Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomaterials 27 (2006) 2599–2608
Biomaterials
www.elsevier.com/locate/biomaterials

pH triggered release of protective poly(ethylene glycol)-b-polycation copolymers from liposomes

Debra T. Auguste[a], Steven P. Armes[b], Krystyna R. Brzezinska[c], Timothy J. Deming[c], Joachim Kohn[d], Robert K. Prud'homme[a,*]

[a]Department of Chemical Engineering, Princeton University, Engineering Quadrangle, Princeton, NJ 08544, USA
[b]Department of Chemistry, Dainton Building, The University of Sheffield, Brook Hill, Sheffield, South Yorkshire S3 7HF, UK
[c]Department of Material Science, University of California at Santa Barbara, Santa Barbara, CA 93106-5050, USA
[d]Department of Chemistry, Rutgers University, Piscataway, NJ 08854-8058, USA

Received 7 April 2005; accepted 23 August 2005
Available online 27 December 2005

Abstract

Triggered release of adsorbed polymers from liposomes enables protection against immune recognition during circulation and subsequent intracellular delivery of DNA. Polycationic blocks, poly[2-(dimethylamino) ethyl methacrylate] (DMAEMA) (0.8, 3.1, 4.9, or 9.8 kg/mol) or polylysine (K) (3 kg/mol), act as anchors for poly(ethylene glycol) (PEG) (2 or 5 kg/mol) protective blocks. In addition, a copolymer with 15 strictly alternating blocks of PEG (2 kg/mol) and cationic amine sites was evaluated as a protective coating. Incorporation of 1,2-dioleoyl-3-dimethylammonium-propane, a titratable lipid with a *p*Ka of ~6.7, allows the liposome's net charge to increase as the pH shifts from 7.4 in the bloodstream to 5.5 in the endosome. The increased net liposome cationicity results in decreased cationic polymer adsorption. The EMPEG113–DMAEMA31 and EMPEG113–DMAEMA62 association constants decrease from 3.1 and 6.2 $(mg/m^2)/(mg/ml)$ at pH 7.4 to 1.7 and 3.2 $(mg/m^2)/(mg/ml)$ at pH 5.5, respectively. However, EMPEG45–DMAEMA5, EMPEG45–DMAEMA20, and EMPEG45–N–DP15 did not show a strong response to changes in pH. Cationic polymer adsorption exceeds calculated values for liposome neutralization, resulting in adsorption profiles in the brush regime.

Keywords: Adsorption; Drug delivery; Controlled drug release; Gene therapy; Polyethylene oxide; Liposomes

1. Introduction

Liposomes, or lipid bilayer spheres, have been investigated for drug and gene delivery because they mimic natural cell membranes, noncovalently encapsulate molecules, and are distributed in sites of inflammation and tumors [1,2]. The challenges that must be overcome for successful implementation in gene delivery include: (1) packaging of DNA into a condensed, biocompatible form, (2) protection from uptake by the mononuclear phagocytic system (MPS) during circulation, (3) transfer across the cell membrane (i.e. endocytosis), and (4) release of the DNA within the cell and avoidance of enzymatic degradation. One advantage of using liposomes for gene delivery is that the various design elements can be optimized separately. In its final formulation, an ideal liposome gene delivery vehicle may contain condensed, encapsulated DNA, an adsorbed protective polymer layer, targeting moieties and a mixture of lipids that induces fusion with cell membranes.

In this paper, we focus on poly(ethylene glycol) (PEG) protective layers that prevent uptake by the MPS and yet can be removed to allow intracellular DNA delivery. Liposome technology has focused on evading the immune system to improve passive delivery to tumors. Polymers [3], carbohydrates [4], and receptors [5] have been investigated as a means to coat liposomes to protect against removal by the MPS and/or to improve the localization of liposomes at a specific site. Blume et al. [6] demonstrated that a protective layer of PEG adsorbed on liposomes can increase circulation times in vivo from 30 min to 8 h in mice. Recently, we have shown that adsorption of multiply

*Corresponding author. Tel.: +1 609 258 4577; fax: +1 609 258 0211.
E-mail address: prudhomm@princeton.edu (R.K. Prud'homme).

0142-9612/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2005.08.036

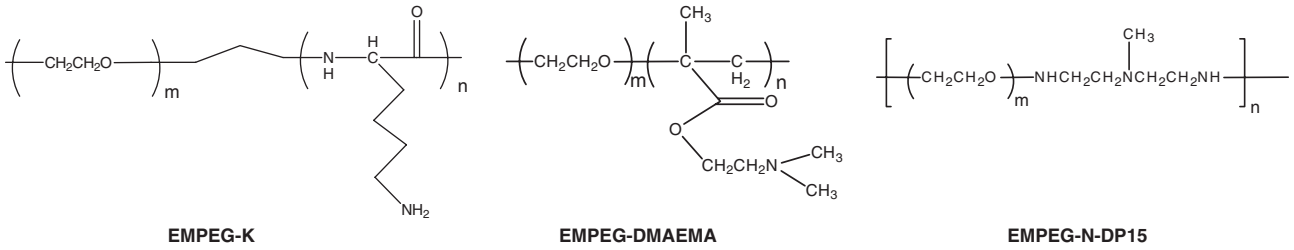

Fig. 1. Chemical structure of electrostatically modified PEG block copolymers and a strictly alternating PEG–amine copolymer.

attached hydrophobically modified PEG polymers on liposomes can inhibit liposome–protein interactions more effectively than the conventional, singly anchored PEGylated lipids [7]. Protein interactions have been shown to correlate with liposome removal by the immune system [8,9]. While PEG layers affect protection from MPS uptake, they do not prevent endocytosis and transport across the cell membrane [10].

