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Association of hydrophobically-modified poly(ethylene glycol) with fusogenic liposomes

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Abstract

We present results on using cooperative interactions to shield liposomes by incorporating multiple hydrophobic anchoring sites on polyethylene glycol (PEG) polymers. The hydrophobically-modified PEGs (HMPEGs) are comb-graft polymers with strictly alternating monodisperse PEG blocks (M_w=6, 12, or 35 kDa) bonded to C18 stearylamide hydrophobes. Cooperativity is varied by changing the degree of oligomerization at a constant ratio of PEG to stearylamide. Fusogenic liposomes prepared from *N*-C12-DOPE:DOPC 7:3 (mol:mol) were equilibrated with HMPEGs. Affinity for polymer association to liposomes increases with the degree of oligomerization; equilibrium constants (given as surface coverage per equilibrium concentration of free polymer) for 6 kDa PEG increased from 6.1±0.8 (mg/m^2)/(mg/ml) for 2.5 loops to 78.1±12.2 (mg/m^2)/(mg/ml) for 13 loops. In contrast, the equilibrium constant for distearoylphosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG5k) was 0.4±0.1 (mg/m^2)/(mg/ml).

The multi-loop HMPEGs demonstrate higher levels of protection from complement binding than DSPE-PEG5k. Greater protection does not correlate with binding strength alone. The best shielding was by HMPEG6k-DP3 (with three 6 kDa PEG loops), suggesting that PEG chains with adequate surface mobility provide optimal protection from complement opsonization. Complement binding at 30 min and 12 h demonstrates that protection by multi-looped PEGs is constant whereas DSPE-PEG5k initially protects but presumably partitions off of the surface at longer times.

Keywords: Fusogenic liposome; Hydrophobically modified PEG; Liposome protection; Complement assay; Comb-graft; Protein shielding

1. Introduction

Liposomes are spheres made up of lipid bilayers, enclosing aqueous volumes [1]. They act as models for biological membranes and show great potential as drug delivery vehicles, especially in cancer and gene therapy [2–10]. Certain fusogenic liposomes, such as those incorporating *N*-acyl phosphatidylethanolamines (NAPEs), have the ability to deliver dextran to erythrocyte ghosts [11] and transfect ovarian cancer cells with DNA encoding a GFP protein [12]. Nonetheless, the use of fusogenic liposomes and other liposomal formulations is often limited by their rapid uptake by the mononuclear phagocytic system (MPS) [9,13–18].

The concept of sterically stabilized or long circulating liposomes was first realized in 1987 when lipids modified with different polymers and polysaccharides were incorporated into the bilayer to increase the circulation half-life of liposomes [3,4,7–10,15,17,19–30]. The most effective coating was formed by integrating distearoylphosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG5k) with a 5 kDa molecular weight PEG chain [6,7,10, 17,22,26,29,31]. Addition of 2.5 to 10 mol% DSPE-PEG5k into liposomes made up of distearoylphosphatidylcholine (DSPC) increases the circulation half-life of liposomes from 0.47 to 8.4 h [17].

It is postulated that increased circulation is a result of PEG producing a steric barrier to protein binding. The structure of DSPE-PEG5k, as described by Woodle and Lasic [3], has the lipid embedded within the hydrophobic bilayer and the hydrophilic PEG chain protruding into the

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aqueous medium. The preference of PEG for water allows PEG to extend even at low grafting densities [32] and thus creates a repulsive, steric barrier. Senior [14] observed a decrease in the rate of protein adsorption onto liposomes incorporating DSPE-PEGs. These results were confirmed by Chonn et al. [33] who showed that greater protein binding, which occurred on bare liposomes, correlated with shorter circulation half-lives [33]. Liu et al. [16] also concluded that recognition and uptake of liposomes was determined by adsorption of specific proteins associated with opsonization, namely the complement proteins. Therefore, we developed an automated in vitro assay to measure liposome complement depletion.

In vitro binding of complement to liposomes has been shown to correlate inversely with circulation lifetime [20]. The complement system consists of a collection of serum proteins that act as a cascade for recognition of foreign agents. The most important of these proteins is C3, which is present in the bloodstream at levels similar to some immunoglobulins (1–2 mg/ml). Two consequences of complement activation are opsonization and lysis of target cells [16].

In this paper, we present the concept of *multiply attached* polymer chains as a mechanism for preparing protected liposomes. The approach exploits recent interest in cooperative binding [34] where multiple relatively weak binding interactions can lead to strong overall association. The concept is demonstrated by a series of new PEG-based comb copolymers with concatenated PEG chains having hydrophobic anchoring groups between the linked PEG chains. These polymers allow the comparison of binding vis à vis protection with polymers having exactly the same ratio of PEG to hydrophobe, but with varied cooperativity by varying the concatenation or degree of polymerization. These constructs overcome several limitations with the previous method of stabilizing liposomes with PEG-lipids: (1) the PEG-lipids must be incorporated during the liposome formation process rather than adding them to preformed liposomes [7,9], and (2) the single lipid anchoring limits the size and amount of PEG polymer that can be attached [22]. The multiply attached PEGs allow the addition of soluble polymer to the preformed liposomes, which permits greater flexibility in processing and tailoring liposome formulations. The second limitation arises because the singly attached PEG will partition off of the liposome surface if the PEG is too soluble. Experimentally, loss of protection is observed for PEG molecular weights above 5 kDa [22]. With multiple attachments, higher molecular weight PEGs can be attached to the liposome with high binding affinities. The hydrophobically modified PEG polymers (HMPEGs) used in this study are comb-graft polymers with strictly alternating monodisperse PEG blocks (M_w 6, 12 or 35 kDa) bonded to C18 stearylamide hydrophobes, with 2 to 13 hydrophobe anchors per polymer chain [35,36]. We report the ability of these polymers to associate with fusogenic liposomes at equilibrium and the ability of the polymers to shield liposomes from complement binding. We compare our results to DSPE-PEG5k, which has been studied previously [15,17,21,26,33].

