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Amphiphilic networks

VIII. Reduced in vitro thromboresistance of amphiphilic networks

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ABSTRACT

Platelet adhesion to and blood coagulation activation by amphiphilic networks (i.e., water soluble acrylates crosslinked by hydrophobic polyisobutylene) have been investigated in reference to polyethylene (PE) and poly(vinyl chloride) (PVC) controls in vitro. Among the amphiphilic networks examined, that containing poly(N,N-dimethylacrylamide) exhibited lowest thrombogenicity and showed significantly lower platelet adhesion than PVC, i.e., the reference with the lowest thrombogenicity. The amphiphilic network containing poly(2-dimethylaminoethyl methacrylate) exhibited less thromboresistance as judged by the adherence of a large number of procoagulant platelets. According to these findings precisely tailored amphiphilic networks may give rise to materials with blood compatibility superior to those currently in use.

INTRODUCTION

Conventional synthetic polymers, such as PE, PVC, poly(ethylene oxide), polyurethanes, are widely used modern biomaterials. However, their application is limited by intrinsically thrombogenic surfaces. Thus, medical devices in contact with circulating blood of these polymers (i.e., catheters and extracorporeal circuits) cannot be used without systemic anticoagulation.

Surface modification of various conventional polymers by heparin has been reported to improve blood compatibility to a degree that systemic heparin can be reduced or even omitted in some clinical settings, i.e., extracorporeal circulation with oxygenator (1). The rate of success of this approach is quite satisfactory, but only for a limited number of applications. For instance, the occlusion of small diameter prosthetic vascular grafts cannot be prevented by surface modification with heparin. The lack of biocompatibility arises because even surfaces that are modified with bioactive substances like heparin, cannot exert the needed active biological functions of endothelial cells lining the walls of blood vessels (2).

Different materials may exhibit greatly different effects in regard to activating biological functions, such as blood coagulation, immune response, etc. According to current thinking, the introduction of various chemical and physical surface characteristics determine biological response of surfaces, i.e., the nature and extent of preferential protein adsorption, conformational changes leading to biological activation (3). Precisely tailored polymers with well-defined surfaces may give rise to synthetic materials with desirable bio- and hemocompatibility for biomaterials applications. Thus it may be possible to design/develop surface microarchitectures which may preferentially bind specific plasma proteins that lead to the passivation of blood contacting surfaces. A series of well-defined macromolecular composites, so called amphiphilic networks, have recently been prepared

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and have been found to exhibit promising thromboresistance (4-6), specifically, for the preparation of small diameter prosthetic vascular grafts (7).

The present study concerns in vitro thrombogenicity testing of various amphiphilic networks consisting of a hydrophilic main chain of poly(2-dimethylaminoethyl methacrylate), poly(2-hydroxyethyl methacrylate), and poly(N,N-dimethyl acrylamide) (abbreviated by D, H, and A in the network code) crosslinked with a hydrophobic methacrylate-capped polyisobutylene chain. In line with earlier publications of this series (4), the network code also shows the molecular weight of the polyisobutylene crosslinker (4500 g/mol in this work), and the weight percent of polyisobutylene (50 wt.%). We found significant differences in platelet adhesion and blood clotting activity among the amphiphilic networks examined, and found A-4.5-50 to exhibit significant thromoresistance.

EXPERIMENTAL

The controls PE (batch MöS) 2790, and PVC (T; batch 29-859 containing tri(ethylhexyl)trimellitate as plasticizer) in the form of 50μ films were kindly provided by W. Lemm, Eurobiomat, Berlin. The preparation and characterization of amphiphilic networks A-, D-, and H-4.5-50 have been described (5,8). Prior to experiments the materials were pretreated (swollen) in phosphate-buffered saline (PBS) for 3 days at room temperature. The blood coagulation factors were isolated and quantitated as previously described (9). All other reagents were of the highest commercially available purity.

Plasmas

Blood was obtained from healthy males free of medication known to influence platelet functions at least 14 days prior to the donation. Blood samples were collected by the use of disposable syringes with one part of 3.8% sodium citrate to nine parts of blood. The preparation of citrated platelet free plasma and citrated platelet rich plasma have been described (10).