Removal of the protective polymer layer is necessary once inside the cell to allow the liposome to fuse with the endosome and release the DNA. The pH change from the bloodstream (pH 7.4) to the endosome (pH ~5.5) allows for triggered deprotection and release of the polymer from the liposome surface [11]. Incorporation of a titratable lipid results in the liposome's net charge being pH dependent. We demonstrate the ability of polycation–PEG conjugates to bind negatively charged liposomes and to dissociate upon the physiologically relevant pH shift, from pH 7.4 to 5.5.

In this work, we formulate liposomes with variable surface charge by varying the composition of a zwitterionic lipid (phosphatidylcholine, PC) and an anionic lipid (phosphatidylglycerol, PG). To induce a pH-dependent liposome surface charge, we incorporate a titratable lipid (dimethylammonium propane, DAP) with a $p\mathrm{Ka} = 6.7$ [12]. Electrophoretic mobility measurements are used to monitor the change in net charge of the liposomes as lipid concentration and pH are varied.

The pH-dependent polymer binding is studied for two distinct polymer architectures: a block copolymer and a comb-graft copolymer. The affects of total cationic charge, type of cationic anchor, and PEG molecular weight on adsorption are investigated.

2. Experimental protocol

2.1. Materials and methods

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (PG), and 1,2-dioleoyl-3-dimethylammonium-propane (DAP) 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 with a 1.0 cm path-length, sodium citrate, and 10 × 75 mm borosilicate glass culture tubes were obtained from VWR (Bridgeport, NJ, USA). Nanosep tubes (100k MWCO) were purchased from Fisher Scientific (Suwanee, GA, USA). All electrostatically modified PEG polymers are designated as EMPEG–(polycation). The poly(ethylene glycol)-b-poly[2-(dimethylamino)ethyl methacrylate] (EMPEG–DMAEMA) polymers, were synthesized at the University of Sheffield (Sheffield, South Yorkshire, UK) as described by Deshpande et al. [13]. The block copolymers are designated as EMPEG"X"–DMAEMA"Y", where X is the number of PEG monomers and Y is the number of cationic monomers. The poly(ethylene glycol)-b-poly(lysine) (EMPEG122–K21, with 122 monomers PEG and 21 monomers K) polymers were prepared at the University of California, Santa Barbara (Santa Barbara, CA, USA) as described by Yu et al. [14]. The strictly alternating PEG–amine copolymer, poly(polyethylene glycol (2 kg/mol, 45 monomers)–3,3′-diamino-N-methyldipropylamine) (EMPEG–N-DP15), was made by D. Bolikal at Rutgers University (Piscataway, NJ, USA). Fig. 1 depicts the chemical structure of the polymers.

2.2. Preparation of liposomes

Pure PC and mixtures of PC:PG:DAP (8:1:1, 4:2:4, 7:0:3, mol:mol:mol) large unilamellar vesicles (LUVs) were prepared as described by Shangguan et al. [15], with some modifications given below. The anionic charge density on the liposome was varied by changing the ratio of PC (zwitterionic) to PG (anionic) lipid. Incorporation of the titratable lipid, DAP, induced a pH-dependent nonionic to cationic response. The lipids were mixed in chloroform and dried for 8 h under reduced pressure (house vacuum) at room temperature to remove residual solvent. The lipid film was hydrated with a TES buffer solution (1 mM TES, 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 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. Sizes of the liposomes were determined by quasi-elastic light scattering using a Lexel argon ion laser (Fremont, CA, USA) and a Brookhaven Instruments goniometer and correlator (BI-2030) (Holtsville, NY, USA) at $\theta = 90^\circ$ and $\lambda = 514.5$ nm. The liposomes have a number-averaged diameter of 146 ± 24 nm.

The concentration of lipid in solution was determined by the phosphate assay as described by Chen et al. [16]. Diluted liposome samples were added to 10 × 75 mm borosilicate glass culture tubes with 0.2 ml of 10 wt% sulfuric acid and heated for 1 h at 200 °C. Addition of 50 μl of 30% hydrogen peroxide was followed by heating for 1 h at 200 °C. Samples were cooled followed by the addition of 480 μl deionized water and 0.5 ml color reagent (0.5% ammonium molybdate, 2% ascorbic acid). The samples were vortexed and heated at 45 °C for 20 min. Samples were diluted to within an optimal adsorption range ($0.1 < \mathrm{AU} < 1.0$). The absorbance ($\lambda = 820$ nm) was recorded using a Beckman DU-64 spectrophotometer (Fullerton, CA, USA).