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy polyethylene glycol (DSPE-PEG5k), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-dodecanoyl (*N*-C12-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) was acquired from Molecular Probes (Eugene, OR, USA). The 0.2-μm polycarbonate membrane filters, semi-micro disposable cuvettes, and 96-well plates were obtained from VWR (Bridgeport, NJ, USA). The HMPEG polymers were synthesized at Rutgers University (Piscataway, NJ, USA) as described by Heitz et al. [35]. Slide-A-Lyzer cassettes with a 10 000 MWCO membrane were procured from Pierce Biotechnology (Rocksford, IL, USA). Sheep erythrocytes and hemolysin rabbit anti-sheep erythrocyte stromata serum were purchased from Biowhittaker (Walkersville, MD, USA). Lyophilized rat sera, 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) and gelatin veronal buffer with EDTA (GVB-EDTA: in addition to the ingredients in GVB^{2+} it contains 10 mM EDTA, pH 7.4) were acquired from Sigma (St. Louis, MO, USA).

2.2. Preparation of liposomes

N-C12-DOPE:DOPC (7:3, mol:mol) large unilamellar vesicles (LUVs) were prepared as described by Shangguan et al. [11] with some modifications. The lipids were mixed in chloroform, dried under reduced pressure using a Büchi RE 111 Rotovapor and 461 water bath (Büchi Labortechnik AG, Flawil, Switzerland), then left under vacuum overnight to remove any residual solvent. The lipid film was hydrated with a TES buffer solution (10 mM TES, 150 mM NaCl, 0.1 mM EDTA, 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.

To aid in separation from unbound HMPEG polymer, the liposome density was increased by encapsulating a sucrose solution and a fluorescent marker (DiD) was added for visibility. Sucrose-encapsulating liposomes (*N*-C12-DOPE:DOPC:DiD 7:2.9:0.1 mol:mol:mol) were prepared as described above except in a sucrose buffer solution (10 mM TES, 250 mM sucrose, pH 7.4). After the liposomes were

made, non-encapsulated sucrose was removed by dialysis against the TES buffer (as described above) using a Slide-A-Lyzer cassette with a 10 000 MWCO. Dialysis occurred at 4 °C overnight. Sucrose-encapsulating liposomes incorporating DSPE-PEG5k were made using the same method described. The amount of DSPE-PEG5k (x) added was subtracted from the moles of DOPC ($2.9-x$), keeping the amount of DiD and *N*-C12-DOPE constant. Thus, the composition of the liposomes containing DSPE-PEG5k is written as *N*-C12-DOPE:DOPC:DSPE-PEG5k:DiD ($7{:}(2.9-x){:}x{:}0.1$, mol:mol:mol:mol).

The concentration of liposomes in solution was determined by the phosphate assay as described by Chen et al. [37]. Sizes of the liposomes were determined by quasi-elastic light scattering using a NICOMP 270 submicron particle sizer (NICOMP Instruments, Goleta, CA). Sucrose encapsulating and buffer encapsulating liposomes had number-averaged diameters of 62.1 ± 16.4 and 112.8 ± 28.5 nm, respectively.

2.3. Adsorption

Liposomes were equilibrated with polymer at a final concentration of 1.4 mM lipid in TES buffer. Hydrophobically modified polymer was added, either solubilized in TES buffer or as dry mass, to achieve the desired concentration. For DSPE-PEG5k-containing liposomes, four liposome compositions were prepared with increasing amounts of polymer coverage (0.14, 0.28, 0.70, and 2.40 times Γ^*, i.e. full surface coverage as calculated from the dimensions of the PEG loops [38]). The DSPE-PEG5k-containing liposomes were diluted as described above. All samples were vortexed and allowed to equilibrate overnight at room temperature in an Eppendorf 5436 Thermomixer (Brinkman Instruments, Westbury, NY, USA). After a 24-h equilibration, an initial fraction of 200 μl was removed. The remaining fraction was then centrifuged at $21\,000 \times g$ in the Eppendorf 5417r Centrifuge (Brinkmann Instruments, Westbury, NY, USA) at 4 °C. The supernatant was removed. Phosphate and PEG assays were performed on the supernatant and initial fractions to detect the amount of PEG per lipid in each sample. The association constant, K, was calculated by determining the initial slope (first four data points) of each adsorption profile, such that

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

2.4. PEG Assay

The amount of PEG was quantified by an assay described by Baleux [39], wherein 25 μl of an iodine-potassium iodide solution (0.04 M I_2, 0.12 M KI) was added to 1 ml of a diluted sample. Samples were diluted to an optimal adsorption range ($0.1 < \text{AU} < 1.0$). The diluted sample and color reagent were mixed in a disposable semi-micro cuvette with a 1.0-cm path-length. After 5 min, the optical density (OD) of the solution was determined at ambient temperature by a UV-2101PC adsorption spectrophotometer (Shimadzu Scientific Instruments, Princeton, NJ, USA) in the visible region, $\lambda = 500$ nm. Because the lipid in the assay could affect the OD, multiple calibration curves were required. Table 1 describes the correlation between the HMPEG concentration and OD for 0, 10, 30, and 50 μM lipid. Calibration curves were completed for DSPE-PEG5k with varying lipid concentrations; however, there was negligible dependence in lipid concentration. The slope given is an average of all of the calibration data taken at different lipid concentrations. The variation in the assay with different polymer architectures is due to the differences in hindrance to helix formation, which is the origin of the colored complex.

2.5. In vitro complement depletion assay

The complement assay is an in vitro assay that measures the depletion of complement protein from serum by the ability of the treated serum to achieve complement-mediated lysis of activated sheep red blood cells (RBCs). The complement assay was performed as described by Ahl et al. [20]. Activation of the sheep erythrocytes was performed

Table 1
HMPEG correlation values for quantification of PEG (mg/l) as a function of the optical density at increasing concentrations of lipid in μM

Polymer	0 μM lipid		10 μM lipid		30 μM lipid		50 μM lipid	
	$d[OD]/d[C_p]$	R^2	$d[OD]/d[C_p]$	R^2	$d[OD]/d[C_p]$	R^2	$d[OD]/d[C_p]$	R^2
HMPEG6k-DP3	0.038	0.987	0.041	0.980	0.043	0.980	0.046	0.973
HMPEG6k-DP13	0.022	0.971	0.023	0.980	0.022	0.998	0.023	0.989
HMPEG12k-DP2.5	0.054	0.996	0.053	0.982	0.056	0.982	0.057	0.988
HMPEG12k-DP5	0.046	0.973	0.050	0.982	0.048	0.997	0.053	0.992
HMPEG35k-DP2.5	0.049	0.976	0.048	0.978	0.052	0.979	0.053	0.992
DSPE-PEG5k	0.046	0.931	–	–	–	–	–	–

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 [53]:

$$R^2 = \frac{n(\Sigma XY) - (\Sigma X)(\Sigma Y)}{\sqrt{[n\Sigma X^2 - (\Sigma X)^2][n\Sigma Y^2 - (\Sigma Y)^2]}}.$$

by first washing the RBCs three times by adding GVB^{2+}, centrifuging at $8000 \times g$ for 4 min, and removing the supernatant. The RBCs were resuspended at 10^8 cells/ml, determined by hemacytometry. Next, the RBCs were incubated at 37 °C for 30 min with hemolysin rabbit anti-sheep erythrocyte stromata serum at 1:500 (v/v). The activated RBCs were washed three times and resuspended at 10^8 cells/ml. Activated RBCs were stored at 4 °C and were used within 1 week.