Partial Thromboplastic Time (PTT)

Citrated plasma (470 μL) in contact with various polymers was incubated with 10 μL phospholipid (25/75 mol% phosphatidylserine/phosphatidylcholine, 2 nM) for 15 mins at 37°C. Clotting was induced by the addition of 20 μL aqueous $CaCl_2$ (0.5 M).

Platelet Medicated Clotting Time (PMT)

Microplates (Costar, Cambridge, UK) containing the polymer films exposed to citrated platelet rich plasma (480 μL) for 15 mins were placed in a plate reader (SLT Lab Instruments, model 340 ATCC, Salzburg, Austria) at 37°C. Clotting was initiated by the addition of 20 μL of a 0.5 M $CaCl_2$ solution. The final free Ca^{2+} concentration was 4 mM. The optical density was recorded at 405 nm every 15 s for each well. The clotting time was taken as the peak value of the first derivative of the tracing.

Thrombin Generation

Samples (2 μL) were removed from the recalcified platelet rich plasma in the wells containing the polymers and assayed for thrombin by the use of the chromogenic substrate S2238 (Chromogenix, Mölhdal, Sweden). The curves of thrombin generation thus obtained were analyzed for free thrombin according to Hemker et al. (11).

Platelet Adhesion

Polymer surfaces exposed to citrated platelet rich plasma or citrated whole blood were rinsed with PBS. The number of platelets adhering to surfaces were quantitated by determining the lactate dehydrogenase (LDH) content. To this end, the washed polymer surface was incubated with a solution of nicotinamide adenine dinucleotide (NADH) (240 μM) and 1% Triton X100 in PBS for 30 s. Pyruvate (0.5 mg/mL) was added and the

change in optical density at 340 nm was recorded. A reference plot was constructed by using known amounts of platelets; the slope of the plot was 8.4 mU LDH/10^5 platelets.

Platelet Procoagulant Activity

Preswollen samples of tubes (5 cm long, 2-4 mm ID) were incubated with citrated whole blood or citrated platelet rich plasma for 20 mins at 37°C. Then the tubes were rinsed twice with 1 mL of 20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 0.19 M NaCl of pH=7.5 and incubated with a mixture consisting of factor Xa (50 pM), factor Va (100 pM) and prothrombin (500 nM). Aliquots were removed at desired time intervals and assayed for thrombin activity.

RESULTS AND DISCUSSION

Blood Clot Promoting Activity

Segments (1 cm length, 2-4 mm ID) of swollen tubing were incubated with citrated platelet free plasma for 20 mins at 37°C, aliquots were removed and assayed for a global test (PTT) for contact activation. Table I shows clotting times of amphiphilic networks in reference to glass, the positive control. All networks exhibited longer clotting times than glass, with A-4.5-50 showing the highest thromboresistance.

Table I. Partial thromboplastin time (PTT) of platelet free plasma exposed for 20 mins to test surfaces. Tests were performed in triplicate and results are given as the mean value ± SD.

Material	Clotting time (s)
Glass	333 ± 45
A-4.5-50	715 ± 74
D-4.5-50	508 ± 47
H-4.5-50	515 ± 48

Table II. Global clotting tests (PTT and PMT) and corresponding thrombin generation data of plasma exposed to surfaces

Material	Global Clotting Test		Thrombin Generation			
	PTT	PMT	Peak value		Peak position	
			PRP*	PFP**	PRP	PFP
	(s)	(s)	(nM)		(s)	
PE	534	288	148	220	510	750
PVC	558	726	55	238	1080	780
A-4.5-50	680	852	82	235	1200	840

* platelet rich plasma
** platelet free plasma

We then determined the clotting times of recalcified platelet rich plasma exposed to A-4.5-50 in reference to PE and PVC. According to the results compiled in Table II A-4.5-50 exhibits superior thromboresistance to PE or PVC. Clearly, PE exhibited the highest clot promoting activity by this test which employs platelets rather than artificial phospholipid procoagulant surfaces.

Thrombin Generation in Recalcified Plasma

Rates of thrombin generation in plasma exposed to experimental materials provide important insight into the intrinsic thrombogeniety of their surfaces. According to the results shown in Figure 1A, thrombin generation on PE and PVC incubated with citrated platelet free plasma in the presence of phospholipids prior to calcium addition is within experimental error; the onset of thrombin generation occurred at 534 s and 558 s, respectively. In contrast to the controls, A-4.5-50 shows clearly retarded thrombin generation, indicating a somewhat slower contact activation. Detectable amounts of thrombin were seen around 660 s. It should be emphasized that in the absence of exogenous phospholipids the onset of thrombin generation is dramatically delayed (> 30 mins) in these tests.