2.3. Adsorption

Liposomes were prepared at a concentration of 1.0 mM lipid in 0.5 ml TES buffer. Polymer, solubilized in either a 1 mM TES (pH 7.4) or a 1 mM sodium citrate (pH 5.5) buffer, was added to achieve the desired

Table 1
Description of electrostatically modified PEG polymers

Polymer	MW (kg/mol)	Full mushroom coverage (mg/m^2)	Neutralization of liposome (mg/m^2)	Baleux calibration	
				d[OD]/dC_p	R^2
EMPEG113–DMAEMA31	9.9	0.7	0.8	0.0185	0.918
EMPEG113–DMAEMA62	14.7	1.1	0.6	0.0134	0.913
EMPEG45–DMAEMA5	2.8	0.5	1.3	0.0135	0.844
EMPEG45–DMAEMA20	5.1	0.9	0.6	0.0139	0.892
EMPEG122–K21	8.6	0.6	11.9	0.0372	0.989
EMPEG45–N-DP15	75.0	0.9	1.0	0.0341	0.998

Full mushroom coverage is defined as the mass of polymer required to cover an area of lipid, with the PEG chain area defined by the Flory radius of the polymer ($A = \pi R^2$). Liposome neutralization is the mass of polymer per lipid area required to neutralize the lipid charge exposed on the surface. We assume each phosphate has a negative charge that can be neutralized by a single cation. The Baleux calibration curve yields the slope (change in optical density (OD) to change in polymer concentration (C_p)) used to determine the polymer concentration in solution. The R^2 value describes the uncertainty of the slope.

concentration. The effect of salt concentration was examined by adding 150 mM NaCl to the TES buffer. All samples were vortexed and allowed to equilibrate overnight at room temperature with gentle shaking (American Rotator V, Miami, FL, USA). The samples were added to Nanosep tubes and centrifuged at 1000g for 10 min. The supernatant was then assayed for phosphate and polymer content.

The polymer concentration was quantified by an assay described by Baleux [17], 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 supernatant sample. Samples were diluted to an optimal adsorption range ($0.1 < AU < 1.0$). After 5 min, the optical density (OD) of the solution ($\lambda = 500$ nm) was determined at ambient temperature. Liposomes were retained by the Nanosep membrane and did not influence the Baleux assay. The variation in the calibration curve with different polymer architectures is due to the differences in hindrance to helix formation, which is the origin of the colored complex. Table 1 reports the slope and error associated with the calibration profiles.

Γ is the mass of polymer adsorbed per square meter lipid. Table 1 provides a description of the polymers investigated and the mass of polymer required to neutralize or coat 1 m^2 of liposome surface. To determine the mass of polymer required to neutralize the liposome surface, we first calculate the number of polymer chains needed by dividing the number of DAP molecules on the liposome surface by the number of cationic anchors of the polymer. Once the number of polymer chains is converted to mass by using the molecular weight and Avogadro's number, we then divide by the liposome surface area. Full surface coverage is calculated by dividing the polymer mass by the lipid surface area. The polymer mass is determined by dividing the total lipid surface area by the area of one polymer and converting to mass.

To determine the amount of polymer needed to coat the surface, we determine the surface area occupied by a polymer. PEG chains obey random-walk statistics and occupy an area at the interface given by a sphere of diameter [18]

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

where m_b is the molecular weight of the chain. From the sphere diameter, we determine the area occupied at the surface from $\pi(\xi/2)^2$. Thus, EMPEG113–DMAEMA31 has a diameter of 54 Å, and occupies an area of 2270 Å^2 per polymer. The quantity of polymer to coat 1 m^2 lipid, Γ^*, is 73 nmoles, or 0.7 mg, of EMPEG113–DMAEMA31. The association constant, K, is determined from the initial slope (first four data points) of each adsorption profile, such that

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

2.4. Electrophoretic mobility

The electrophoretic mobility was measured on a Coulter DELSA 440 (Langley Ford Instruments, Amherst, MA, USA). Liposome solutions were prepared (1 mM lipid) in 5 ml TES buffer with and without polymer at varying concentrations. The solutions were allowed to equilibrate overnight at room temperature under gentle shaking. The cell reservoir was rinsed thoroughly three times with deionized water and three times with buffer before loading with sample. Inspection of the cell ensured no bubbles were present. The system was calibrated using a Fisher conductivity standard (1015 mS/cm). Measurements were taken at 25 °C averaged over four laser beam angles (8.9°, 17.6°, 26.3°, and 35.2°). Each 60 s run was measured at 500 Hz and 10 mA, with a voltage time of 2.5 s and a 0.5 s relaxation period.

3. Results and discussion

Triggered release of two polymer architectures has been examined: block copolymers (a PEG chain covalently bound to a polycationic anchor) and a strictly alternating PEG–amine copolymer (with 15 repeating units of a PEG (45 monomers, 2 kg/mol)–amine conjugate). Fig. 2 depicts the two-dimensional attachment of the electrostatically modified PEG polymers to a lipid bilayer. We tune the affinity of adsorption by varying the polymer architecture, hydrophilicity (PEG molecular weight) and number and type of cationic anchors.

