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Amphiphilic networks

V. Polar/nonpolar surface characteristics, protein adsorption from human plasma and cell adhesion*

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ABSTRACT

The surface energetics, specifically the balance of polar/nonpolar forces on the interfaces of various amphiphilic networks comprising hydroxyethyl methacrylate (HEMA) or N,N-dimethylacrylamide (DMAAm) hydrophilic chains linked by polyisobutylene (PIB) hydrophobic chains (for brevity H and A networks) in contact with water have been studied by dynamic contact angle measurements. Both networks show large contact-angle hysteresis due to surface heterogenity and surface rearrangements. The balance of the polar/nonpolar forces of the dry networks, as quantitated by the I_p/W_A^d ratio is much below unity; upon equilibrating in water the I_p/W_A^d ratio increases but remains below unity. Protein adsorption from human plasma and human monocyte adhesion to A and H amphiphilic networks possessing polar/nonpolar ratios lower than unity have been investigated. Both networks adsorb less fibrinogen, albumin and Hageman factor (factor XII) than glass, polyethylene (PE), and polydimethylsiloxane (PDMS). The extent of adsorption of factor VIII on A, H and glass are very similar. Adsorption of IgG on A was appreciable, however, on H it was less than on any of the other surfaces studied. Monocyte adhesion was significantly inhibited on both networks and glass, relative to a positive adhesive surface such as tissue culture polystyrene (TCPS). Evidently both the A and the H networks exhibit reduced protein adsorption and cell adhesion which indicates biocompatibility of these networks at blood contacting surfaces. The ratio of the polar/nonpolar forces expresed by the I_p/W_A^d ratio may be useful to predict low protein adsorption and cell adhesion on polymer surfaces.

INTRODUCTION

Amphiphilic networks are randomly crosslinked hydrophobic-hydrophilic polymer chains that swell both in water and hydrocarbon solvents (1). We have prepared and characterized a series of amphiphilic networks by copolymerizing methacrylate-ditelechelic polyisobutylene (MA-PIB-MA) of various molecular weights with various water-soluble monomers, (e.g., HEMA, DMAAm) (1-3). Recently we have studied the diffusional and drug (theophilline) -release characteristics of PHEMA-*l*-PIB, and PDAAm-*l*-PIB (abbreviated respectively by H and A) networks of various compositions immersed in water and in *n*-heptane (2-4). We have discovered that these amphiphilic networks within a certain compositional range (i.e., close to 50/50 wt% hydrophilic/hydrophobic compositions) exhibit excellent biocompatibility and biostability *in vivo* in rats (5).

Orienting research carried out jointly with several groups of investigators over a period of years (see Acknowledgements) have

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also suggested that our amphiphilic networks (particularly the A and H networks) were hemocompatible. Encouraged by this, we have investigated the surface characteristics of our amphiphilic networks in some detail with the aim of correlating these with hemocompatibility. This problem of course, has also been studied by others (6-8).

Our approach was to prepare well-defined A and H networks, characterise their surfaces by dynamic contact angle measurements to obtain the overall polar/nonpolar characteristics of the surfaces, (i.e., I_p/W_A^d ratios, see below) and to correlate this quantity with protein adsorption determined by ^{125}I radioimmunoassay, as well as with blood monocytes adhesion. Ratner et al. first suggested that a balance of polar/apolar sites at a polymer surface is needed for blood compatibility (9).The premise of this hypothesis is that certain for hemocompatibility beneficial proteins will be adsorbed and remain adherent if the polar and apolar (i.e., the hydrophilic and hydrophobic) character of the surface is properly balanced. Baszkin and Lyman related quantitatively the I_p/W_A^d ratio of the surface-water interfaces to protein adsorption (10,11). A comparison of surface energetics with results gleaned from the literature support the I_p/W_A^d ratio concept (10,12,13). Thus, it seems that the level of irreversible protein adsorption is reduced when $I_p/W_A^d>1$ or $I_p/W_A^d<1$, while protein adsorption is maximum when the polar/nonpolar forces are balanced on the surface. Protein adsorption on biomedical materials is critical for blood compatibility (14,15). Vroman et al. have demonstrated that protein adsorption occurs rapidly and follows a specific sequence (16,17): Initially fibrinogen, immunoglobulin G (IgG), and albumin are adsorbed which are later replaced by high molecular weight kininogen and, to a lesser extent, by factor XII. (Hageman factor). Ziats et al. demonstrated that trace proteins adsorb simultaneously with other proteins and may play a significant role in thrombus formation (18). They also demonstrated that radioimmunoassay can be used for protein adsorption determination. The advantages of this method are the ability to detect proteins adsorbed from a complex fluid (such as blood) to surfaces, and the detection of virtually any protein provided an antibody to the protein of interest is available. The adhesion (antiadhesion) of blood cells, in particular leukocytes and platelets, is also important for biocompatibility (19,20).