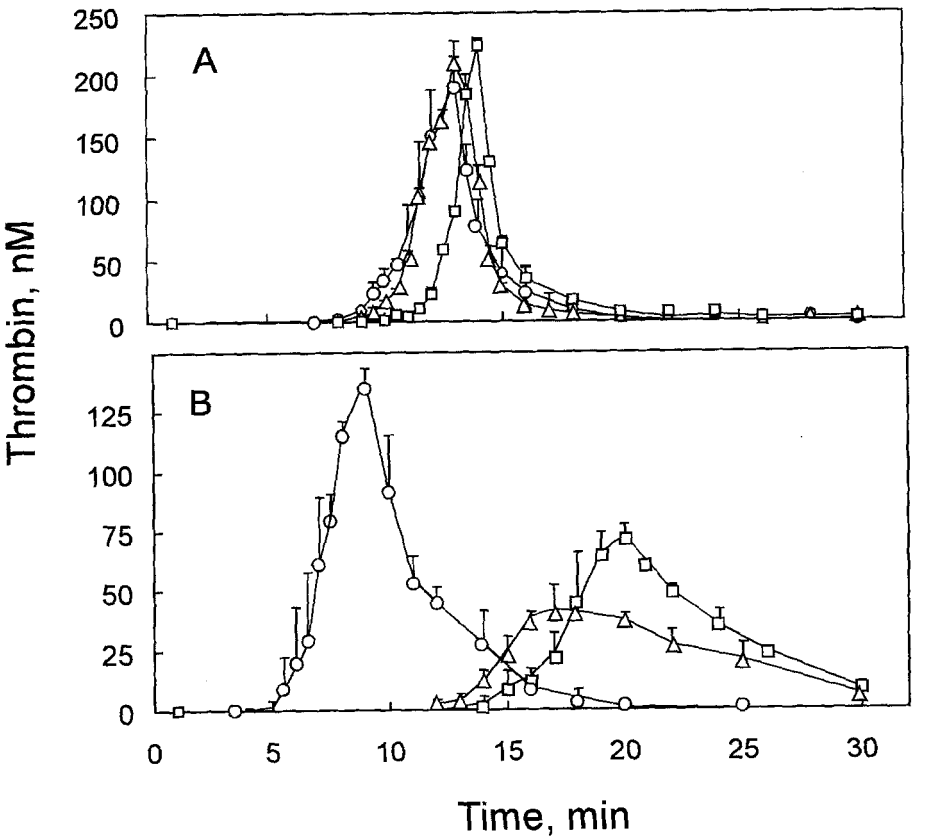

Figure 1. Thrombin formation on PE (O), PVC (Δ), and A-4.5-50 (❑). Surfaces exposed to (A) recalcified platelet free plasma containing 50 μM phospholipid; (B) platelet rich plasma. Tests were performed in triplicate; error bars indicate standard error of the mean.

After blood clotting initiation, propagation of thrombin formation (and thus thrombus formation) requires procoagulant phospholipids provided by activated blood platelets (12). We anticipated that different extent of platelet activation by different surfaces will produce marked differences in thrombin formation in platelet rich plasma. Figure 1B shows thrombin formation in recalcified platelet rich plasma incubated with PE, PVC and A-4.5-50. In contrast to the results of experiments with platelet free plasma shown in Figure 1A, in the experiments with platelet rich plasma and the rate of thrombin generation by PE is much higher than that by PVC. Importantly, the onset of thrombin generation is most delayed in conjunction with A-4.5-50 although the thrombin peak is somewhat higher than that of PVC.

These findings are reinforced by the results of global clotting tests shown in Table II. Evidently, the clotting times determined by both PTT and PMT indicate superiority of A-4.5-50, in reference to PE and PVC, in regard to thromboresistance. We propose that the reduction of the extent of platelet adhesion and subsequent activation (which result in the exposure of procoagulant sites) is critically important for thromboresistance to develop.