3.1. Tuning liposome surface charge

The electrophoretic mobility of liposomes incorporating different mole ratios of PG, PC, and DAP were determined. Pure PC liposomes have a mobility of -0.01 ± 0.1 (μm/s)/(V/cm), which is essentially zero within experimental error. The electrophoretic mobility of PC:DAP liposomes at pH 7.4 is also zero within experimental uncertainty and is independent of DAP concentration. Upon decreasing the pH to 5.5, the mobility of PC:DAP (7:3, mol:mol) liposomes increased to 1.2 ± 0.1 (μm/s)/(V/cm). Liposomes incorporating anionic PG exhibited smaller pH-dependent mobility changes when DAP was

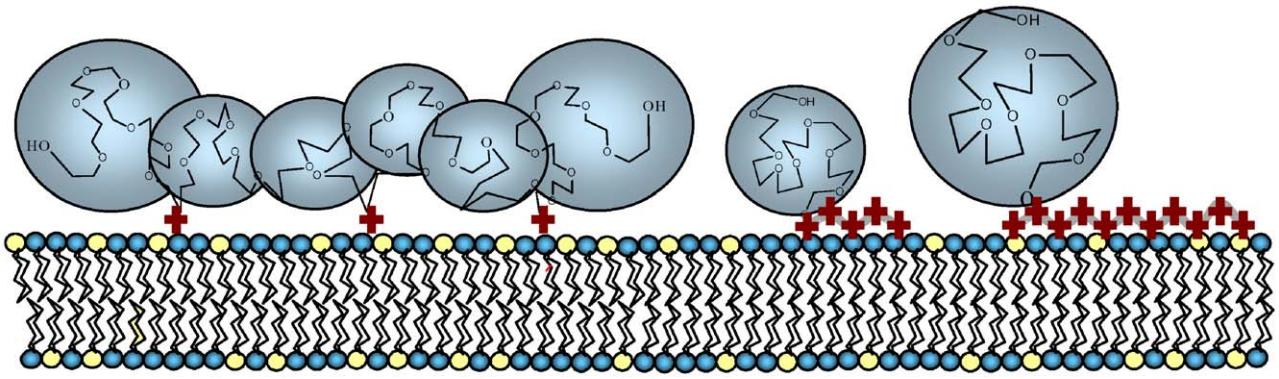

Fig. 2. Schematic of a strictly alternating PEG–amine copolymer bound to a lipid bilayer (left) and electrostatically modified PEG block copolymers (PEG-b-poly(cation)) (right). The block copolymers vary in number of monomers of PEG and number and type of cationic anchors.

added. PG:DAP (7:3, mol:mol) liposomes had an initial mobility of -3.7 ± 0.2 (μm/s)/(V/cm) at pH 7.4 which decreased to -3.0 ± 0.1 (μm/s)/(V/cm) when the pH shifted to 5.5. This demonstrates the ability to trigger an increase in liposome cationicity with pH shifts by incorporating DAP into the liposome formulation.

3.2. Influence of salt on polymer adsorption

Adsorption of electrostatically modified polymers to PG:PC (7:3, mol:mol) liposomes, shown in Fig. 3, is compared in the presence and absence of 150 mM NaCl. Overall the trend is a decrease in adsorption when the salt content is increased to physiological conditions. This is the expected ion exchange competition that weakens ionic associations [19].

In the absence of salt, the strongest binding EMPEG polymers are EMPEG113–DMAEMA62 and EMPEG45–DMAEMA20, both having an association constant of 3.0 (mg/m^2)/(mg/ml). The ratio of PEG monomers to cationic anchors (PEG:CA) is equal to approximately two for these polymers. We observe a trade-off in the hydrophilicity of the PEG polymer chain and strength of anchoring. In the presence of 150 mM NaCl, the association constant for EMPEG113–DMAEMA62 and EMPEG45–DMAEMA20 decreased by 46.7% and 10.0%, respectively. Increasing the molecular weight of PEG, at the same ratio of PEG:CA, results in greater polymer desorption in the presence of 150 mM NaCl. This shows that the binding strength of EMPEG113–DMAEMA62 and EMPEG45–DMAEMA20 at physiological conditions is not only dependent on the PEG:CA ratio but also the hydrophilicity of the PEG chain.

Increasing the PEG:CA ratio at constant PEG length results in lower polymer adsorption. In the absence of salt, the equilibrium constants for EMPEG113–DMAEMA31 (PEG:CA~4) and EMPEG45–DMAEMA5 (PEG:CA~9) are 2.5 and 2.1 (mg/m^2)/(mg/ml), respectively. These polymers exhibit a decrease in their equilibrium constants in the presence of salt, 61.5% for EMPEG113–DMAEMA31 and 55.0% for EMPEG45–DMAEMA5. Higher PEG:CA ratios, at constant PEG length, have lower binding affinities and greater sensitivity for desorption with respect to salt concentration.