This paper concerns the results of the first phase of our investigations directed toward the design and development of novel hemocompatible materials. Specifically, it indicates, that the I_p/W_A^d ratio may provide guidance in selecting surfaces for biomaterials use, and that the protein adsorption and cell adhesion characteristics of amphiphilic networks may hold promise for the synthesis of blood-compatible surfaces.

EXPERIMENTAL

The synthesis of networks and their characterization by solubility studies, swelling and diffusional experiments, have been described (1-4). Crosslinked PIB and PHEMA were prepared by radical polymerization of MA-PIB-AM and copolymerization of HEMA with 5 mol% of ethylene glycol dimethacrylate, respectively. Conditions of the synthesis were the same as described above (1-3).

Table I summarizes some characteristics of the networks used in these studies.

Table I. Characteristics of Amphilic Networks Used

Abbreviation	Symbol	Hydrophobe*/ hydrophile wt%	$\bar{M}_n$ of PIB g/mol	$\bar{M}_c$** of hydrophile g/mol
PHEMA	PHEMA	0/100	-	-
PHEMA-l-PIB	H-4.5-40	40/60	4,500	3,400
	H-4.5-50	50/50	4,500	2,300
	H-4.5-60	60/40	4,500	1,500
PIB	PIB	100/0	4,500	-
PDMAAM-l-PIB	A-4.5-50	50/50	4,500	2,300

*PIB

**Calculated from $\bar{M}_c = W_h \times \bar{M}_n / 2W_{PIB}$, where W_h and W_{PIB} are the weight fraction of the hydrophilic polymer and the PIB, respectively, $\bar{M}_n$ is the number average molecular weight of the MA-PIB-AM (which is the $\bar{M}_c$ for the hydrophobic component).

Contact Angle Measurements

Dynamic contact angle measurements were made by using a Cahn DCA-312 instrument. Both advancing and receding angles were obtained at a platform speed of 120 μm/sec. Averages of five measurements were taken for each liquid (i.e., water, glycerol, diiodomethane, ethylene glycol, dimethyl sulfoxide, and hexadecane) and polymers. The solid-liquid work of adhesion (W_A) was calculated by

$$W_A = \gamma_1(1 + \cos \theta) \qquad (1)$$

where γ_1 is the surface tension of the test liquid and θ the contact angle of the same liquid measured on the polymer (equation (1) was obtained combining the basic Dupre and Young equations). The dispersive (W_A^d) and polar (I_p) terms of solid-liquid work of adhesion were obtained by Baszkin et al.'s procedure (10,11): Plotting cos θ versus $(\gamma_1^d)^{1/2}/\gamma_1$ yields a straight line whose intercept gives $(\gamma_s^d)^{-1/2}$ (γ_1^d and γ_s^d are the dispersive force contributions to the surface free energy of the liquid and the solid, respectively). W_A^d and I_p are obtained from equations (2) and (3), respectively (10,21,22).

