Surface Rheology of Hydrophobically Modified PEG Polymers Associating with a Phospholipid Monolayer at the Air−Water Interface

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Surface rheology of irreversibly bound hydrophobically modified poly(ethylene glycol) (PEG) polymers (HMPEG) on a dipalmitoylphosphatidylcholine (DPPC) monolayer is investigated to determine attributes that may contribute to immune recognition. Previously, three comb-graft polymers (HMPEG136-DP3, HMPEG273-DP2.5, and HMPEG273-DP5) adsorbed on liposomes were examined for their strength of adsorption and protection from complement binding. The data supported an optimal ratio between the hydrophilicity of the PEG polymer and the number of hydrophobic anchors. The HMPEG polymers have different polymer brush thicknesses (4.2−5.9 nm) and levels of cooperativity (2.5−5 hydrophobes). The results indicate that an increased viscous force (above 0.25 mN s/m) at the surface may enable the polymers to shield liposomes from protein interactions. Similar rheological behavior is shown for all polymer architectures at low polymer surface coverage (0.5 mg/m^2, in the mushroom regime), whereas at high surface coverage (>0.5 mg/m^2, in the brush regime), we observe a structural dependence of the surface viscous forces at 40 mN/m. This threshold correlates with a 92% decrease in complement protein binding for liposomes coated with 1 mg/m^2 HMPEG273-DP5. This may suggest that surface viscous forces play a role in reducing complement protein binding.

Introduction

Amphiphilic polymers are unique in that they can self-assemble and change the rheology of solutions.[1,2] Their behavior relies on the formation of intricate networks governed by thermodynamics. The attributes of these polymers have been leveraged to control chemical reactions, reduce or increase the viscosity of solutions, produce molecular networks to control protein delivery, modify phase equilibria (i.e., solubility, melting depression, etc.), and change surface properties. In biomedical applications, amphiphilic polymers, such as poly(ethylene glycol) (PEG) covalently bound to a lipid (e.g., 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)−PEG), are useful in increasing the solubility of drugs, maintaining a dispersion in solution, and as a method to modify surfaces.

Surface modification is important in biomedical applications to evade immune recognition. In particular, PEG has been studied because of its biological inertness. PEG is a unique polyether because it is water soluble, which results in specific ordering of water molecules along the polymer chain in solution.[3] It is also highly soluble in organic solvents and has been studied at interfaces, illustrating its amphiphilic behavior.[4,5] Adsorption of PEG in high concentrations (to produce a dense brush configuration) sterically protects the surfaces against protein interactions.[6,7,8] For drug delivery, liposomes incorporating 10 mol % DSPE−PEG are able to circulate in the bloodstream longer than bare liposomes.[9,10]

We have previously reported the ability of HMPEG polymers to adsorb on liposome surfaces and protect against complement binding.[11] These comb-graft copolymers are described as HMPEG"X"-DP"Y", which denotes the number of PEG monomers "X" between hydrophobic anchoring groups and the average of PEG loops "Y" per chain. Adsorption of these polymers on liposomes show reduced complement protein binding. Complement proteins are a series of proteins that activate an immune response.[12] Evidence that immune recognition was a result of complement protein adsorption was demonstrated by Liu et al.[7] Additonally, complement protein binding correlated with shorter in vivo circulation times.[8] Use of PEGylated lipids reduce the rate of protein adsorption.[6] The ability to inhibit complement protein interactions by adsorption of PEG may help evade the immune system and allow for increased systemic circulation times.

Two polymers, HMPEG136-DP3 and HMPEG273-DP5, exhibited similar adsorption profiles and protection against

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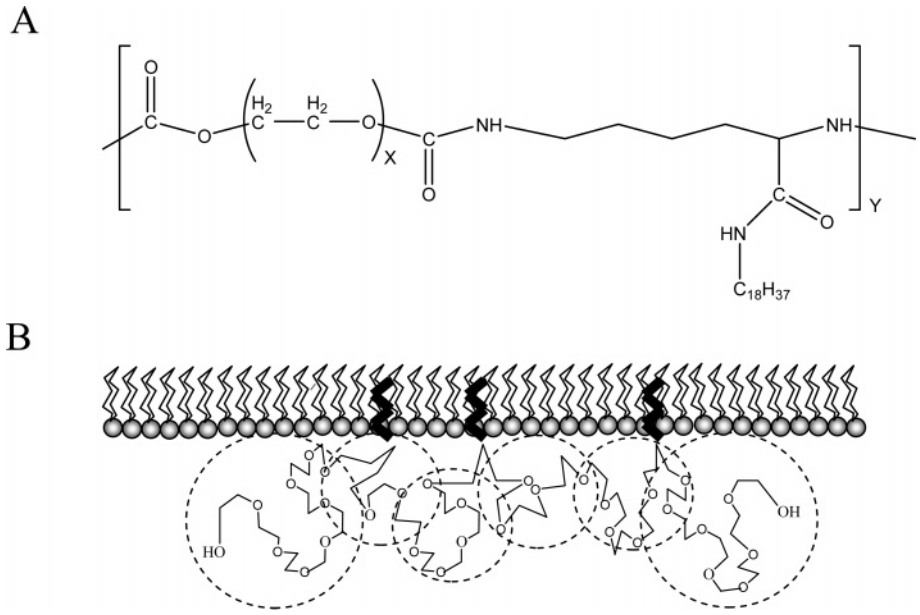

Figure 1. Schematic of (A) a hydrophobically modified poly(ethylene glycol) (HMPEG) polymer, where X is the number of PEG monomers and Y is the degree of polymerization and (B) incorporation of an HMPEG into a monolayer of DPPC at the air−water interface.

complement protein interactions.[11] These polymers are multiply attached polymers that exploit current interest in cooperativity, where multiple, relatively weak binding leads to strong overall association. The results revealed that there exists a tradeoff between the hydrophilicity of PEG and the number of hydrophobic anchors. However, differences between the two polymers' architecture lead to speculation as to how they prevent protein interactions. In particular, the thickness, level of cooperativity, and surface mobility may contribute to reduced immune recognition.

PEG covalently bound to a single lipid has been shown to influence the surface properties of a lipid monolayer. A two-dimensional gel transition occurs when the PEG cross-sectional area exceeds the lipid area;[13] therefore, there is a molecular weight dependence on the transition. Further work has shown that strong van der Waals anchoring interactions are necessary to sustain 2D gelation.[14] The surface viscoelastic behavior of a monolayer with mixtures of lipid and the lipid-linked PEG derivative has revealed insights into the nature of gelation transitions.[15] The interfacial rheological behavior of PEG adsorbed to a lipid monolayer can be used to describe the surface properties that arise due to molecular interactions.