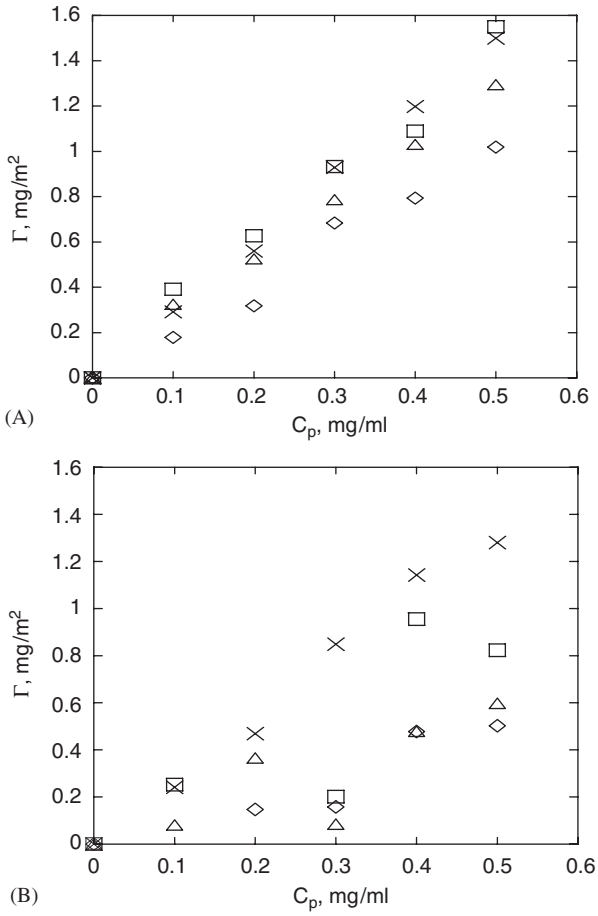

Fig. 3. Effect of salt concentration on the adsorption of EMPEGs to PG:PC (7:3, mol:mol) liposomes. Graphs depict the adsorption profiles in the absence (graph A) and in the presence (graph B) of 150 mM NaCl of EMPEG113–DMAEMA31 (△), EMPEG113–DMAEMA62 (□), EMPEG45–DMAEMA5 (◇), and EMPEG45–DMAEMA20 (×) bound to 1.0 mM lipid in 10 mM TES buffer, pH 7.4. The uncertainty comes from the precision of the Baleux assay (see Table 1) and the precision of the phosphate assay ($R^2 = 0.992$).

For similar PEG chain lengths, additional cations contribute to a stronger overall association. Four times the number of cationic DMAEMA units (from 5 to 20), with 45 monomers of PEG, results in roughly a 40% increase in binding affinity. For 113 PEG monomers, increasing the number of cations from 31 to 62 results in a 20% increase in the strength of binding. This correlates roughly to a 10% increase in binding strength for each doubling of the anchor length.

Although there is a reduction in polymer adsorption at physiological conditions, we are able to achieve adsorption in excess of 0.9 mg/m^2 for EMPEG45–DMAEMA20 and 1.0 mg/m^2 for EMPEG113–DMAEMA62. This allows us to conclude that we can obtain coverage in the brush regime at physiological conditions. A dense polymer layer is necessary to protect against protein interactions that contribute to immune recognition [20]. Optimization of the polymer architecture will allow improved adsorption at high salt concentrations.

3.3. Polymer adsorption

The adsorption isotherms demonstrate triggered release of electrostatically modified PEG polymers from pH-dependent (PC:DAP, 9:1, mol:mol) liposomes. These experiments were conducted in a 1 mM TES, 0 M NaCl, pH 7.4 buffer that has a Debye length of 8.7 nm. For 2 and 5 kg/mol PEG, the polymer layer thickness corresponds to 3.4 and 5.4 nm, respectively. Therefore, the Debye length extends past the adsorbed, unconstrained PEG layer. The experiments were conducted far below the overlap concentration of the polymer in solution (C^*~0.1 g/ml) [21,22]. We observe similar adsorption profiles as a result of a pH shift after a minimum of 30 min.

The adsorption isotherms of polymers to PC:DAP (9:1, mol:mol) liposomes (Figs. 4 and 5) show evidence of deprotection (decreased polymer binding affinity) as a result of a pH shift. The adsorption parameters are summarized in Table 2. Among the diblock copolymers, very strong binding of EMPEG113–DMAEMA62 and EMPEG45–DMAEMA20 correlated with their low PEG:CA ratios. These polymers had association constants at pH 7.4 of 6.2 and 3.2 (mg/m^2)/(mg/ml), respectively. The ability of the polymers to desorb upon a pH shift was dependent on their hydrophilic chain length. EMPEG113–DMAEMA62 desorbed 48% while EMPEG45–DMAEMA20 only desorbed 3%. Although their association constants at pH 7.4 differed, similar amounts of these copolymers were adsorbed at pH 5.5. Given their similar structures, we conclude that a longer hydrophilic PEG chain is necessary for destabilizing the electrostatic attraction to achieve polymer desorption. The polymer architecture can be optimized to be released or to remain bound. This would enable selective desorption where vesicles with two mixed polymer chain lengths has a barrier (the longer PEG chain) that can be displaced exposing the shorter PEG chain, which may incorporate a targeting moiety at the PEG terminus.