$$W_A^d = 2(\gamma_s^d \gamma_1^d)^{1/2} \qquad (2)$$

$$I_p = W_A - W_A^d \qquad (3)$$

Protein Adsorption from Human Plasma

Networks, H and A were stored in phosphate buffered saline solution (PBS, Sigma #1000-3) containing sodium azide until evaluation. Other materials included PDMS (Mercor-Thoratec, Inc.), PE (Abiomed Corp.), and alcohol cleaned glass coverslips (#3550, Gold Circle, Thomas Scientific). Round disks, ~15mm in diameter, punched from the materials, were placed into Falcon (#3047) 24 well tissue culture plates and secured by placing a silicone rubber ring (#06411-80, 16mm O.D.,Cole-Palmer) over the material to prevent floating. The samples were rehydrated at 4°C for two days and then prehydrated for one hour with PBS at room temp-erature prior to experimentation. Human citrated (0.01M) platelet poor plasma was obtained from eight fasted healthy donors and the prepared plasma pooled and frozen until use. The samples were rinsed once with PBS, and then incubated for sixty minutes with 1 mL of either 100% plasma or 1% plasma (diluted with PBS) at 37°C. The plasma was removed and the materials rinsed 3x with PBS followed by addition of 2 mL of a milk solution (15% nonfat dry milk in PBS with 2mL Na_2EDTA). The samples were refrigerated at 4°C overnight, then rinsed 3x with PBS. The methods for determination of protein adsorption by radioimmunoassay have been described (18,27,). Primary antibodies, rabbit antihuman IgG fractions (10mg/mL stock), were

diluted 1:500 in PBS containing 1% chicken egg albumin (ovalbumin) and 1 mL was added to each material for 60 minutes at 37°C. The samples were again rinsed with PBS 3x and the milk solution added for overnight at 4°C. The samples were rinsed with PBS (3x) and 1 mL of ^{125}I goat anti-rabbit IgG (7 uCi/mL, 250,000 CPM) was added to each material for 60 minutes at 37°C. After incubation the samples were rinsed 3x with PBS, the silicon rubber sleeve removed, the materials placed into test tubes and counted in a gamma counter.

The data are expressed as CPM of ^{125}I binding/2cm^2 area of polymer and of the second antibody to the primary antibody of the protein of interest including: FB=fibrinogen, IgG=immunoglobulin G, ALB=albumin, HF=Hageman factor (factor XII), VIII=factor VIII/von Willebrand, AP=alphafetoprotein control and PBS=1% ovalbumin/PBS control. AP represents the background control for the primary antibodies while PBS represents the control for background binding of ^{125}I to the materials. The bar graphs represent the mean values of two experiments ±SD. In each experiment duplicate samples of material for each protein were determined.

Human Monocyte Adhesion

The methods for monocyte isolation have been previously described but modifications were used (20). Briefly, human monocytes were isolated from peripherial blood of healthy individuals (30mLs) using sodium citrate (0.01M) as the anticoagulant. Mononuclear cells were isolated and separated from other cells using standard Ficoll-Hypaque gradients as described (20). The separated mononuclear cells were further purified to monocytes using a 73% percoll/PBS gradient to obtained an 80% purified cell population. The cells were resuspended at a concentration of 2 x 10^5 cells/mL into RPMI 1640 medium containing 1% heat inactivated bovine serum albumin. The suspended cells (2 mLs) were added to 24 well plates containing polymer disks as described above for the protein adsorption experiments. In addition, control surfaces included glass coverslips added to the wells or plain tissue culture treated 24 well plates (Falcon #3002, Becton Dickinson). Cell adhesion to the various surfaces was determined after four hours incubation at 37 C in a humidified, 5% CO incubator. The non-adherent cells were removed by aspiration and the surfaces rinsed with PBS. The materials or wells were fixed with 100% methanol and the cells stained with May-Grunwald and Giemsa stains. The adherent cells were observed with an image analyzing system (Java, Jandel Scientific) connected to a light microscope. The number of cells were recorded (per ten high microscopic fields, 100x) and the mean and standard deviations determined.

RESULTS AND DISCUSSION

Dynamic contact angle analysis

Advancing and receding water contact angles for dry and hydrated polymers are shown in Table II. The contact angle hysteresis observed can be explained by proposing that in air (i.e.,in a very hydrophobic medium) the hydrophobic PIB segments and the hydrophobic part of the PHEMA (i.e., mainly the CH_3-groups) dominate the surfaces, while in water the hydrophilic $-CH_2CH_2OH$ side chains of the methacrylic segments are dominant (23-25). Analysis of dynamic contact angles of a polymer provides insight into the conformational mobility of the surface in different environments. The large contact angle hysteresis observed may be due to the rearrangement of surface polymeric chains and their side groups, or to surface roughness or heterogenity (25,26).

Table II. Contact angles of water measured on dry and wet amphiphilic networks.