This work focuses on the ability of multiply attached polymers to change the surface properties of a lipid monolayer. Our aim is to define the surface viscoelastic properties of HMPEG136-DP3, HMPEG273-DP5, and HMPEG273-DP2.5 bound to a DPPC monolayer at the air−water interface. Evaluation of these three polymers will determine the affect of the following: (1) the PEG chain length at approximately constant number of loops (HMPEG136-DP3 vs HMPEG273-DP2.5), (2) the number of loops at constant PEG chain length (HMPEG273-DP2.5 vs HMPEG273-DP5), and (3) the ratio of PEG monomers to hydrophobes (HMPEG136-DP3 and HMPEG273-DP5). Knowledge of the interfacial rheological behavior provides information on molecular interactions under dynamic conditions. This may identify interfacial rheological properties that result in reduced protein adsorption.

Experimental Protocol

Materials. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The hydrophobically modified poly(ethylene glycol) (HMPEG) polymers were synthesized at Rutgers University (Piscataway, NJ) as described by Heitz et al.[16,17] The comb-graft copolymers are designated as HMPEG"X"-DP"Y", where X is the number of PEG monomers and Y is the degree of oligomerization. See Figure 1A for the chemical structure of HMPEGs. Sheep erythrocytes and hemolysin rabbit anti-sheep erythrocyte stromata serum were purchased from Biowhittaker (Walkersville, MD). Gelatin veronal buffer (GVB^{2+}: 0.15 M $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% gelatin, 1.8 mM sodium barbital, 3.1 mM barbituric acid, 141 mM NaCl, pH 7.4), GVB^{2+}-EDTA (GVB-EDTA: in addition to the ingredients in GVB^{2+} it contains 10 mM EDTA, pH 7.4), and lyophilized rat sera were acquired from Sigma (St. Louis, MO).

Interfacial Surface Rheology. Interfacial surface rheology (ISR) measurements were conducted at Stanford University (Stanford, CA). The ISR is used to measure molecular interactions of polymers on a monolayer by applying a shear stress to a Langmuir monolayer and monitoring the time-dependent response of the probe.[18] The trough, rod, flow chamber, glass slide, and barriers were cleaned thoroughly by rinsing with butyl acetone, ethanol, and deionized water. The trough was aligned perpendicular to the Helmholtz coils. The barriers were positioned at either end of the trough. The flow chamber was placed over the glass slide, which was situated over the quartz window (centered in the trough). The trough was filled with deionized water, which was subsequently removed using a vacuum (to further clean contaminants from the trough). The trough was refilled with deionized water, and the needle was placed in the center of the flow chamber. The microscope was adjusted to focus on the edge of the rod. The Wilhelmy balance was put in position to read the surface pressure. The instrument was then calibrated to the force required to move the needle at the air−water interface. The applied strain was between 0.019 and 0.024, i.e., the amplitude of

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Table 1. Description of Hydrophobically Modified PEG Polymers[a]

polymer	MW, kg/mol	N_b	loop radius $\bar{\xi}$, Å	calcd polymer area, $\times 10^{17}$ m^2	measured polymer area, $\times 10^{17}$ m^2	full coverage, mg/m^2	K, (mg/m^2)/(mg/mL)
HMPEG136-DP3	42	4	50	7.9	5	0.88	6.1 ± 0.8
HMPEG273-DP2.5	48	3	75	13.3	8	0.60	1.9 ± 0.1
HMPEG273-DP5	106	8	65	26.5	8	0.66	16.4 ± 2.5
DSPE-PEG113	5.8	1	54	2.3	0.3	0.42	0.4 ± 0.1

[a] Full mushroom coverage is defined as the mass of unconstrained polymer required to cover 1 m^2 of lipid. Error is given as one standard deviation from the mean.

the needle movement (between 65 and 85 μm) divided by the distance of the needle to the wall of the channel (3.5 mm).

Lipid or lipid and HMPEG polymer were dissolved in chloroform and added dropwise from a syringe onto the surface of the water. After the chloroform evaporated, the barriers were moved to achieve the desired starting pressure. After equilibration (by observing pressure stability), the strain is measured as a function of frequency. The rheometric measurement can be performed at constant pressure or at constant barrier position. In order to investigate the polymer/lipid monolayers until failure, additional material was added to the surface which resulted in nonzero surface pressure. We observed a shallow, steadily increasing surface pressure when starting at zero surface pressure, which overlaps with the data shown.

The ISR consists of 4 main features: (1) the Langmuir trough, (2) the Helmholtz coils (or the magnetic field), (3) the magnetic rod and flow cell, and (4) the light microscope. A commercial Langmuir trough (33.0 cm $\times$ 7.5 cm) made from Teflon with a quartz window (30 mm in diameter) was purchased from KSV Instruments (Helsinki, Finland).[18] The position of the rod was monitored as a function of time using an inverted microscope procured from Nikon (Model TMS-F). The image of the rod was projected onto an image sensor with a 512-pixel photodiode array (Hamamatsu, Model C4350 multichannel detector with Model S3902-512Q image sensor).[18] Each pixel is 50 μm long $\times$ 0.5 mm wide. The output of the array was analyzed to detect the edge of the magnetic rod. The two parameters that are measured are the amplitude ratio, defined as the ratio of the amplitude of the rod displacement to the force amplitude, and the phase difference, the phase angle difference between the rod response and the applied force. These parameters were procured from the frequency spectrum acquired from the fast Fourier transform of the applied force and the response of the rod.[18]

Polymer Adsorption. Experiments were conducted at increasing surface coverage of polymer. We consider full surface coverage to occur when the lipid surface area (approximately one-half of the total lipid area) is equivalent to the unconstrained polymer area (approximately the unconstrained PEG area). The amount of polymer added to the subphase to reach full surface coverage on liposomes was determined from the association constant data as reported previously (see Table 1).[11] The association constant, K, was determined from the initial slope (first four data points) of each adsorption profile such that

$$K = \frac{d\Gamma}{d[C_p(f\text{free})]} \equiv \frac{(\text{mg/m}^2)}{(\text{mg/mL})} \quad (1)$$

The idea of coverage is based on the mass of polymer needed to cover the exterior surface area of liposomes. From the random walk approximation described previously[11] and verified by neutron scattering for HMPEG chains,[19] we are able to approximate the area occupied by the HMPEG polymers (reported in Table 1). Because the polymers differ greatly in molecular weight and number of subchains, we find this the most practical means for comparison.