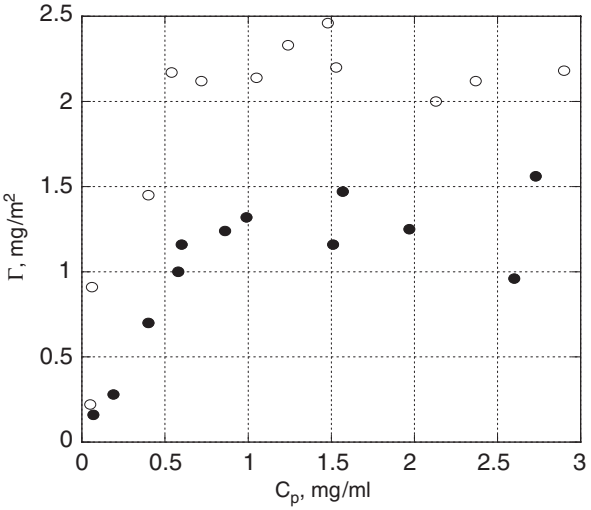

Fig. 4. Adsorption isotherms for the electrostatically modified PEG polymer EMPEG113–DMAEMA31 on PC:DAP (9:1, mol:mol) liposomes in either 1 mM TES buffer (pH 7.4, ○) or 1 mM sodium citrate buffer (pH 5.5, ●). EMPEG113–DMAEMA31 is a block copolymer with 113 monomers of PEG bound to 31 cationic anchors of DMAEMA. The graph depicts surface coverage, Γ, versus the free polymer in solution, C_p. Each sample contained 1.0 mM lipid and 0.1–3.5 mg/ml polymer. Samples were equilibrated for 24 h before separating the polymer-coated liposomes from free polymer in solution.

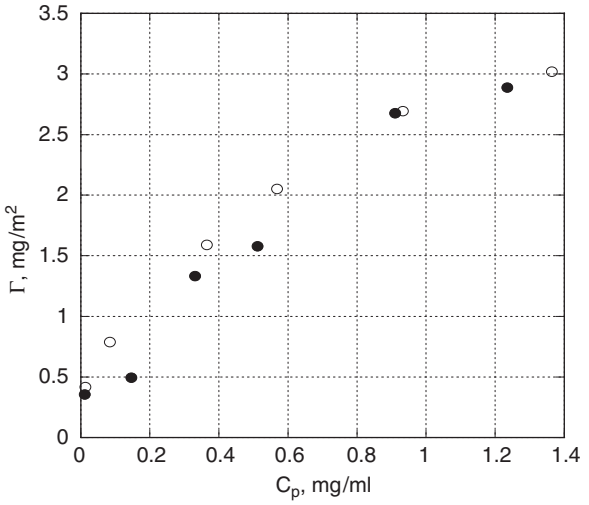

Fig. 5. Adsorption isotherms for the electrostatically modified PEG polymer EMPEG45–DMAEMA20 on PC:DAP (9:1, mol:mol) liposomes in either 1 mM TES buffer (pH 7.4, ○) or 1 mM sodium citrate buffer (pH 5.5, ●). EMPEG45–DMAEMA20 is a block copolymer with 45 monomers of PEG bound to 20 cationic anchors of DMAEMA. The graph depicts surface coverage, Γ, versus the free polymer in solution, C_p. Each sample contained 1.0 mM lipid and 0.1–3.5 mg/ml polymer. Samples were equilibrated for 24 h before separating the polymer-coated liposomes from free polymer in solution.

We have also compared block copolymers with identical PEG chain length and different number of anchoring cations. EMPEG113–DMAEMA31 has an association

Table 2
Comparison of adsorption parameters for EMPEG polymer adsorption on PC:DAP (9:1, mol:mol) liposomes

Polymer	PEG:CA	K, pH 7.4 (mg/m^2)/(mg/ml)	K, pH 5.5 (mg/m^2)/(mg/ml)	Saturation, pH 7.4 (mg/m^2)/(mg/ml)	Saturation, pH 5.5 (mg/m^2)/(mg/ml)
EMPEG113–DMAEMA31[a]	4	1.3	1.3	2.5	NA
EMPEG113–DMAEMA31	4	3.1	1.7	2.2	1.4
EMPEG113–DMAEMA62	2	6.2	3.2	2.6	1.5
EMPEG45–DMAEMA5	9	4.8	4.3	4.5	4.0
EMPEG45–DMAEMA20	2	3.2	3.1	3.0	3.0
EMPEG122–K21	6	1.2	0.2	0.8	NA
EMPEG45–N-DP15	45	1.4	1.4	NA	NA

We define the PEG:CA ratio as the monomers of PEG divided by monomers of cationic anchors. The equilibrium constants (K) are given for pH 7.4 and 5.5.
[a]Data reflect EMPEG113–DMAEMA31 adsorbed on latex spheres.

constant of 3.1 (mg/m^2)/(mg/ml) at pH 7.4. This is 50% less than the association constant for EMPEG113–DMAEMA62. At pH 5.5, the binding affinity of EMPEG113–DMAEMA31 is reduced by 45%. By tuning the PEG:CA ratio at constant PEG chain length, we can control the amount of polymer adsorbed.

Desorption of the EMPEG polymers is independent of the PEG:CA ratio but depends on PEG chain length. An exception to this observation is EMPEG45–DMAEMA5, which has an association constant of 4.8 (mg/m^2)/(mg/ml) at pH 7.4 that decreases by 10% when the pH shifts to pH 5.5 (data not shown). In comparison to EMPEG45–DMAEMA20, EMPEG45–DMAEMA5 has a 50% higher association constant but a lower PEG:CA ratio. This occurs due to the complementary packing of the polycation on the surface and the PEG chain in solution, where larger polycation anchors cause polymer–polymer repulsion that is greater than the steric repulsion of the PEG chain. The radius of gyration of DMAEMA5 (19 Å) is less than the radius of gyration of PEG45 (33 Å), whereas DMAEMA20 (38 Å) is twice that of DMAEMA5 and exceeds the radius of gyration of PEG45.