Each individual complement assay consisted of the following six samples that were prepared in 200-μl volumes: TES buffer (the negative control, without liposomes), 8 mM unmodified liposomes in TES buffer (the positive control), and four test samples containing 8 mM liposomes in TES buffer with increasing amounts of polymer. The samples were equilibrated overnight at 4 °C in the thermomixer. Each sample was then incubated with 100-μl reconstituted rat sera, diluted 1:1 (v/v) with GVB^{2+}, at 37 °C for 30 min with gentle shaking. Addition of 300 μl of GVB^{2+} was followed by vortexing and centrifugation at $8000 \times g$ for 4 min. We employed a Biomek® 2000 Laboratory Automation Workstation (Beckman Coulter, Brea, CA, USA) to perform the pipetting for the rest of the assay. A 100-μl volume of supernatant was diluted 1:1 (v/v) followed by eight successive serial dilutions in GVB^{2+}. Next, 100 μl of activated sheep RBCs was added to each dilution and allowed to incubate for 30 min at 37 °C with gentle shaking. Hemolysis was quenched by addition of 300 μl of GVB^{2+}-EDTA. RBCs that were not lysed were sedimented by centrifuging the samples at $8000 \times g$ for 4 min. A 200-μl aliquot from the supernatant of each sample was transferred to a 96-well plate. Hemolysis was determined by measuring the optical density of 200 μl of each sample well at 415 nm using a 3550-UV spectrophotometer plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Buffer encapsulating *N*-C12-DOPE:DOPC:DiD (7:2.9:0.1, mol:mol:mol) liposomes and sucrose encapsulating *N*-C12-DOPE:DOPC:DSPE-PEG5k:DiD (7:(2.9−*x*):*x*:0.1, mol:mol:mol:mol) were used in the complement assays. Sucrose encapsulation enables pelleting of the liposomes but does not interfere with the complement assay.

The complement assay results are plotted as the percent hemolysis versus the log of the inverse of the serum dilution. The CH50, utilized in related literature [40–42], is the serum dilution required to achieve 50% hemolysis and is directly proportional to the amount of active complement in the serum. The CH50 value for each hemolysis curve was obtained by a linear fit to a log–log version of the von Krough equation [43]. The inhibition of liposome complement binding or surface "protection" mediated by HMPEG or DSPE-PEG5k addition can be quantitatively described as the percentage of the maximum expected CH50 shift from the CH50 of the bare liposome to the CH50 of the buffer, i.e. no liposome sample. This can be calculated using the following equation, where B is buffer, BL is bare liposomes, and PL is polymer coated liposomes:

$$\%\ \text{Protection} = \frac{\text{CH50}_{\text{PL}} - \text{CH50}_{\text{BL}}}{\text{CH50}_{\text{B}} - \text{CH50}_{\text{BL}}} \times 100 \tag{2}$$

3. Theory and models

3.1. Defining coverage, Γ*

The HMPEGs consist of PEG backbones with $C_{18}H_{37}$ hydrophobes that are described by the size of the PEG spacer (molecular weight of PEG between hydrophobes) and by the number of loops (or degree of polymerization, denoted DP) [35,36]. The polymers are designated: HMPEG″*X*″-DP″*Y*″, where *X* is the molecular weight of the PEG spacers in Daltons and *Y* is the average number of PEG spacers.

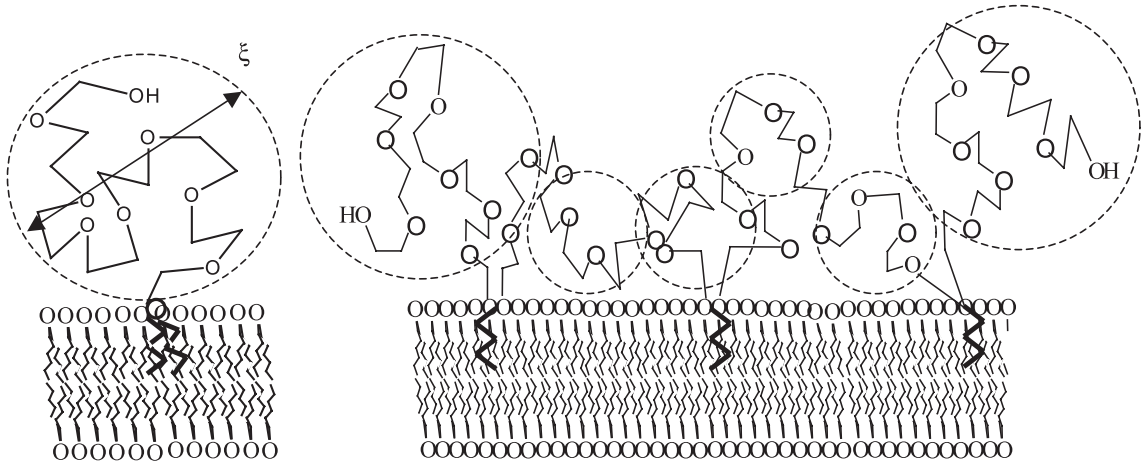
The conformation of the entire PEG polymer chain tethered to the lipid membrane is shown schematically in Fig. 1. Our previous studies have shown [38] that the chain can be treated as a series of subchains, having one-half the molecular weight of the PEG unit between hydrophobes. These subchains obey random-walk statistics and occupy an area at the interface given by a sphere of diameter [44]

$$\xi_{m_b} = 0.76 m_b^{1/2}\ [\text{Å}], \tag{3}$$

where m_b is the molecular weight of the subchain. The terminal PEG segments of the chain are treated differently because they are free and not bound at each end; therefore, they occupy a size corresponding to a sphere of diameter twice the molecular weight of the internal subchains (see Fig. 1). The number of spheres, N_b, is equal to two times the degree of polymerization minus the two ends ($N_b = 2D_p - 2$). The number-averaged diameter for spheres of a polymer chain is given as follows [38]:

$$\bar{\xi} = \frac{2}{N_b}(\xi_{2m_b}) + \frac{N_b - 2}{N_b}(\xi_{m_b}). \tag{4}$$