Preparation of Liposomes. DOPC liposomes were prepared as described previously.[11] Briefly, DOPC was mixed in chloroform, dried under vacuum, and rehydrated in a buffered sucrose solution (10 mM TES, 250 mM sucrose, pH 7.4). After vortexing, the lipid solution underwent five cycles of freezing in liquid nitrogen and thawing in a room-temperature water bath. The sample was then extruded 10 times through a 0.2 μm polycarbonate membrane filter at 250 psi using a 10 mL Lipex extruder (Northern Lipids, Inc., Vancouver, BC, Canada). The liposomes were stored at 4 °C under nitrogen. The concentration of liposomes was determined by the phosphate assay.[20] The liposome size was determined by dynamic light scattering using a ZetaPALS instrument (Brookhaven Instruments Co., Holtsville, NY). Sucrose encapsulating DOPC liposomes have a number-averaged diameter of 82.4 $\pm$ 12.2 nm.

In vitro Complement-Mediated Hemolysis Assay. The depletion of complement protein from serum has been shown to correlate with in vivo *immune response*.[21,22,23] The complement assay was conducted as described by Ahl et al.[21] Activation of sheep erythrocytes was performed by first washing the cells three times in 10 mL GVB^{2+}, centrifuging at 8000*g* for 4 min, and removing the supernatant. The cells were resuspended at 10^8 cells/mL, determined by hemacytometry, and incubated with hemolysin rabbit anti-sheep erythrocyte stromata serum at 1/500 (v/v). Excess hemolysin was removed by rinsing three times in GVB^{2+} and resuspended at 10^8 cells/mL. Activated cells were stored at 4 °C and used within 7 days.

Each individual complement assay consisted of six samples prepared in 200 μL volumes: TES buffer (the negative control, no liposomes, 0% complement activation), 8 mM unmodified liposomes in TES buffer (the positive control, 100% complement activation), and four test samples containing 8 mM liposomes in TES buffer with increasing amounts of polymer. The samples were equilibrated overnight at 4 °C with gentle shaking. Each sample was incubated at 37 °C for 30 min with 100 μL reconstituted rat sera, diluted 1:1 (v/v) with GVB^{2+}. Subsequently, 300 μL of GVB^{2+} was added followed by vortexing and centrifugation at 8000*g* for 4 min. A 100 μL aliquot of the supernatant was diluted 1:1 (v/v) preceding eight successive serial dilutions in GVB^{2+}. To each diluted sample of treated serum, 100 μL of activated sheep cells was added and incubated for 30 min at 37 °C. Hemolysis was quenched by the addition of 300 μL of GVB^{2+}-EDTA. (Cells that were intact were sedimented by centrifuging the samples at 8000*g* for 4 min.) A 200 μL aliquot from the supernatant of each sample was placed into a 96 well plate. The optical density of each sample well was determined at 415 nm using a 3550-UV spectrophotometer plate reader (Bio-Rad Laboratories, Hercules, CA). Sucrose encapsulation enables pelleting of the liposomes however it does not affect the complement assay.

The results are plotted as the percent hemolysis versus the log of the inverse of the serum dilution [-log(1/SD)]. The CH50, commonly utilized in related literature,[22,24,25] is the serum dilution necessary to achieve 50% hemolysis and is directly related to the level of active complement in the serum. The CH50 of each hemolysis curve

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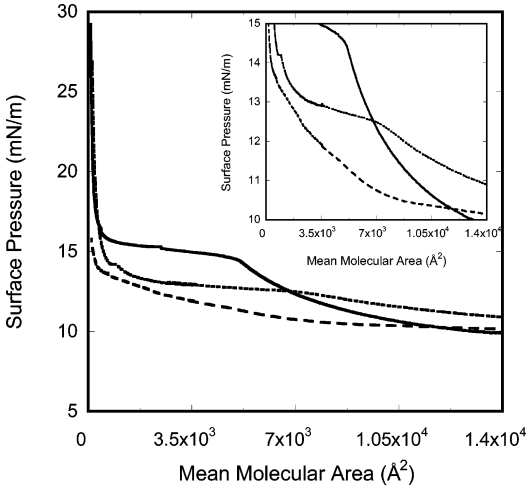

Figure 2. $\pi-$A isotherms of three different HMPEG polymers over a water subphase: HMPEG136-DP3 (solid line), HMPEG273-DP2.5 (dashed line), and HMPEG273-DP5 (dotted line) at 22 °C.

was acquired by a linear fit to a log−log version of the von Krough equation.[26] The surface "protection" mediated by HMPEG adsorption can be quantitatively described using the following equation:

$$\%\ \text{protection} = \frac{\text{CH50}_{\text{PEG-Liposomes}} - \text{CH50}_{\text{BareLiposomes}}}{\text{CH50}_{\text{Buffer}} - \text{CH50}_{\text{BareLiposomes}}} \times 100 \quad (2)$$

Results

We have investigated the viscoelastic behavior of multiply attached HMPEG polymers on a DPPC monolayer to identify attributes that may correlate with reduced complement protein binding. Three polymers were chosen to evaluate: (1) the PEG chain length at approximately constant number of loops (HMPEG136-DP3 vs HMPEG273-DP2.5), (2) the number of loops at constant PEG chain length (HMPEG273-DP2.5 vs HMPEG273-DP5), and (3) the ratio of PEG monomers to hydrophobes (HMPEG136-DP3 and HMPEG273-DP5). As liposomes comprise a lipid bilayer, we have employed a DPPC monolayer to measure the effect of HMPEG polymer adsorption on the interfacial rheological behavior of the membrane.

First, π vs A isotherms for pure HMPEG136-DP3, HMPEG-273-DP2.5, and HMPEG273-DP5 monolayers over a water subphase were measured to determine the polymer surface properties in the absence of a lipid monolayer (Figure 2). Second, we observed π vs A isotherms for DPPC and DPPC with 0.5, 1.0, and 2 mg/m^2 each of of HMPEG136-DP3, HMPEG273-DP2.5, and HMPEG273-DP5 (Figure 3). We report dynamic moduli as a function of frequency at three different surface concentrations (0.5, 1.0, and 2 mg/m^2) and three different surface pressures (20, 30, and 40 mN/m) for HMPEG273-DP2.5 adsorbed on a DPPC monolayer (Figure 4). An exponential fit of this data is tabulated (Table 2). We compare the dynamic viscosity as a function of surface pressure, polymer surface density, and polymer architecture for all three polymers (HMPEG136-DP3, HMPEG-273-DP2.5, and HMPEG273-DP5) at a fixed frequency (Figure 5). Finally, we assess the interfacial rheology and its relationship to the ability of HMPEG polymers to protect against complement protein binding (Figure 6).