Platelet Adhesion and Formation of Procoagulant Sites

Samples of amphiphilic networks and references were incubated for 30 mins with citrated platelet rich plasma and whole blood. The number of platelets adhering to the surfaces was determined from the LDH content. Table III shows the results. The correlation between the number of adhering platelets and thrombogenicity of surfaces is evident as assessed by thrombin formation in platelet rich plasma (Table III and Figure 1). Compared with the reference materials only few platelets were found to adhere to A-4.5-50.

Table III. Platelet adhesion by different materials exposed to platelet rich plasma.

Material	10^3 Platelets/cm^2
Glass	2700
PE	1572
PVC	320
D-4.5-50	200
H-4.5-50	50
A-4.5-50	30

To support our proposition that adhering platelets are procoagulant and promote thrombin generation, we have determined the rate of thrombin formation on surfaces pretreated with whole blood and then incubated with mixtures of the purified blood coagulation factors prothrombin, factor Xa and factor Va. Figure 2 shows the amount of thrombin formed at the various surfaces examined as a function of time. As expected, the correlation between the number of adhering platelets and thrombin formation is excellent. According to this study both networks A-4.5-50 and H-4.5-50 are highly thromboresistant. In fact, we observed for these networks that the same activity as that of the negative control, i.e., glass exposed to platelet free plasma.

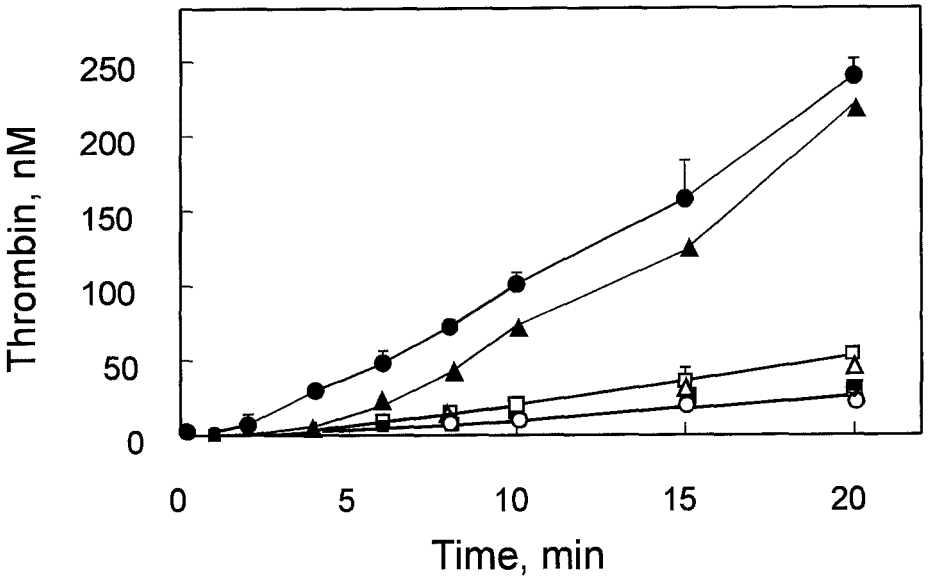

Figure 2. Thrombin formation at surfaces of tubes of PE (●), D-4.5-50 (▲), PVC (Δ), A-4.5-50 (□), and H-4.5-50 (O). Tubes were incubated with citrated blood and rinsed with 20 mM Hepes, 190 mM NaCl, pH=7.5. Thrombin generation was monitored upon incubation of the surfaces with mixtures of prothrombin (500 nM), factor Va (0.1 nM) and Factor Xa (0.05 nM) in 20 mM Hepes, 190 mM NaCl, 5 mM $CaCl_2$, pH=7.5, containing 0.5 mg bovine serum albumin/mL. Thrombin generation at the surface of glass incubated with platelet free plasma prior to the incubation with prothrombin, factor Va and factor Xa is indicated by ■.

CONCLUSION

On the basis of our platelet-dependent coagulation tests, platelet adhesion and platelet activation (leading to procoagulant sites) studies we conclude that the amphiphilic network A-4.5-50 consisting of poly(N,N-dimethylacrylamide) crosslinked by polyisobutylene is less thromogenic than PE and PVC, i.e., materials commonly used in blood contact applications.

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