In addition to the DMAEMA anchor block, Fig. 6 illustrates anchoring PEG with lysine (K). Although EMPEG122–K21 has a similar number of EG segments in the soluble tail and cationic anchoring groups as EMPEG113–DMAEMA31, it has a lower affinity for binding at pH 7.4, 1.2 (mg/m^2)/(mg/ml). At pH 5.5, the association constant of EMPEG122–K21 has decreased by 83%. Polylysine forms an α-helix that is stabilized by hydrogen bonding; however, below its pKa (~10.0–10.54) Coulomb repulsion prevents hydrogen bond formation and the helix becomes a random coil [23]. We hypothesize that the steric nature of the polylysine's peptide backbone ($l_p \approx 30$ Å [24]) hinders its ability to orient and bind tightly to the liposome surface. The methylacrylic backbone of the DMAEMA block ($l_p \approx 21$ Å [25]) is more flexible, allowing for rearrangement on the liposome surface. A recent study on the influence of chain stiffness on the adsorption of polyelectrolytes to oppositely charged micelles found that stiffer chains bind more weakly, an effect that was increased at higher ionic strengths [26]. Polylysine is able to anchor a dense PEG layer at pH 7.4 and is able to be displaced almost completely from the liposome surface at pH 5.5.

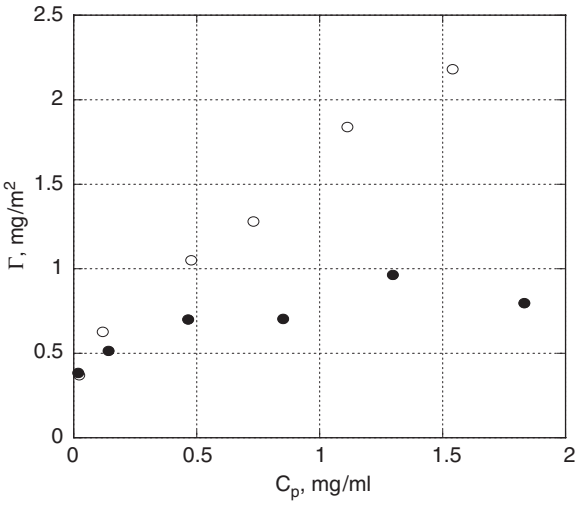

Fig. 6. Adsorption isotherms for the electrostatically modified PEG polymer EMPEG122–K21 on PC:DAP (9:1, mol:mol) liposomes in either 1 mM TES buffer (pH 7.4, ○) or 1 mM sodium citrate buffer (pH 5.5, ●). EMPEG122–K21 is a block copolymer with 122 monomers of PEG bound to 21 cationic anchors of polylysine. The graph depicts surface coverage, Γ, versus the free polymer in solution, C_p. Each sample contained 1.0 mM lipid and 0.1–3.5 mg/ml polymer. Samples were equilibrated for 24 h before separating the polymer-coated liposomes from free polymer in solution.

We observe triggered release of PEG block copolymers with either a DMAEMA or K anchoring block. We now introduce a strictly alternating PEG–amine copolymer that utilizes multiple, discrete, and relatively weak electrostatic interactions to achieve strong adsorption. This polymer, EMPEG45–N-DP15, exhibited an association constant at pH 7.4 of 1.4 (mg/m^2)/(mg/ml) (data not shown). Upon shifting the pH to 5.5, the affinity of binding was unaffected. The cumulative effect of multiple weak