HMPEG Polymer Monolayers. We investigated the surface viscoelastic behavior of HMPEG136-DP3, HMPEG273-DP2.5, and HMPEG273-DP5 at the air−water interface in the absence of a lipid monolayer (Figure 2). Our aim was to identify the phase behavior of the pure polymer alone and establish the area per polymer.[18] The experiments began at a polymer surface density, where the free chain coils would pack as spheres. This enabled us to probe the transition between the mushroom and brush regime; that is, the transition from closely packed spheres with radius $\bar{\xi}$ to compressed and extended layers at high PEG surface density. The mass of the polymer added to the surface is calculated based on the association constants obtained from previous adsorption measurements.[11]

Figure 2 depicts phase transitions that occur as HMPEG136-DP3, HMPEG 273-DP2.5, and HMPEG273-DP5 monolayers are slowly compressed. Each isotherm shows a characteristic film pressure transition between 10.5 and 14.5 mN/m. HMPEG136-DP3 undergoes a transition at 14.5 mN/m and 5000 Å^2/molecule. A similar transition is detected for HMPEG273-DP2.5 and HMPEG273-DP5, where the transition occurs at 8000 Å^2/molecule but at different surface pressures, 10.5 mN/m and 12.8 mN/m, respectively.

The polymers differ in the molecular weight of the PEG chain and the number of hydrophobes. A comparison of increasing the PEG chain (HMPEG136-DP3 vs HMPEG273-DP2.5) shows a shift in the isotherm when molecules begin to interact (5000 Å^2/molecule vs 8000 Å^2/molecule). Two polymers have a similar ratio of PEG monomers to hydrophobes (HMPEG136-DP3 vs HMPEG273-DP5), approximately 50:1 PEG:hydrophobe. The isotherms of HMPEG136-DP3 and HMPEG273-DP5 are similar, though the coexistence region of HMPEG273-DP5 is more pronounced. Additionally, the slope (pressure/mean molecular area) in the coexistence region of the isotherms is significantly higher for HMPEG273-DP2.5 than for the other two polymers. The $\pi-$A isotherms of polymers with similar ratios of PEG: hydrophobe have similar characteristics (HMPEG136-DP3 and HMPEG273-DP5), whereas those of polymers with similar PEG chain length have similar transition regions (HMPEG273-DP2.5 and HMPEG273-DP5). The calculated polymer area (calculation described in ref 11 from the average area of each loop × number of loops) is higher than the measured polymer area (Table 1). The calculated area assumes an ideal nonsolvent condition, which may not accurately predict the experimental result.

HMPEG Polymers Adsorbed on a DPPC Monolayer. $\pi-A$ *Isotherms.* The $\pi-$A isotherms depicted in Figure 3 show the effect of compressing HMPEG polymers in the presence of DPPC with 0.5, 1, and 2 mg/m^2 polymer adsorbed. Each experiment was performed with the same mass of DPPC lipids at the surface (5.4 mg/m^2). Due to the high compressibility of the polymers, a higher mass (lipid and polymer) was added to the surface which initiated an increase in the baseline surface pressure from 0 to 10 mN/m. We observe (not reported) a slowly increasing, linear response within this domain.

The isotherms show a similar transition between a disorganized and organized phase between 14 and 18 mN/m. Increasing the polymer surface concentration shifts the isotherm to the right. The shift between 0.5 and 1 mg/m^2 however is markedly less than that between 1 and 2 mg/m^2. The isotherms with 2 mg/m^2 polymer show a decrease in their slopes in comparison to those of 0.5 and 1 mg/m^2 polymer. The isotherms for HMPEG136-DP3 and HMPEG273-DP2.5 come close to the water surface tension value (73 mN/m).

As the surface concentration increases, we observe a nonlinear interchain response. The transitions of HMPEG136-DP3 are at 520, 600, and 800 Å^2/molecule for 0.5, 1, and 2 mg/m^2, respectively. Similarly, HMPEG273-DP2.5 has transitions at 275, 320, and 480 Å^2/molecule for 0.5, 1, and 2 mg/m^2, respectively.

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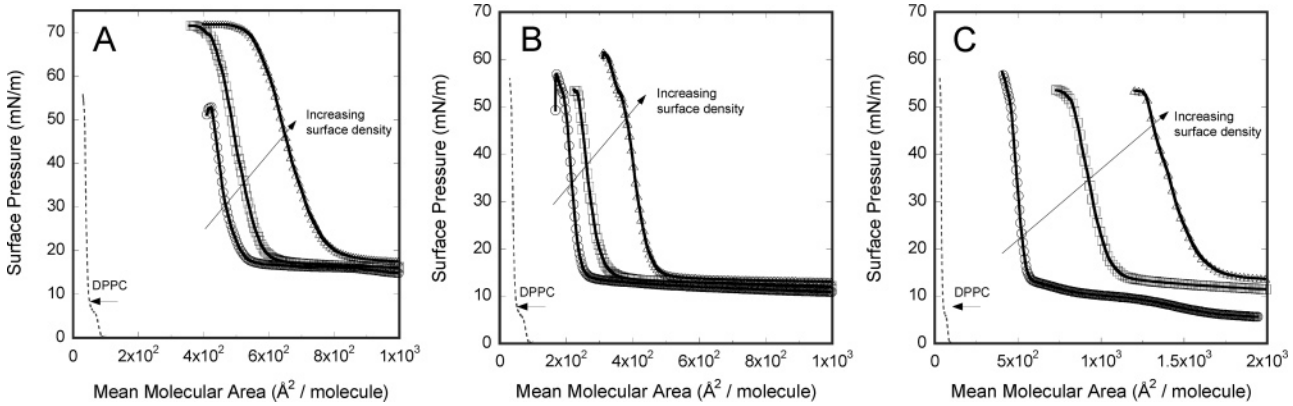

Figure 3. $\pi-$A isotherms for DPPC monolayers at the air−water interface incorporating (A) HMPEG136-DP3, (B) HMPEG273-DP2.5, and (C) HMPEG273-DP5 at 0.5 (○), 1 (□), and 2 mg/m2 (△) surface coverage at 22 °C. For reference, the $\pi-$A isotherm for a DPPC monolayer at the air−water interface at 22 °C is depicted.

