bind AT III. This binding was dependent on the type of heparin (low and high affinity for AT III) used. As expected, glass and PDMS coated with $PS_5E_2H_1$ bound a significant amount of AT III when exposed to PBS solutions containing different concentrations of AT III (1 IU ml^{-1} and 0.067 IU ml^{-1}) or to plasma (Figs 1 and 2). Surprisingly, glass and PDMS coated with PS_5E_2 also bound AT III when exposed to PBS solutions containing different concentrations of AT III or to plasma. This is probably due to preferential adsorption, or perhaps preferential binding of AT III onto the PS matrix of the PS_5E_2 coated surfaces. As was already reported by Hennink *et al.* [23], a significant adsorption of AT III from PBS solution onto polystyrene was observed. When plasma or plasma dilutions were used as a source of AT III, no adsorption was detected.

When glass and PDMS coated with PS_5E_2 and $PS_5E_2H_1$ were exposed to undiluted plasma (Fig. 2), significant differences in the adsorption of AT III were observed. For both substrates, the $PS_5E_2H_1$ coated surfaces showed the highest amounts of AT III adsorbed as compared to those on PS_5E_2 coated surfaces. This indicates that there is a specific interaction of AT III with the heparin moiety. The low adsorption of AT III from plasma as compared to that from PBS

solutions onto the copolymer coated surfaces presumably has to be attributed to the preferential adsorption of another plasma component. Grainger [31] investigated the surface effects of PS–PEO multiblock and PDMS–PEO–Hep triblock copolymers on the adsorption of proteins from buffer and plasma. It was demonstrated that surfaces coated with PS–PEO (containing 29 mol % PEO) showed an affinity for albumin > γ-globulin > fibrinogen from plasma. Protein adsorption from plasma onto PDMS–PEO–Hep coated surfaces demonstrated high levels of albumin and γ-globulin adsorption and a low level of fibrinogen adsorption.

4.3. Plasma recalcification times

The results obtained from the recalcification time studies, using method (a), clearly show that the recalcification times of plasma in contact with glass coated with heparin-containing block copolymers were strongly prolonged (Fig. 3). This prolongation is possibly due to the exposure of covalently coupled heparin to the plasma, thereby neutralizing activated clotting factors. The PS–PEO–NH_2 diblock copolymers coated on glass also cause prolonged clotting times as compared to those of plasma in contact with uncoated glass and PS coated on glass. Coatings of diblock copolymers which are composed of heterogeneous microphase-separated microdomains have an effect on the adsorption of plasma proteins [32] and possibly on the adsorption of clotting factors, thereby retarding the clotting process. These findings are in close agreement with observations made by Grainger *et al.* [9, 31].

The results obtained with method (b) demonstrate that, as compared to the uncoated materials, the recalcification times of plasma exposed to glass, Biomer®, PDMS and PS coated with heparin-containing block copolymers are significantly prolonged, except for $PS_1E_2H_2$ and $PS_5E_2H_2$ coated on PS coated glass tubes. Related to this last observation is the fact that the recalcification times of plasma in contact with the $PS_2E_2H_1$ and $PS_5E_2H_1$ coating tend to be longer than those in contact with the $PS_1E_2H_2$ and $PS_5E_2H_2$ coating. This is possibly due to differences in bioactivity of the coupled heparins [H_1 (165 U mg^{-1}) versus H_2 (65 U mg^{-1})]. From Fig. 4 it appears that the highest inhibitory effect on the surface-induced coagulation is reached when heparin-containing block copolymers are coated on Biomer® coated glass, whereas those coated on PS coated glass show only minor effects. Heparin-containing block copolymers coated on glass and PDMS coated glass demonstrate intermediate inhibitions.

Also of importance is the observation that plasma removed from tubes coated with heparin-containing block copolymers (after an incubation time of 15 min) showed recalcification times comparable to those of plasma exposed to uncoated tubes. This indicates that heparin or heparin-containing block copolymers had not leached from the coating and that the immobilized heparin was able to interact with the coagulation proteins.

In conclusion, coatings, of heparin-containing block copolymers on different materials (glass, Biomer®, PDMS and PS) are very effective in inhibiting the surface-induced coagulation, as measured by plasma recalcification times. The prolongation of recalcification times of plasma was not caused by the release of heparin or heparin-containing block copolymers from the coating into the plasma.