Sample	Contact Angles, Θ			
	Dry		Wet	
	Adv.	Rec.	Adv.	Rec.
PHEMA	92.7	54.6	72.4	49.5
H-3.8-40	99.2	32.7	79.2	26.1
H-3.8-50	103.3	28.9	82.2	29.4
H-3.8-60	103.8	33.2	85.3	31.0
A-3.8-50	115.5	17.4	96.9	34.2
PIB	109.8	36.3	106.5	33.6

In view of microdomain separation inherited from the synthesis, surface conformational rearrangements and surface heterogenity are proposed to be mainly responsible for contact-angle hysteresis. Since all polymer samples were optically clear, surface roughness was considered to be of limited significance. Not surprisingly, the crosslinked PIB (which except at the vicinity of the crosslink is hydrophobically homogeneous), can only respond structurally by side chain rotations (ester groups at the crosslinks); thus PIB will show little reduction in advancing contact angle after immersion in water. In contrast, the phase-separated networks show large decreases in the advancing angle because they can conformationally restructure at a large scale and thus minimize their interfacial energy.

Contact angle measurements on these networks allow the calculation of the solid-liquid work of adhesion and the calculation of its dispersive and polar components. Data are listed in Table III and are plotted in Figure 1. Interestingly, the I_p/W_A^d ratio of the relatively less hydrophilic H network is higher than that of the A network of the same overall composition. Increasing the hydrophilic component in the H series increases also the I_p/W_A^d ratio, but all values remain less than unity. The I_p/W_A^d ratio increases upon equilibrating the networks in water, but except for the H-3.8-40 and PHEMA networks, the ratios remain below unity. Apparently, the I_p/W_A^d ratio is affected by the overall composition of the networks and by the chemical structure of the hydrophilic component, rather than by the nature of the hydrophilic moiety. Others found, that polymers having I_p/W_A^d ratios below or above unity exhibit low

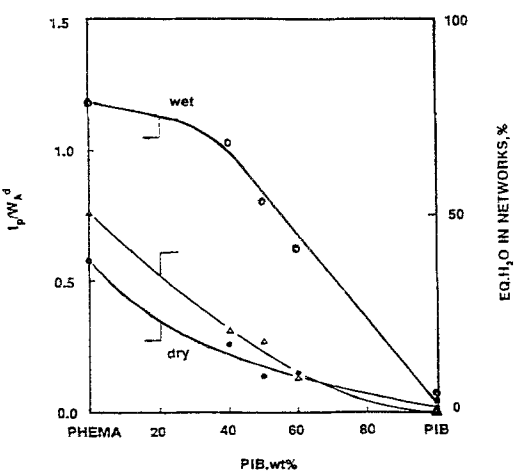

Fig.1. Equilibrium water content and I_p/W_A^d for dry and wet H-networks

protein adsorption (10,12). Based on our calculation of I_p/W_A^d values we have selected networks A-3.8-50 and H-3.8-50 as promising candidates for low protein adsorption and cell adhesion studies.

Table III. Surface energetics for polymer-water systems

Samples	Eq. water content %	γ_s^d dyn/cm		W_A dyn/cm		W_A^d dyn/cm		I_p dyn/cm		I_p/W_A^d	
		dry	wet	dry	wet	dry	wet	dry	wet	dry	wet
A-3.8-50	75.2	26.2	22.5	48.8	64.1	47.1	44.3	1.7	19.8	0.04	0.45
H-3.8-50	18.4	27.9	25.6	56.1	85.1	49.3	47.2	6.8	37.9	0.14	0.80

Protein Adsorption from Human Plasma

Protein adsorption from human plasma (100% and 1%) was evaluated on hydrated A and H networks and on the reference materials glass, PE and PDMS using radioimmunoassay (Figure 2A, B). The amphiphilic networks adsorbed less fibrinogen and albumin than glass, PE and and PDMS from 100% plasma (Figure 2A). Significantly less fibrinogen, Hageman factor (factor XII), and albumin were adsorbed to A and H from 1% plasma (Figure 2B) than the reference surfaces. The extent of adsorption of factor VIII on A and H and glass was very similar. Controls to determine nonspecific adsorption of primary antibodies (AP) and labelled ^{125}I antibody (PBS) exhibited low background binding, indicating specificity and sensitivity of the assay. The detection of IgG from either 100% or 1% plasma on A was appreciable: greater or