From the mean diameter, we determine the area occupied at the surface from $N_b\pi(\bar{\xi}/2)^2$. The total area of one polymer is then the number of subchains times the average area occupied by a subchain. Thus, HMPEG6k-DP3 has four subchains, a mean diameter of 50 Å, and requires an area of 7900 $Å^2$ per polymer. The amount of polymer to cover 1-m^2 lipid, Γ*, is 21 nmol, or 0.88 mg, of HMPEG6k-DP3. Assuming that each lipid head area is approximately 70 $Å^2$ [45] and that one half of the lipid is on the exterior, there are 4.7 μmol of lipid per square meter. Alternately, we can base the coverage on the number of hydrophobes required to cover 1-m^2 of lipid



a. DSPE-PEG5k b. Multi-loop PEG polymer

Fig. 1. Diagram of (a) a covalently bound PEG to a lipid and (b) a hydrophobically modified PEG associating with a lipid membrane.

bilayer or 4.7 μmol of lipid. See Table 2 for polymer details.

The idea of coverage is based on the amount of polymer needed to cover the exterior surface area of liposomes. From the random walk approximation described above, which we have verified by neutron scattering for the HMPEG chains [38], we are able to approximate the area occupied by the HMPEG polymers and the DSPE-PEG5k polymer. Because the polymers differ greatly in molecular weight and number of subchains, we find this the best means for comparison.

3.2. Partitioning model

A partitioning model can describe the association of a hydrophobe to a phospholipid bilayer. Consider the bilayer and aqueous solution as two phases. It is assumed that there is a partition coefficient which relates the amount of bound polymer per lipid area, [PL]/[L] (in mg/m^2), with the concentration of free polymer, [P] (in mg/ml) such that

$$\frac{[PL]}{[L]} = \frac{\Gamma}{\gamma}[P], \tag{5}$$

where Γ is the partition coefficient determined by the difference in free energy of the polymer between the two phases and γ is the activity coefficient, representing the deviation from ideality [46]. The plot of [PL]/[L] vs. [P] yields an association isotherm that is linear at low polymer concentrations and has a decreasing slope due to non-ideal interactions (γ>1) which depends on the polymer type. A strict thermodynamic analysis will define γ as the ratio of the polymer activity coefficients in both the aqueous and bilayer phases, $\gamma = \gamma_P^L/\gamma_P^A$. Porcar shows how this partitioning model relates to a simple binding model where [P]+[L]⇆[PL]. The slope, Γ/γ, is equivalent to the association constant divided by the number of available sites, K/N [46].

Table 2
Description of hydrophobically modified PEG polymers

Polymer	N_b	ξ, Å	MW, kDa	Polymer area, $\times 10^{17}$ m^2	Γ*, mg/m^2	Γ_{hm}*, hydrophobe mol%
HMPEG6k-DP3	4	50	42	7.9	0.88	0.89
HMPEG6k-DP13	24	43	112	35.0	0.53	1.21
HMPEG12k-DP2.5	3	75	48	13.3	0.60	0.40
HMPEG12k-DP5	8	65	106	26.5	0.66	0.53
HMPEG35k-DP2.5	3	128	138	38.8	0.59	0.14
DSPE-PEG5k	1	54	5781	2.3	0.42	1.55

To convert Γ* (mg/m^2) to Γ_{hm}*, determine the moles polymer per 4.7×10^{-6} mol of lipid then multiply by the number of hydrophobes (DP-1) and 100 to obtain the mol% hydrophobes per lipid, or C_{hm}*.

3.3. Cooperativity

We define cooperativity as the increased probability of the polymer to remain adsorbed due to its multiple attachment sites. It is our hypothesis that increasing the number of hydrophobic anchors on a PEG polymer increases the cooperativity of the polymer binding to the liposome membrane while still maintaining the ability to add the protective polymer after the formation of the vesicles. The cooperativity of a set of polymers can be evaluated by comparing the equilibrium constants, which assesses the ability of a polymer to leave the liposome surface. Thus, a polymer's cooperativity relies on its architecture. For a single hydrophobic anchor attached to

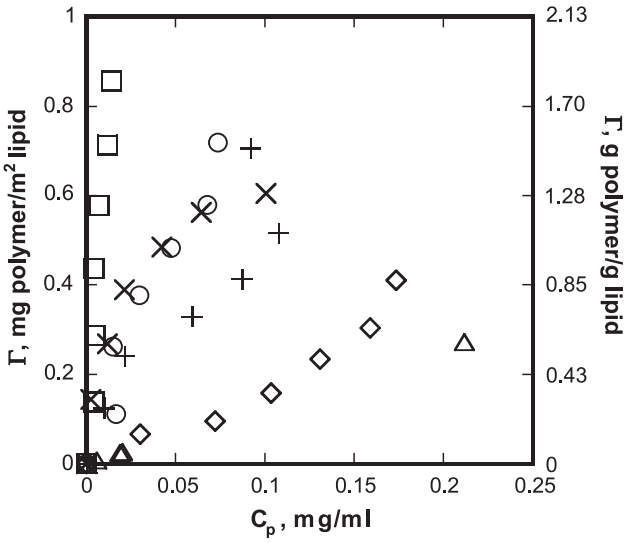


Fig. 2. Association of hydrophobically modified PEG polymers to sucrose encapsulating fusogenic liposomes. Graph depicts surface coverage, Γ, versus the free polymer in solution, C_p, for HMPEG6k-DP3 (○), HMPEG6k-DP13 (□), HMPEG12k-DP2.5 (◇), HMPEG12k-DP5 (×), HMPEG35k-DP2.5 (+), and DSPE-PEG5k (△). Each sample contained 1.4 mM liposomes and 0.1 to 0.6 mg/ml polymer in 1-ml TES buffer. Sucrose encapsulating liposomes incorporating 0.19, 0.37, 1.11, and 3.70 mol% DSPE-PEG5k were equilibrated in TES buffer. Samples were equilibrated for 24 h. Uncertainty comes from the precision of the Baleux assay (see Table 1) and the precision of the phosphate assay ($R^2 = 0.992$).