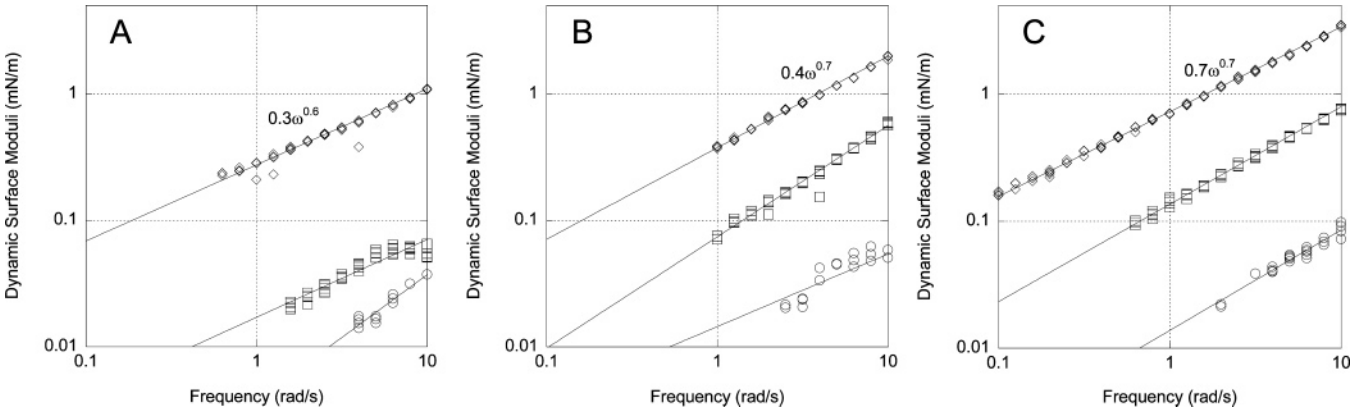

Figure 4. Frequency dependence of the dynamic surface moduli for a DPPC monolayer at the air−water interface incorporating HMPEG273-DP2.5 at (A) 0.5, (B) 1, and (C) 2 mg/m^2 at 22 °C. Frequency sweeps were performed at surface pressures of 20 (○), 30 (□), and 40 (◊) mN/m. G_s'' is proportional to the frequency, implying that the surface is shear thinning.

Table 2. Fitting Parameters for the Equation $G_s'' = C\omega p + 1$, Where *C* Is a Constant and *p* Describes the Frequency Power Law Dependence[a]

		surface Pressure					
		20 mN/m		30 mN/m		40 mN/m	
polymer	fraction of coverage	C	p	C	p	C	p
HMPEG136-DP3	0.5						
	1	0.03	−0.33	0.10	−0.28	0.36	−0.34
	2	0.04	−0.28	0.12	−0.27	0.53	−0.40
HMPEG273-DP2.5	0.5	0.004	0.02	0.01	−0.20	0.25	−0.31
	1	0.02	−0.57	0.07	−0.05	0.35	−0.26
	2	0.01	−0.23	0.12	−0.16	0.67	−0.27
HMPEG273-DP5	0.5	0.01	0.05	0.11	−0.10	0.33	−0.14
	1	0.003	0.50	0.09	−0.19	0.33	−0.17
	2	0.003	0.7	0.04	0.02	0.27	0.09
DSPE-PEG45[b]	100 mol %	2	−1				
	40 mol %	0.01	0.6				

[a] Fitting the data resulted in error no less than $R^2 = 0.98$. The square of the Pearson product moment correlation coefficient, R^2, interprets the proportion of the variation in Y attributable to the variation in X. It is given as follows, where a value of one indicates that the estimated value is equal to the actual value (ref 25): $R^2 = n(\Sigma XY) - (\Sigma X)(\Sigma Y)/\sqrt{[n\Sigma X^2-(\Sigma X)^2][n\Sigma Y^2-(\Sigma Y)^2]}$. [b] For comparison, rheology of mixtures of DSPE-PEG45 with DMPC is expressed in mol % at approximately 20 ± 2 mN/m.

Comparison of HMPEG136-DP3 and HMPEG273-DP2.5 (panels A and B of Figure 3), which have a similar number of hydrophobes but different number of PEG monomers, shows the elasticity of the PEG chains. Though the PEG chain is double in size, the transition occurs at approximately half the mean molecular area (MMA). The slope in the ordered phase however is much steeper for HMPEG273-DP2.5 than for HMPEG136-DP3.

The HMPEG273-DP5 polymer has a similar ratio of PEG: hydrophobe to HMPEG136-DP3 but also has 2.5 times the overall molecular weight. HMPEG273-DP5 has transitions at 520, 1100, and 1650 Å^2/molecule for 0.5, 1, and 2 mg/m^2 polymer, respectively. The low-pressure transition of HMPEG273-DP5 at 0.5 mg/m^2 is identical to that of HMPEG136-DP3. As we double the polymer surface concentration of HMPEG273-DP5 (from 0.5 to 1 mg/m^2), we see a doubling of the onset MMA. Increasing the surface concentration of HMEPG273-DP5 from 1 to 2 mg/m^2 results in approximately the same ∼500 Å^2 growth in the MMA, as seen on going from 0.5 to 1 mg/m^2.

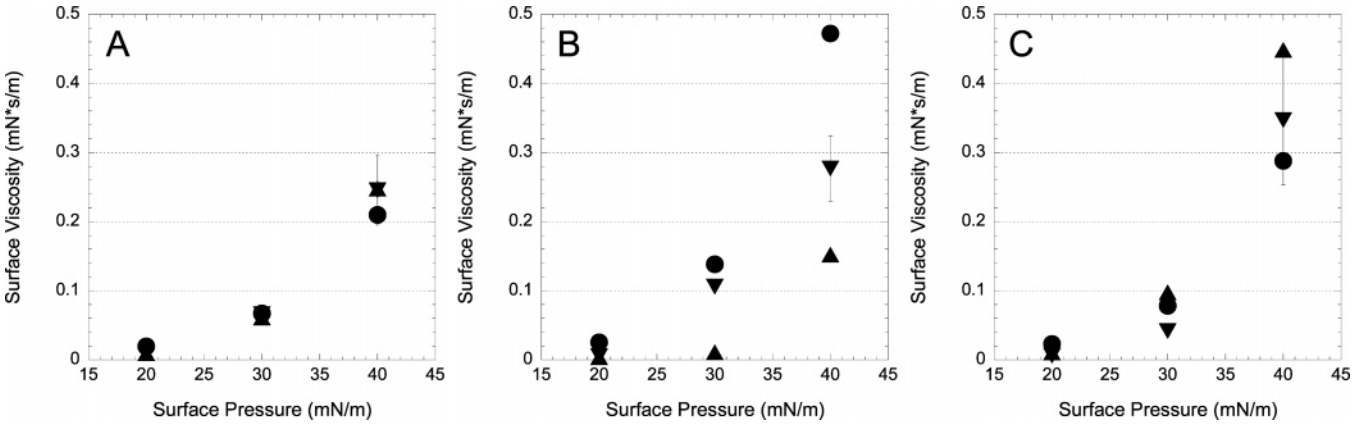

Figure 5. Dynamic viscosity of a DPPC monolayer as a function of surface pressure with (A) 0.5, (B) 1, and (C) 2 mg/m^2 adsorbed polymer at 22 °C and a frequency of 3.9 rad/s for HMPEG136-DP3 (●), HMPEG273-DP2.5 (▲), and HMPEG273-DP5 (▼). The error is one standard deviation of the mean, and if not visible, then the error is smaller than the size of the mark.