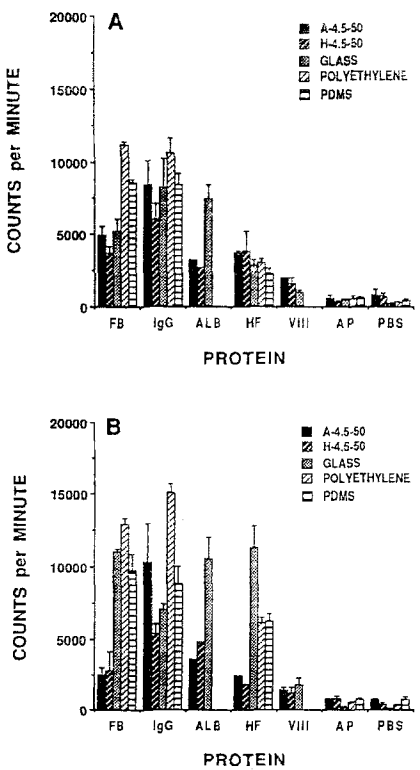

Fig.2. Protein adsorption from 100% human plasma (A) or 1% human plasma (B) after 60 minutes to polymer surfaces using antibodies to human proteins: fibrinogen (FB), immunoglobulin G(IgG), albumin (ALB), Hageman factor or factor XII (HF) and factor VIII/von Willebrand factor (VIII). Controls include alphafetoprotein (AP) and phosphate buffered saline (PBS). Results are expressed as surface counts per minute ± standard deviation of two separate experiments with duplicate polymers evaluated for each protein.

equal to than glass, less than PE, and equivalent to PDMS. Polymer H showed less adsorption of IgG than any of the other materials. Overall, these data suggest reduced protein adsorption to our amphiphilic networks with significant reduction in adsorbed fibrinogen and Hageman factor. These proteins are important in coagulation while fibrinogen is particularly important as an adhesive protein for blood leukocytes and platelets. Thus reduction of adsorption of these proteins may reduce thrombus formation. The findings with IgG show reduced adsorption to H but greater adsorption to A, however, IgG adsorbs to surfaces readily, regardless of surface composition (18,20,27).

Human Monocyte Adhesion

Another index of the biocompatibility is the evaluation of cell adhesion or antiadhesion (19,20). Thus, we have determined the adhesion of one circulating blood cell type, the human monocyte. According to the results shown in Figure 3, there was significant inhibition of monocyte adhesion after four hours to networks A and H, and glass as compared to a positive adhesive surface, TCPS. These data further support the biocompatibility of these materials at blood contacting surfaces. Evidently, our amphiphilic surfaces show reduced protein adsorption and cell adhesion as compared to other surfaces of known surface composition and biological activity.

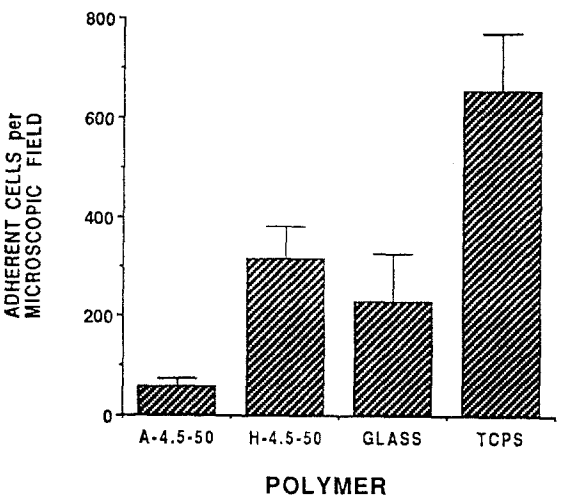

Fig.3. Human blood monocyte adhesion to polymer surfaces after 4 hours incubation in medium RPMI 1640 containing 1% BSA. Initial monocyte concentration per well is $2x10^5$/ml. The bar graphs depict the mean number of adherent cells per microscopic field (1 field = 0.43 mm^2) ± standard deviation of two separate experiments.

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