PEG, an association constant can be written for the relationship between the anchored polymer and the free polymer as follows [47]:

$$K = \frac{[\mathrm{PL}]}{[\mathrm{P}][\mathrm{L}]}. \quad (6)$$

The probability of one anchor desorbing can then be written in terms of the equilibrium constant such that

$$P_{\mathrm{desorption},1} = \frac{1}{K+1}. \quad (7)$$

If K is large, meaning a high affinity for the polymer being bound, then $P_{\mathrm{desorption},1}$ goes to zero. On the other hand, if K is small, corresponding to a low affinity of adsorption, the $P_{\mathrm{desorption},1}$ goes to 1. Assuming that there are no correlations between the anchors, the probability of a multi-loop polymer leaving the surface is the probability of all of the anchors being desorbed simultaneously, which for a chain of x anchors gives:

$$P_{\mathrm{desorption}} = (P_{\mathrm{desorption},1})^x. \quad (8)$$

Therefore, for the multi-looped PEGs in this study, with 2 to 13 anchors, the effect of cooperative interactions should be pronounced.

4. Results and discussion

4.1. Association of HMPEGs with fusogenic liposomes

Polymer binding involves the interplay between hydrophilicity (molecular weight of PEG) and hydrophobicity (number of anchors) that controls the HMPEG polymers' association to liposomes. Fig. 2 and Table 3 are the binding isotherms and association constants for the HMPEG polymers and DSPE-PEG5k on *N*-acyl liposomes. We have chosen to observe adsorption of HMPEGs to bare liposomes and desorption of DSPE-PEG5k from liposomes incorporating DSPE-PEG5k. Attempts were made at producing liposomes containing HMPEGs similar to the method of liposomes containing DSPE-PEG5k, i.e. incorporation of the polymer into the dry lipid before hydration; however, the mixture became a gel. HMPEGs were added to liposomes at the appropriate concentrations and allowed to equilibrate overnight with gentle shaking. For DSPE-PEG5k liposomes, four different lipid compositions were prepared into liposomes. These liposomes were diluted and equilibrated overnight with gentle shaking. The uncertainty comes from the precision of the Baleux assay (see Table 1) and the precision of the phosphate assay ($R^2 = 0.992$).

The polymer with the greatest association to the lipid membrane is HMPEG6k-DP13 with an association constant of $K = 78.1 \pm 12.2$ (mg/m^2)/(mg/ml). In comparison, HMPEG6k-DP3, which has the same molecular weight spacer and same ratio of PEG loop hydrophobic anchors, has an association constant that is 1/13th as strong. Increasing the cooperativity by ~4-fold increases the binding 13-fold. A qualitatively similar response is observed for HMPEGs with a 12-kDa spacer, HMPEG12k-DP2.5 and HMPEG12k-DP5.

At constant number of loops, increasing the molecular weight of the PEG spacer reveals the effect of the polymer's hydrophilicity. We expect that increasing the PEG molecular weight will reduce the affinity for binding. This is observed when we compare HMPEGs with 6k and 12k spacers, with approximately three loops. However, the HMPEG35k-DP2.5 polymer has an association strength between HMPEG6k-DP3 and HMPEG12k-DP2.5. This suggests that

Table 3
Association constants of HMPEGs to fusogenic liposomes

Polymer	Association constant, K (mg/m^2)/(mg/ml)
HMPEG6k-DP3	6.1 ± 0.8
HMPEG6k-DP13	78.1 ± 12.2
HMPEG12k-DP2.5	1.9 ± 0.1
HMPEG12k-DP5	16.4 ± 2.5
HMPEG35k-DP2.5	4.3 ± 0.5
DSPE-PEG5k	0.4 ± 0.1

Error is given as one standard deviation.

there may be a range of PEG molecular weights between 6k and 35k where polymers are not able to pack efficiently, resulting in a minimum in K.

The adsorption isotherms in Fig. 2 show multi-layer coverage at high polymer concentrations. Full surface coverage for HMPEG6k-DP13 is calculated to be $\Gamma^* = 0.53$ mg/m^2 and we find experimentally a surface coverage Γ of 0.85 mg/m^2 in equilibrium with $C_p = 0.02$ mg/ml. Sunamoto et al. [30] studied the adsorption of hydrophobically modified polysaccharides to liposomes and observed binding levels greater than theoretical coverage and adsorption increasing with the number of hydrophobic anchors [30].

An important observation is that DSPE-PEG5k partitions off of the surface of *N*-acyl liposomes in TES buffer. After overnight equilibration in TES buffer, 21% of the added DSPE-PEG5k is in the supernatant. PEG covalently bound by one lipid has the lowest association constant $\{K = 0.41 \pm 0.08(\text{mg/m}^2)/(\text{mg/ml})\}$ of all the polymers tested.

4.2. In vitro complement depletion assay

The complement assay measures how well the HMPEGs and DSPE-PEG5k inhibit in vitro complement binding and thus "protect" the liposomes from complement opsonization. The complement assay results are presented as the percent hemolysis versus the log of the inverse of the serum dilution, i.e. Log(1/SD). The typical complement assay hemolysis curve such as Fig. 3 shows a sigmoidal curve

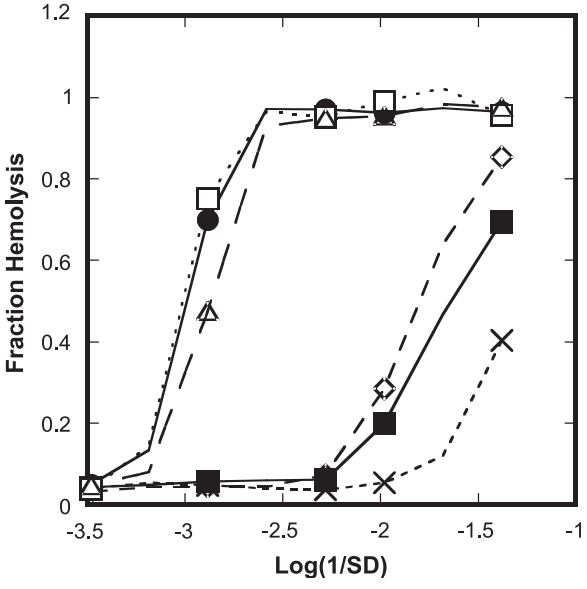


Fig. 3. Complement assay for *N*-C12-DOPE:DOPC 7:3 (mol:mol) liposomes modified with HMPEG6k-DP3. Graph depicts buffer (●), liposomes corresponding to 0.34 m^2 lipid area (■), liposomes with 0.05 mg polymer (◇), 0.15 mg polymer (×), 0.50 mg polymer (□), or 1.50 mg polymer (△). Γ^* is 0.88 mg/m^2. Addition of 0.15 mg HMPEG6k-DP3 shifts the curve to the right instead of towards 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. To obtain the surface coverage (mg/m^2), divide the amount of polymer added by the lipid area, i.e. 1.50 mg HMPEG6k-DP3/0.34 m^2 lipid is 5 mg/m^2. 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.