4.4. *In vitro* platelet studies

To investigate the influence of heparin, as part of the heparin-containing block copolymers, on the adhesion and aggregation of platelets in contact with a polymer surface, platelet adhesion experiments were performed. The platelet adhesion test was designed to measure the number of platelets remaining in the PRP (3×10^5 platelets ml^{-1}) solution after various incubation times with glass beads coated with heparin-containing block copolymers. Fig. 5 and also Fig. 6 to some extent, shows that glass surfaces coated with Biomer®, PEO and PS showed significantly higher degrees of platelet adhesion as compared to those of glass coated with PS–PEO and PS–PEO–Hep block copolymers, after 1 h incubation time. Furthermore, the percentages of adhered platelets to PS–PEO coated glass beads were not significantly different from those to PS–PEO–Hep coated glass beads.

The effects of hydrophilic and hydrophobic microdomains on the mode of interaction between ABA-type block copolymers, composed of HEMA and styrene and platelets was studied by Okano *et al.* [33]. In the case of homopolymers and random copolymers, a significant degree of platelet adhesion and aggregation was observed, using a microsphere column method. The degree of platelet adhesion and deformation was suppressed on the surfaces of the block copolymers, whose microdomains were hydrophilic–hydrophobic lamellae and isolated hydrophilic islands in hydrophobic matrices, respectively. It was concluded that the microphase-separated structures were antithrombogenic and prevented platelet adhesion and deformation. In an earlier report, Okano *et al.* [34] found that plasma proteins selectively adsorbed to the microdomain surface of the HEMA–styrene block copolymers. Serum albumin selectively adsorbed to HEMA domains and γ-globulin or fibrinogen to styrene domains. It was considered that the "microphase-separated" protein layer formed on the surface of the block copolymer played a significant role in the suppression of platelet adhesion and shape change. The possible preferential adsorption of AT III to neutralize thrombin, instead of adsorption of albumin, γ-globulin and fibrinogen, to PS–PEO and PS–PEO–Hep coated surfaces may have accounted for the decrease in platelet adhesion.

As discussed by Kim *et al.* [35] the effect of heparin, both in solution and immobilized at surfaces, on the aggregation and the adhesion of platelets is still not fully understood. Hennink *et al.* [36] showed that precoating of polymers with an albumin–heparin conjugate led to a significant decrease in blood platelet adhesion, whereas pre-adsorption of albumin resulted

in a slight reduction of platelet adhesion. The results indicated that complexes of AT III and conjugate formed at the material surface after contact with blood reduced platelet adhesion. These results supported the observation made by Ebert and Kim [37] that immobilized heparin was covered with a layer of absorbed proteins, probably AT III, which prevented direct contact between immobilized heparin and platelets. Mori *et al.* [38] reported on the adsorption of blood elements onto polyvinylchloride-*graft*-methoxypoly(ethyleneglycol)monomethacrylate (PVC- *g*-M_nG), with PEO chains of various chain lengths (n = 4–100). *In vitro* as well as *in vivo* studies showed that the number of adhered platelets significantly decreased with an increase in PEO chain length (n), to an almost negligible value at $n = 100$. Long term peripheral vein implantation studies (longer than one day) demonstrated depositions of a double layer of plasma proteins on the surface of PVC-*g*-$M_{100}G$. Although the mechanism for generation of the double layer was not clarified, it seemed that the PEO chains ($n = 100$) protracting from the surface into the blood in some way constructed the double layer [4 nm $\approx$ end-to-end distance for PEO ($n = 100$) chain]. It was concluded that this double layer of long chain PEO played a role as buffer, due to the superior flexibility, hydrophilicity and biocompatibility of the long chain PEO, to minimize the denaturation of blood elements.

From the above considerations it is concluded that the synthesized PS–PEO and PS–PEO-Hep block copolymers coated onto material surfaces show no adverse effect on the adhesion and aggregation of platelets.