Interfacial Rheology Measurements. The frequency dependence of the dynamic surface moduli for HMPEG136-DP3, HMPE273-DP2.5, and HMPEG273-DP5 was determined at different surface pressures. The elastic surface moduli were found to be small, and thus the dynamic surface modulus is primarily a result of the loss surface modulus, which can be thought of as a surface dynamic viscosity, η^*_s. Figure 4 illustrates the typical (frequency vs dynamic surface modulus) profile as a function of surface pressure and polymer surface concentration for HMPEG273-DP2.5.

We observe that the surface dynamic viscosity, η^*_s, has a power law dependence with frequency, ω, of the form $\eta^*_s = A\omega^p$, where A is a constant and p is the power law exponent. The surface dynamic viscosity can be converted to a surface viscous modulus, G_s'', using the relationship $G_s'' = \eta^*_s\omega$. The surface dynamic modulus will likewise obey a power law relationship to the frequency given by $G_s'' = C\omega^{p+1}$, where C is a constant. The fitted constants C and p are shown at 40 mN/m in Figure 4 and show that the fluids are shear thinning, non-Newtonian monolayers at high surface pressure ($p < 0$).

Fitting parameters (C, p) for the dynamic surface modulus as a function of frequency for all three polymers are presented in Table 2. For the polymer surface coverage and surface pressures measured, HMPEG136-DP3 is consistently shear thinning ($p < 0$). As the values in Table 2 indicate, the value of p is not consistently negative for HMPEG273-DP5. At high surface concentration (2 mg/m^2) of HMPEG273-DP5 and high surface pressures (30 and 40 mN/m), we observe $p \sim 0$. At 20 mN/m and either 1 or 2 mg/m^2 polymer surface coverage, we observe frequency thickening surface viscosity, where p is greater than zero.

The dynamic viscosity is depicted as a function of surface pressure for the HMPEG polymers at a fixed frequency (3.9 rad/s, Figure 5). At low polymer density (0.5 mg/m^2, Figure 5A), the unconstrained polymers exhibit an increase in surface viscosity as the surface is compressed. In this regime, the polymers exist as separate islands adsorbed at the air−water interface and slide past one another when under shear. At 1 mg/m^2 (Figure 5B), the polymers are moderately constrained resulting in significant differences in the surface viscosity for the different polymer architectures. The highest surface viscosity is exhibited by HMPEG136-DP3 (0.47 ± 0.01 mN s/m), followed by HMPEG-273-DP5 (0.28 ± 0.05 mN s/m) and HMPEG273-DP2.5 (0.15 ± 0.003 mN s/m) at 1 mg/m^2 and 40 mN/m, respectively.

We observe differences in the dependence of surface viscosity on surface pressure between moderately constrained polymers (1 mg/m^2) and highly constrained polymers (2 mg/m^2). At the highest surface density (2 mg/m^2, Figure 5C), the profile shown in Figure 5B becomes inverted in Figure 5C at 2 mg/m^2 and 40 mN/m. The highest surface viscosity corresponds to HMPEG273-DP2.5 (0.45 ± 0.01 mN s/m), followed by HMPEG273-DP5 (0.35 ± 0.09 mN s/m) and HMPEG136-DP3 (0.29 ± 0.01 mN s/m). HMPEG273-DP5 lies within one standard deviation of both HMPEG273-DP2.5 and HMPEG136-DP3. However, the latter ones are significantly different from each other.

Consequently, we observe that surface density, surface pressure, and polymer architecture contribute to the surface viscosity. Below full coverage, the surface viscosity is solely dependent on the surface pressure. Dependence on the polymer architecture is apparent when the polymers are constrained. Shorter PEG chains have a higher surface viscosity at 1 mg/m^2 and a lower surface viscosity at 2 mg/m^2. Longer PEG chains show the opposite effect; they have a lower surface viscosity at 1 mg/m^2 and a higher surface viscosity at 2 mg/m^2.

Complement Protein Assay. The objective of this work is to correlate surface rheology of adsorbed HMPEG polymers on a lipid monolayer to complement protein adsorption studies performed on HMPEG-coated liposomes. Significant complement protein adsorption is a precursor for recognition by the immune system. Reducing the amount of complement protein adsorption may be achieved by incorporating HMPEGs on the liposome surface. We attempt to correlate the reduction in complement protein adsorption with interfacial rheological behavior.

The complement protein assay (or hemolysis assay) measures the depletion of complement protein from samples where liposomes or HMPEG-coated liposomes are incubated with the complement protein. The protein that remains in solution (not adsorbed) lyses red blood cells, which may then be read via a spectrophotometer. Figure 6 describes the complement protein assay. Two controls are used to indicate no protein binding (buffer, ideal case) and high protein binding (bare liposome solution, worst case). HMPEG-coated liposomes, at increasing polymer surface densities, provide different levels of protection from complement protein binding. Addition of an HMPEG polymer may shift the curve to the right instead of toward the left. This is a result of being portrayed on a logarithmic scale, where the error at this dilution is large relative to the difference in hemolysis.

We observe similar protection from complement protein binding with HMPEG136-DP3 (not shown) and HMPEG273-DP5 (Figure 6B), which have a similar PEG monomer to hydrophobe ratio. At 1 mg/m^2, we observe a 40% decrease in protein binding when liposomes are coated with HMPEG273-