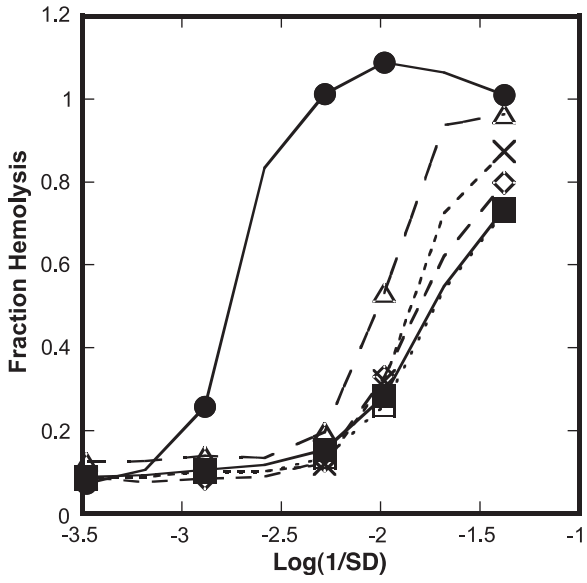


Fig. 4. Complement assay for *N*-C12-DOPE:DOPC 7:3 (mol:mol) liposomes modified with HMPEG6k-DP13. Graph depicts buffer (●), liposomes corresponding to 0.34 m^2 lipid area (■), liposomes with 0.03 mg polymer (◇), 0.09 mg polymer (×), 0.30 mg polymer (□), or 0.90 mg polymer (△). Γ^* is 0.53 mg/m^2.

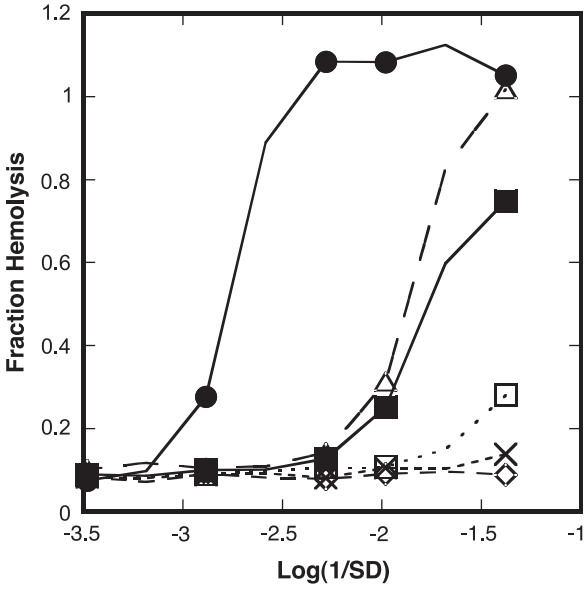


Fig. 5. Complement assay for *N*-C12-DOPE:DOPC 7:3 (mol:mol) liposomes modified with DSPE-PEG5k. Graph depicts buffer (●), liposomes corresponding to 0.34 m^2 lipid area (■), liposomes with 0.02 mg polymer (◇), 0.04 mg polymer (×), 0.10 mg polymer (□), or 0.34 mg polymer (△). Γ^* is 0.42 mg/m^2.

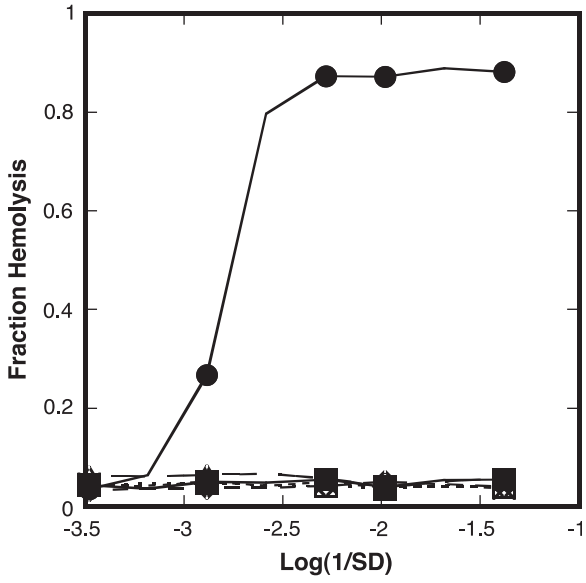


Fig. 6. Complement assay with 12-h incubation with rat sera for *N*-C12-DOPE:DOPC 7:3 (mol:mol) liposomes modified with DSPE-PEG5k. Graph depicts buffer (●), liposomes corresponding to 0.34 m^2 lipid area (■), liposomes with 0.02 mg polymer (◇), 0.04 mg polymer (×), 0.10 mg polymer (□), or 0.34 mg polymer (△). Γ^* is 0.59 mg/m^2. The weak protection shown in (Fig. 5) for a 30-min incubation is eliminated at the 12-h incubation, which shows that DSPE-PEG5k does not protect against complement binding over long periods of time.

with a sharp transition from low hemolysis to complete hemolysis as the SD decreases and the Log(1/SD) increases. The CH50 value is the SD at 50% hemolysis and is directly proportional to the amount of available complement following the initial serum incubation. Shifts in the CH50 value indicate changes in level of complement protection for different liposome formulations. Unmodified *N*-C12-DOPE/DOPE (7:3, mol/mol) liposomes with no PEG protection and high complement binding levels have low CH50 values typically under 100. We will arbitrarily define the CH50 values for unmodified liposomes as 0% protection. In contrast, 100% protection, which we define as the CH50 value of the buffer control, is typically in the 500 to 1000 range. The HMPEG and DSPE-PEG5k liposome formulations we tested in each experiment typically had CH50 values between these two extremes. The precision of each measurement can be evaluated based on 1 standard deviation from the mean for the buffer and liposome controls: 3.1% and 8.9%, respectively. Additional complement assays (with 30-min incubation) of HMPEG6k-DP3, HMPEG6k-DP13, HMPEG12k-DP2.5, and DSPE-PEG5k showed excellent agreement with the data presented.