4.5. *Ex vivo* A–A shunts

The non-thrombogenicity of polyester–polyurethane tubings coated with PS–PEO or PS–PEO–Hep block copolymers in whole blood was evaluated *ex vivo* in rabbits by the newly developed low-flow-rate A–A shunt method of Nojiri *et al.* [12]. In this experiment, the occlusion time (defined as the time required for the formation of a stable, non-embolized thrombus which is large enough to occlude the blood flow in the tubing) was measured by monitoring the blood flow with an ultrasonic flow meter. To minimize non-laminar flow effects, the shunt flow rate was maintained at a constant 2.5 ml min^{-1}.

Fig. 7 shows occlusion times of blood in contact with polyester–polyurethane tubings coated with PS–PEO and PS–PEO–Hep block copolymers. With the heparinized tubings significant prolonged occlusion times were observed, as compared to those of PS–PEO coated tubings. The heparinized surfaces appeared to be effective in suppressing fibrin and platelet deposition to remain patent for a longer time. These findings are in agreement with observations made by Grainger *et al.* [4, 9, 31], who reported that PDMS–PEO–Hep block copolymer coated onto polyester–polyurethane tubing revealed a prolongation of the occlusion time over a control PDMS surface (204 $\pm$ 31 min versus 16 $\pm$ 0 min).

Furthermore, the PS–PEO coated surfaces demonstrated significant prolongation of occlusion times as compared to those of control surfaces. It seems that block copolymer surfaces that exhibit microphase-separated structures of hydrophilic and hydrophobic domains have an influence on the behaviour of blood with these polymer surfaces. Okano *et al.* [34, 39–41] recognized this phenomenon and recently Grainger *et al.* [9, 31] demonstrated that polyester–polyurethane tubings coated with PS–PEO multiblock copolymers showed a marked increase in occlusion times as compared to those of tubings coated with homopolymers (uncoated, with PEO and PS).

In conclusion, coatings of heparin-containing block copolymers onto polyester–polyurethane tubings prevent extensive thrombus formation on the inner surface of these tubings as measured with the *ex vivo* A–A shunt method. These *ex vivo* A–A shunt correlated closely with results obtained with *in vitro* platelet adhesion experiments.

5. Conclusions

The estimation of the heparin activities of surface-immobilized, heparin-containing block copolymers was performed using three different methods. It was demonstrated that heparin was available at the coating surface of all heparin-bound surfaces studied to interact with AT III (as measured with the chromogenic antifactor X_a assay) and thrombin (as measured with a recently developed chromogenic kinetic assay) and to prevent the formation of clots (as measured with the APTT assay). The maximum surface-immobilized heparin activity (5.5×10^{-3} U cm^{-2}) was found on hydrated $PS_5E_2H_1$ coated glass beads.

The adsorption of AT III onto glass and PDMS coated with heparin-containing block copolymers was studied using ELISA. No differences in adsorption of AT III onto glass and PDMS coated with PS–PEO and PS–PEO–Hep were observed, when AT III–buffer solutions were used, due to preferential adsorption of AT III onto the PS matrix of the block copolymer coated surfaces. When AT III was adsorbed from plasma, the highest amounts of adsorbed AT III were found on substrates coated with heparin-containing block copolymers, due to a specific interaction of AT III with the heparin moiety.

Different materials (glass, Biomer®, PDMS and PS) were coated with heparin-containing block copolymers and the recalcification times of plasma exposed to these surfaces were determined. Coated surfaces showed a significant prolongation of the plasma recalcification times as compared with control surfaces, due to surface-immobilized heparin.

The platelet adhesion demonstrated that platelets reacted only minimally with the heparin-containing block copolymers in the test system and that the heparin-containing block copolymers seemed to passify the surface as compared to control surfaces.

In the *ex vivo* A–A shut experiments under low flow and low shear conditions the heparin-containing block copolymers exhibited prolonged occlusion

times, indicating the ability of these heparin-containing block copolymers to inhibit thrombosis in whole blood.

The blood compatibility of biomaterials can be improved through the use of an ABC type block copolymer, consisting of a hydrophobic block of polystyrene, a hydrophilic spacer block of poly(ethylene oxide) and a bioactive block of heparin, as a coating.

Acknowledgements

The authors wish to thank Miss C. Nojiri for carrying out the *ex vivo* experiments and Dr D. Grainger, Mr J. Lin and Mr K.D. Park for their assistance in assaying the platelet adhesion. This work was partially supported by NIH Grant HL-17623-14.

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Received 18 February 1992
and accepted 2 February 1993