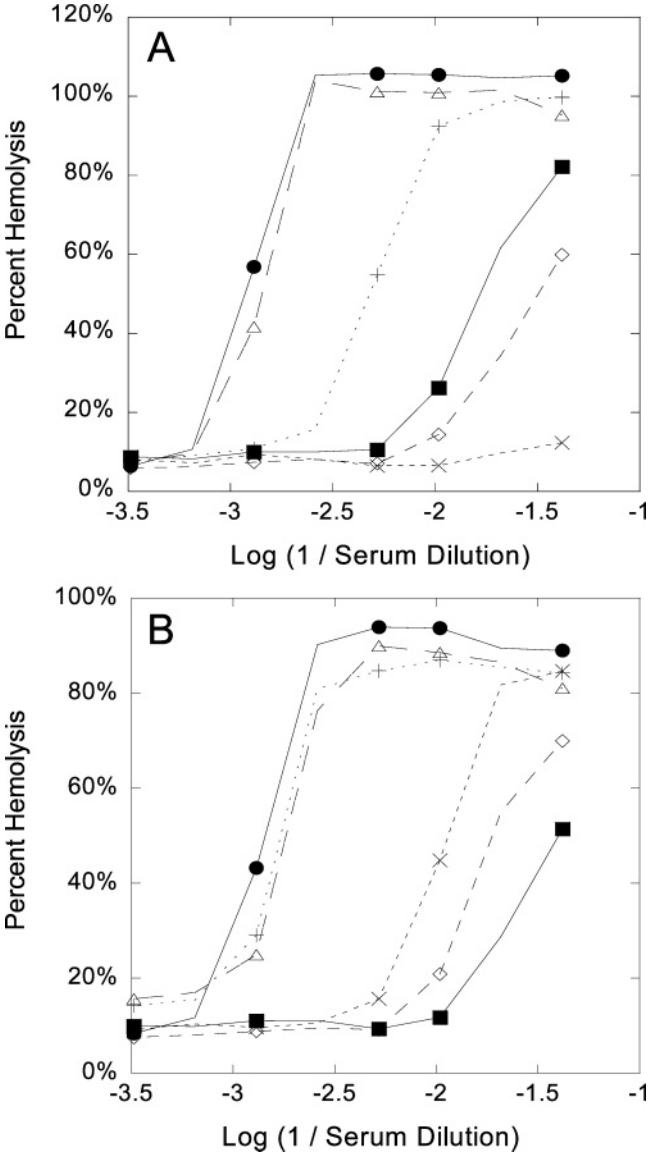

Figure 6. Complement assay for DOPC liposomes modified with (A) HMPEG273-DP2.5 and (B) HMPEG273-DP5. Each liposome sample contained 200 μL of 8 mM (1.6 mg/mL) lipid, having a lipid surface area of 0.3 m^2. Graph depicts buffer (●), bare liposomes (■), liposomes with 0.1 (◊), 0.5 (×), 1 (+), or 2 mg/m^2 (Δ). Addition of an HMPEG polymer may shift the curve to the right instead of toward the left. This is a result of being portrayed on a logarithmic scale, where the error at this dilution is large relative to the difference in hemolysis. The precision of each measurement is evaluated based on one standard deviation from the mean for the buffer and liposome controls, 3.1% and 9.1%, respectively.

DP2.5 (Figure 6A) and a 92% reduction in protein binding when coated with HMPEG273-DP5 (Figure 6B). At 2 mg/m^2, both HMPEG273-DP5 and HMPEG273-DP2.5 protect against complement protein binding (>90%). The precision of each measurement is based on one standard deviation from the mean for the buffer and liposome controls, 3.1 and 9.1%, respectively.

Discussion

HMPEGs are amphiphilic and have the ability to adsorb at the air−water interface, on a lipid monolayer, or on a lipid bilayer (such as a liposome). The effect the polymers have at the surface is influenced by the polymer architecture, specifically the balance of the hydrophilicity and the number of hydrophobic anchors (approximately 50:1 or 100:1, PEG:hydrophobe, mol/mol). Characterization of polymer-coated lipid monolayer systems by interfacial surface rheology may aid in the design of novel drug delivery vehicles that resist protein adsorption and improve tissue perfusion.

π−A isotherms. We have measured the MMA of each polymer (Figure 2), which is defined as the initial onset of pressure increase. We report a single transition from the disordered to interacting phase as seen in typical π−A isotherms of a lipid.[27,28] Our calculation of the polymer area (Table 1), based on the random walk approximation, is higher than the measured value. The lower MMA may be a result of the compressibility of the polymer or polymer−solvent interactions. The complement protein binding assay suggests that a uniform polymer layer exists at 1 mg/m^2. Partial coverage (Figure 6) indicates incomplete protection. The collection of data for Figure 2 required a long trough that was able to compress the surface by 250 mm. The compressibility (C) of the polymer may be described as:[15]

$$C = -\frac{1}{A}\left(\frac{dA}{d\pi}\right) \tag{3}$$

where A is the area. We can infer from the large change in area relative to change in pressure (Figure 2) that the polymer compressibility contributes to the low MMA for the HMPEG polymers.

Polymers HMPEG136-DP3 and HMPEG273-DP2.5 are more compressible than HMPEG273-DP5. This is evident in the π−A isotherms with different polymer surface concentrations in the presence of DPPC (Figure 3). We observe that increasing the polymer concentration shifts the isotherm to the right. For HMPEG273-DP5, the shift is especially large. Figure 3 depicts one first-order phase transition, which shows that the polymers are strongly anchored at the surface and do not desorb, as is the case for a singly-anchored PEG chains (DSPE-PEG45, similar ratio, PEG:hydrophobe = 50:1).[15]

Interfacial Surface Rheology. All three polymers demonstrated a shear thinning behavior (Figure 4 and Table 2). From plots of the dynamic surface moduli vs frequency, we fit parameters to the equation $G_s'' = C\omega^{p+1}$. For HMPEG136-DP3, the value of p was consistently within a narrow negative range, between −0.27 and −0.40, demonstrating a consistent shear thinning behavior. By contrast, p varied for HMPEG273-DP2.5 between +0.02 and −0.57. HMPEG273-DP2.5 demonstrated a Newtonian-like behavior at 0.5 mg/m^2 and 20 mN/m and at 1 mg/m^2 and 30 mN/m.

The behavior of HMPEG273-DP5 exhibits signs of shear thickening, shear thinning, and Newtonian-like behavior. We observe positive values for p at 1 mg/m^2 and 20 mN/m and at 2 mg/m^2 and 20 and 40 mN/m. Shear thinning is demonstrated by p values within the narrow range of −0.1 and −0.19 at low polymer surface densities and at low surface pressures (0.5 and 1 mg/m^2 and 20 and 30 mN/m). Newtonian-like behavior is observed at 0.5 mg/m^2 and 20 mN/m and at 2 mg/m^2 and 30 mN/m. There seems to be a weak correlation in that low polymer density and high surface pressure results in shear thinning behavior. However, the opposite does not hold true. We do not see consistent shear thickening or Newtonian-like behavior at high polymer density or low surface pressure. The transition of HMPEG273-DP5 from Newtonian (at low surface pressure) to shear thinning (at high surface pressure) may be explained by the Deborah number, where deformation occurs faster than

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molecular relaxation. After enough energy has been provided or the PEG layer has been constrained, the molecules move more freely at high surface pressures.

The surface viscosity of HMPEG polymers adsorbed on a DPPC monolayer varies with surface pressure, polymer surface density, and polymer architecture (Figure 5). At low polymer density, all three HMPEG polymers exhibit a similar viscosity increase. We interpret that the rheology of the polymers in this regime is a function of the DPPC and the unconstrained polymers sliding past one another (Figure 5A). Once the network reaches a polymer density where the interchain interactions dominate, we see a divergence in the viscosity dependence on the surface pressure (Figure 5B). At low surface pressure, the viscosity remains low even at high polymer surface density. We believe that this is in part due to the polymer's ability to compress.