The results are presented in the following order: (1) demonstration of how number and molecular weight of the PEG spacers produce cooperativity (Figs. 3 and 4); (2) comparison of DSPE-PEG5k (Fig. 5) versus the multiply attached chains (Figs. 3 and 4); and (3) evaluation of the time dependence of the DSPE-PEG5k (Fig. 6) dissociation in contrast to the stability of the HMPEG protection (Fig. 7).

4.2.1. Number and molecular weight of the PEG spacers

In Fig. 3, HMPEG6k-DP3 shows 98% protection when the polymer concentration exceeds full coverage: Γ^*. For the higher molecular weight spacers, HMPEG12k series, a degree of polymerization of five (DP5) shows 95% protection at 1.8 times the calculated polymer coverage (data not shown); however, it requires five times the calculated polymer coverage for HMPEG12k-DP2.5 to exhibit 98% shielding from complement binding (data not shown). The higher molecular weight PEG group requires more cooperative binding sites to affect protection. The lower molecular weight PEG (6k) with lower cooperativity (DP = 3) shows results that are comparable to the 12k PEG with the higher DP = 5. There is a tradeoff between higher molecular weight PEG that tends to pull the polymer off of the surface and greater cooperativity that keeps the polymer adsorbed. However, greater cooperativity alone is not sufficient to protect against complement binding. Namely, the highly cooperative HMPEG6k-DP13 is unable to shield against complement binding even with the addition of polymer five times Γ^*, as depicted in Fig. 4.

Based on our previous studies on these highly cooperative polymers [35,36], where we saw aggregation of these poly-

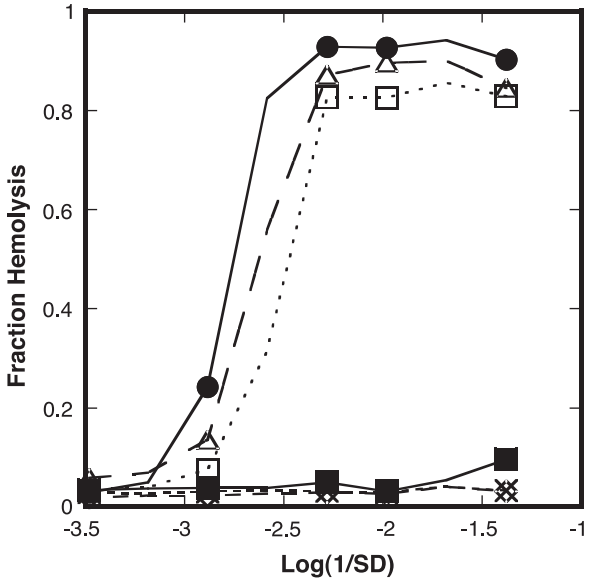


Fig. 7. Complement assay with 12-h incubation with rat sera for *N*-C12-DOPE:DOPC 7:3 (mol:mol) liposomes modified with HMPEG6k-DP3. Graph depicts buffer (●), liposomes corresponding to 0.34 m^2 lipid area (■), liposomes with 0.05 mg polymer (◇), 0.15 mg polymer (×), 0.50 mg polymer (□), or 1.50 mg polymer (△). Γ^* is 0.88 mg/m^2. The results are qualitatively similar to the results with a 30-min incubation (Fig. 3), which show that the polymer layer protects against complement binding over the time interval of 30 min to 12 h.

mers in solution, we suggest that too high a level of cooperativity leads to inter-chain and polymer self-assembly as depicted schematically in Fig. 8. The interactions are so strong that rearrangement of the polymer onto the liposome surface to form an impenetrable layer is frustrated. Although the mass of polymer associated with the liposome is high, the structure of the layer is not optimal and allows diffusion to the liposome surface. After 24 h, the HMPEG6k-DP13 coated liposomes display aggregation as a result of bridging. However, aggregation did not occur in HMPEG12k-DP2.5 and HMPEG35k-DP2.5 after 1 week. This is a kinetic effect, however, and may be controlled using excipients. Further investigation of polymer bridging is needed. The HMPEG35k-DP2.5 (data not shown) shows less than 50% protection at five times Γ^*, which is consistent with the high solubility of the PEG chain and low degree of cooperativity. This leads to the conclusion that strength of binding alone does not dictate complement shielding. The structure of the adsorbed polymer layer also plays a key role in protection.

4.2.2. DSPE-PEG5k versus multiply attached HMPEG polymers

Fig. 5 illustrates that at 2.4 Γ^* DSPE-PEG5k shows only 15% protection—compared to 95% at 1.8 Γ^* for HMPEG12k-DP5. Our previous studies have shown that the 12k PEG loop with attachment sites at each end behaves approximately like two terminally grafted 6k chains [38]. Therefore, the difference in performance between the DSPE-PEG5k and the HMPEG12k-DP5 is not due to the molecular weight of the PEG, but rather due to the cooperativity of the attachment. The DSPE-PEG5k can be thought of as the monomer (DP = 1) in the sequence of HMPEG12k-DP(n).

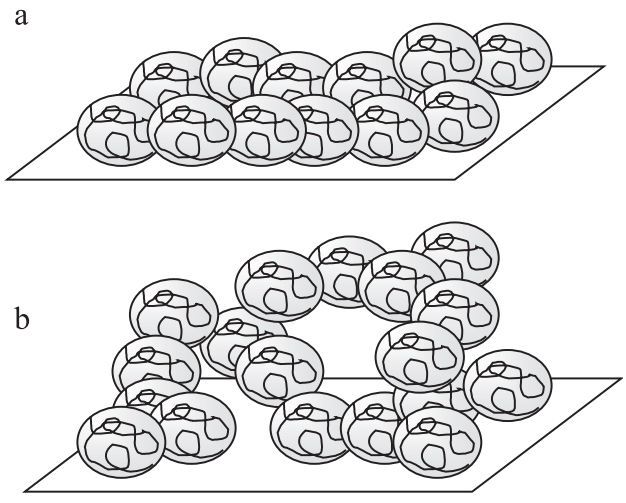


Fig. 8. Schematic of polymer blobs associating with a surface (a) at low levels of cooperativity, where the polymer can distribute over the surface, and (b) high levels of cooperativity, which immobilizes the polymer on the surface and also results in inter- and intra-polymer associations that affect polymer coverage.