Two interesting phenomena occur at 40 mN/m between 1 and 2 mg/m^2 (Figure 5B,C): (1) there is a measured drop in HMPEG136-DP3 viscosity, and (2) there is a striking increase in the viscosity of HMPEG273-DP2.5. At 40 mN/m, the viscosity of adsorbed HMPEG136-DP3 on a DPPC monolayer at 1 mg/m^2 is 0.47 mN s/m. At 40 mN/m and 2 mg/m^2, the viscosity of HMPEG136-DP3 on a DPPC monolayer is 0.29 mN s/m. This difference may be attributed to interchain interactions at 1 mg/m^2. At double the polymer density, intercalation of the chains may alter the viscous behavior. We attribute the rise in viscosity for HMPEG273-DP2.5 to doubling the polymer surface density, which results in increased interchain interactions.

The surface viscosity of DPPC also varies with surface pressure. The surface viscosity of a pure DPPC monolayer as a function of surface pressure is ~0 mN s/m and 0.5 mN s/m at 20 mN/m and 40 mN/m, respectively.[28] Thus, adsorption of HMPEG polymer contributes to reducing the surface viscosity.

Complement Protein Adsorption. The aim of this work is to correlate the physical phenomena with resistance to protein binding. Others have reported a significant reduction in protein adsorption due to PEG shielding.[29,30] Figure 6 depicts the ability of HMPEG-coated liposomes to protect from complement protein binding at different polymer surface densities.

We observe a threshold in the surface viscosity that explains our current data. HMPEG136-DP3 and HMPEG273-DP5 exhibit protection from complement protein binding (and thus immune evasion) at polymer densities exceeding 1 mg/m^2. At 1 mg/m^2, we observe surface viscosities above 0.25 mN s/m for HMPEG136-DP3 and HMPEG273-DP5. HMPEG273-DP2.5 has a viscosity of 0.15 mN s/m at 1 mg/m^2, which is below the threshold and thus does not show protection. At 2 mg/m^2, all three polymers exceed 0.25 mN s/m and exhibit protection from protein binding.

The rheological behavior of a pure DPPC monolayer and a pure HMPEG monolayer at the air−water interface results in negligible surface viscosity at 3.9 rad/s (data not shown). The synergistic effect of associating HMPEGs with the DPPC monolayer results in an increase in the surface viscosity and, as shown in Figure 6, may be correlated with a reduction in protein adsorption.

In our previous work, we hypothesized that high degrees of cooperativity could result in three-dimensional self-assembled structures on the monolayer. Since the polymer was added with the lipid in chloroform, we do not expect to see this phenomenon. However, it still may hold true for liposomes, where the polymers are added to an aqueous phase and adsorbed onto the lipid bilayer.

Surprisingly, the HMPEG polymers do not exhibit a gel transition, where G_s' exceeds G_s''. DSPE-PEG45 and DSPE-PEG113 have been reported to exhibit a gel transition.[15,31] For HMPEGs, the elastic modulus G_s' is approximately zero. Diffusion studies using fluorescence recovery after photobleaching (FRAP) show a reduction in the diffusion of molecules within a monolayer of DSPE-PEG45 incorporated within a 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) monolayer. Reduced diffusion occurred as a result of both an increase in the polymer density on the surface and an increase in the surface pressure (which leads to a gel phase transition for DSPE-PEG45).[32] We believe the rise in surface viscosity seen with HMPEG-coated lipid monolayers is a result of hindered motion within the monolayer as a result of both intra- and interchain interactions.

The differences between HMPEG-coated lipid monolayers and the incorporation of PEGylated lipids within the monolayer are several-fold. First, the DSPE-PEGs are anchored by a lipid with two hydrophobic chains as opposed to the HMPEGs which have a single $C_{17}H_{37}$ hydrophobic anchor. The size of the anchor may play a role in the reduced diffusion of lipids on the surface. Second, DSPE-PEGs have both lateral and rotational freedoms, whereas HMPEGs which are multiply anchored have more limited mobility in comparison. Upon increasing the surface pressure, hindered motion may affect the DSPE-PEGs more than the HMPEGs. Third, HMPEG folding of the PEG loop toward the monolayer may reduce the degrees of freedom of chain orientation. We hypothesize that these inherent differences may contribute to the overall differences in rheological behavior that we have measured.

Other biological membranes are defined by surface rheology behavior. Erthyrocytes are subjected to mechanical stress in blood flow and protein adsorption due to the high protein concentration in plasma (i.e., albumin which is present at 7 g/dL). On the basis of the dimensions of the albumin molecule, it is proposed to result in a 6 nm thick layer (similar to PEG thickness). A decrease in surface viscosity, which relates to erythrocyte deformability as a result of reduced protein adsorption, is shown to correlate with improved tissue perfusion.[33] We hypothesize that HMPEG adsorption may also contribute to the deformability of liposomes, which may enable protein resistance.

Surface viscoelasticity plays an important role in the behavior of cells. It regulates cell shape and couples a dynamic response with gene expression.[34] Mathematical models to predict cell migration speed are based upon cell membrane adhesion and cell membrane mechanics. Cell viscosity and stiffness are essential for mobility.[35] Proteins (like Ras[36]) can also sense surface viscosity. It is hypothesized that changes in cell viscosity may enable protein mobility and localization. The rheological behavior of biological systems has been addressed; however, it is our hope to broaden the spectrum of characterization in bioengineering applications (i.e., drug delivery) by associating a well-defined technique with a physical phenomena.

Conclusions

We have characterized the interfacial surface rheology of irreversibly bound HMPEG polymers on a lipid monolayer at the air−water interface. Three polymers, HMPEG136-DP3,

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HMPEG273-DP2.5, and HMPEG273-DP5, were examined to elucidate the role of the following polymer attributes: (1) the PEG chain length at approximately constant number of loops (HMPEG136-DP3 vs HMPEG273-DP2.5), (2) the number of loops at constant PEG chain length (HMPEG273-DP2.5 vs HMPEG273-DP5), and (3) the ratio of PEG monomers to hydrophobes (HMPEG136-DP3 and HMPEG273-DP5). We find similar rheological behavior of all polymers at low polymer surface coverage (0.5 mg/m^2), whereas at high surface coverage ($>$0.5 mg/m^2), we observe a structural dependence of the surface viscous forces at high surface pressure (40 mN/m). This threshold correlates with reduced protein binding, which may suggest that surface viscous forces play a role in immune recognition. Thus, interfacial surface rheology may be useful in characterizing the effect of polymers in bioengineering applications that include self-assembly. Specifically, liposomal drug delivery vehicles may be easily analyzed using this technique. Engineering of drug delivery systems have focused primarily on the chemical nature of biological systems. This work addresses studying surface rheology to understand how polymer interactions may affect protein adsorption.

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