It has been shown that incorporating DSPE-PEG5k into liposomes extends the in vivo circulation time [17,26]. From the calculation of PEG coil dimensions, 1.55 mol% of DSPE-PEG5k is needed for complete coverage of a liposome. Blume and Cevc [17] have shown that addition of 2.5 mol% DSPE-PEG5k to a liposome formulation shows similar protection to 10 mol%. Incorporation of 1.55 mol% corresponds to having 0.42 mg of DSPE-PEG5k per square meter. The data we present at the highest DSPE-PEG5k coverage 1.0 mg/m^2 is approximately equal to 3.7 mol%. Therefore, while we have not yet conducted the in vivo tests, these preliminary results form the basis of two hypotheses about in vivo experiments: First, HMPEGs with the appropriate architecture should provide even greater protection than DSPE-PEG5k. Second, in vivo tests would provide a substantiation of the ability of the in vitro complement binding assay to predict PEG-liposome protection in vivo. The complement assay has been validated in vivo for liposomes with different lipid compositions and modifications [20], but not for PEG-protected liposomes.

4.2.3. Time-dependent dissociation of the DSPE-PEG5k and stability of HMPEG binding

To broaden our observations to longer circulation times, we modified the complement assay to extend the incubation with rat serum from 30 min to 12 h. As shown in Fig. 6, after an incubation of 12 h, the DSPE-PEG5k shows no protection from complement binding. There are two possible explanations. The first is that the DSPE-PEG5k layer is dynamic enough that even though the PEG chains are overlapped ($\Gamma^*>1$), fluctuations can expose regions of the liposome surface to which proteins adsorb. The second explanation, which we feel is more likely, is that the weaker equilibrium constant of the DSPE-PEG5k allows dissociation of the polymer from the liposome surface in the presence of serum. Electrostatic repulsion may aid in the desorption of DSPE-PEG5k from the negatively charged liposome because it is itself negatively charged. The HMPEG adsorbed layers have slow dynamics as we have shown from Spin Echo Neutron Scattering [38] and therefore we would expect them to be less prone to protein penetration by the first mechanism. The multiple attachment of the HMPEGs slows the dynamics of detachment in the same way that they increase the energetics of attachment. In the literature, there does not appear to have been a thorough study of the dynamics of partitioning of the PEG from the liposome surface during in vivo tests. Experiments of this sort would certainly aid in the understanding and application of long circulating liposomes.

In contrast to the DSPE-PEG5k, the HMPEG6k-DP3 shows the same high level of protection after 30 min of incubation (Fig. 3) or 12 h (Fig. 7). The stability of the HMPEG polymer to resist either displacement from the liposome surface or penetration by complement protein is quite remarkable when compared to the DSPE-PEG5k.

5. Conclusions and summary

We have demonstrated protection from complement binding by adsorption of multi-looped, HMPEG polymers to fusogenic liposomes. A series of polymer samples has been prepared with monodisperse PEG chains in a strictly alternating copolymer with stearylamide hydrophobes. The polymers are designated: HMPEG″*X*″-DP″*Y*″, where *X* is the molecular weight of the PEG spacers in Daltons and *Y* is the average number of polymer chains in the polymer. Cooperativity is defined by the reduced probability that the polymers will desorb from the liposome surface. Multiple attachment sites result in a stronger binding association of the polymer to liposomes, which increases with number of anchors. Increasing the molecular weight of PEG between hydrophobes affects cooperativity by controlling the efficiency of packing. Some polymers, such as HMPEG6k-DP3 and HMPEG35k-DP2.5, exceed the amount of polymer required to occupy a specific area, producing multiple layers.

HMPEGs provide excellent protection against complement binding as measured by the hemolysis assay. However, binding strength alone does not correlate with protection. The most resiliently bound HMPEG, HMPEG6k-DP13, displayed very low levels of protection. This indicates that the structure of the adsorbed PEG layer must play a key role in protection. The highly cooperative HMPEG6k-DP13 may have strong inter- and intra-polymer associations that prevents the chains from being able to redistribute uniformly over the liposome surface [48]. The theories of Rubinstein and Semenov [49] on association of comb-graft polymers indicate strong inter-molecular interactions leading to phase separation for this class of polymers. Further experiments using surface plasmon techniques could address the question of the importance of intra versus inter-chain associations on PEG adsorption and complement protection.

The standard technique to prepare long circulating liposomes has been to incorporate DSPE-PEG5k lipids into the liposome formulation during the initial liposome formation process. An advantage of the HMPEG polymers is that they can be added to the liposome formulation after the liposomes have been formed. This uncouples liposome formulation and preparation from the protection process. Although HMPEG polymers have single acyl chain units (as opposed to two tails of the lipid in DSPE-PEG5k), it is the cooperativity of the acyl chain's binding that results in larger equilibrium constants for the HMPEGs. The individual hydrophobes of the HMPEG can re-equilibrate in solution and distribute on the liposome surface. However, if cooperativity is too great the polymer can become immobile as predicted by Rubinstein and Semenov [49]. With HMPEGs, liposomes with high polymer coverage and large PEG protective layers can be produced than is possible with PEG lipids.

The protection from complement binding provided by HMPEG6k-DP3 is constant over 12 h. In contrast, the protection by DSPE-PEG5k decreases from a moderate level after the normal 30-min assay period to no protection after 12 h of incubation. The temporal instability of the DSPE-PEG5k may arise from partitioning of the lipid off of the liposome surface or fluctuations in surface coverage that expose bare regions on the liposome surface. In either case, the HMPEG has neither of these defects.

This study suggests the following rules for the structures of HMPEG polymers for liposome protection. Equivalent protection was observed with HMPEG chains with lower degrees of polymerization and lower molecular weight PEG (6k) and HMPEG with larger degrees of polymerization and larger PEG (12k). This indicates a tradeoff is possible between solubility of the PEG and cooperativity of anchoring. Polymers with too high a level of cooperativity (DP13) did not perform well, and polymers with too high molecular weight PEG (35 K) did not function well either.

Currently, there is uncertainty as to the role PEG plays on the surface of liposomes to produce decreased liposomal clearance. Although it is assumed that PEG forms a steric barrier that reduces protein binding [29,50,51], Xu and Marchant [52] and Price et al. [29] found similar total protein adsorption profiles on bare liposomes and liposomes incorporating DSPE-PEG5k. Price et al. [29] speculate that the steric barrier may affect the interaction between liposomes and macrophages. Our experiments address complement binding; they do not address steric stabilization between macrophages and liposomes. This would indicate a direction of future research should be in vivo studies. It may result that the 12k PEG provides a thicker steric barrier than the 6k PEG and provides longer circulation times.

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