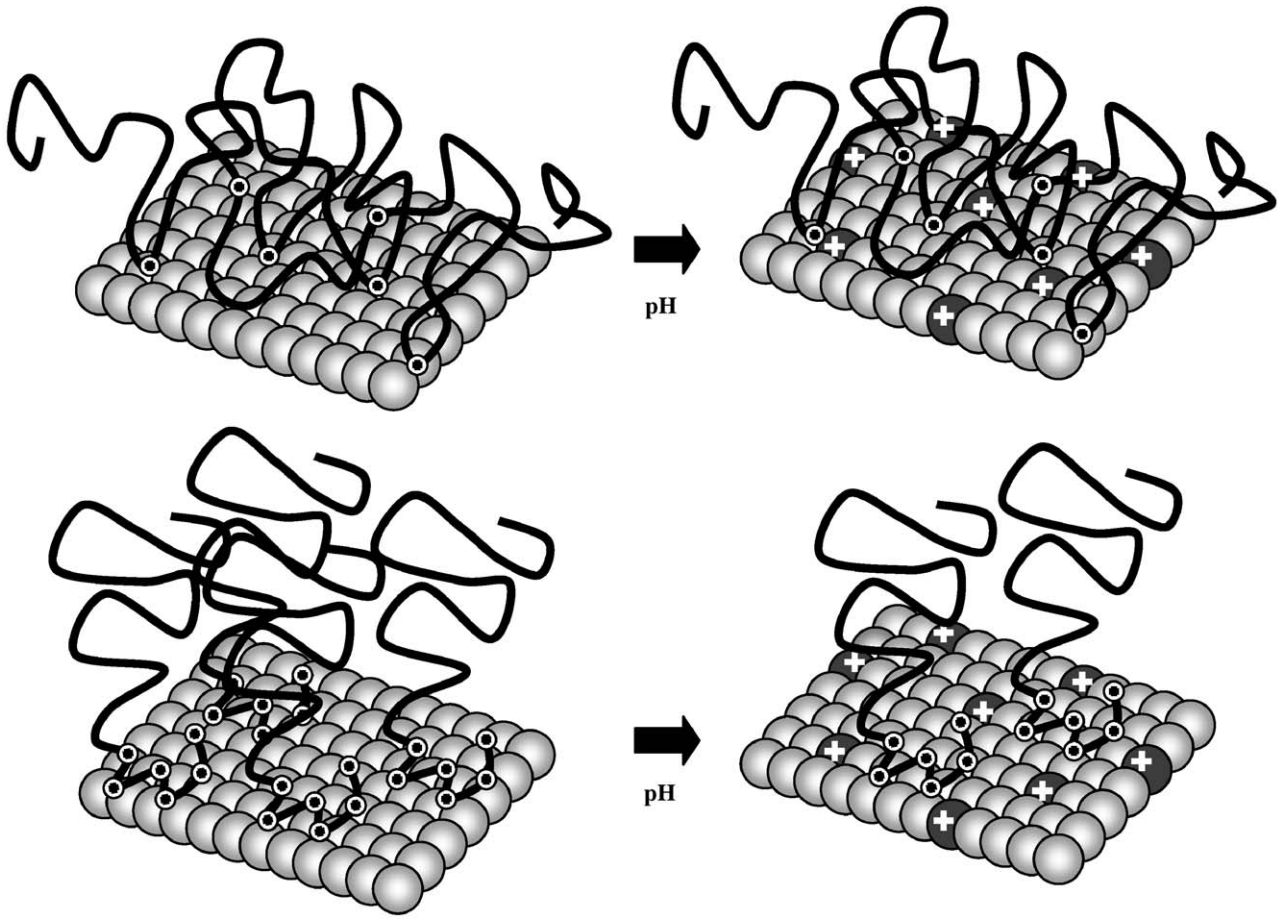

Fig. 7. Illustration of tunable lipid surface and impact on strictly alternating PEG–amine copolymer and PEG-b-polycation block copolymer. The ratio of zwitterionic to pH-sensitive lipids (9:1,mol:mol) and polymer chain masses are representative. The distribution of charge on the strictly alternating PEG–amine copolymer allows rearrangement of the multiple anchors on the surface and results in constant, strong overall association. This contrasts with the PEG-b-polycation block copolymers that have a dense area of cationic charge bound to the surface. The pH shift results in a competition between the anchors for suitable surface adsorption sites, which is limited by the positive-charge of the DAP on the lipid surface.

interactions is a strong overall association. In comparison with the EMPEG45–DMAEMA5 block copolymer (which has five cations per 45 PEG monomers compared with 15 repeating units of 1 cation per 45 PEG monomers for the EMPEG45–N-DP15), the equilibrium constant of EMPEG45–DMAEMA5 is 3.4 times stronger (4.8 mg/m^2) than EMPEG45–N-DP15 (1.4 mg/m^2). The positioning of the associating group in the backbone as opposed to a side-chain or terminal end results in weakened adsorption due to steric constraints that arise from the inflexibility of the polymer. A schematic illustration of this is given in Fig. 7. Evidence of this effect has been reported using hydrophobically modified alkali-soluble polymers [27,28]. The multiply anchoring EMPEG45–N-DP15 polymer is able to distribute more efficiently over the liposome surface resulting in pH insensitive adsorption but is hindered by having the anchoring site on the backbone, which reduces the anchoring efficiency.

Adsorption and triggered release of electrostatically modified PEG copolymers has been demonstrated on pH-sensitive liposomes. Liposomes provide a unique surface for adsorption because the lipids are able to move laterally throughout the bilayer. This allows them to achieve a lower free energy state (higher entropy) than on a surface with fixed charges. Fig. 8 addresses how EMPEG113–DMAEMA31 adsorbs onto polystyrene latex spheres with a fixed mobility of -5.5 ± 0.2 (μm/s)/(V/cm). The latex spheres have a mean diameter of 0.2 μm and were evaluated at a concentration that had an equivalent surface area (0.08 m^2) to our liposomes. The radius of curvature of the polymer is much less than the radius of curvature of the liposomes or latex spheres ($R_{\text{polymer}}/R_{\text{liposome}} = 0.045$); therefore, the curvature does not play an important role in adsorption. As depicted in Fig. 8, the association constant of EMPEG113–DMAEMA31 on latex spheres was 1.3 (mg/m^2)/(mg/ml) and was independent of pH. The maximum amounts of polymer adsorbed on liposomes and latex spheres at pH 7.4 are 2.2 mg/m^2 (where the onset of polymer saturation occurs in equilibrium with 0.5 mg/ml) and 2.5 mg/m^2 (where the onset of polymer saturation occurs in equilibrium with 1.6 mg/ml), respectively. However, the association constant for binding to liposomes is

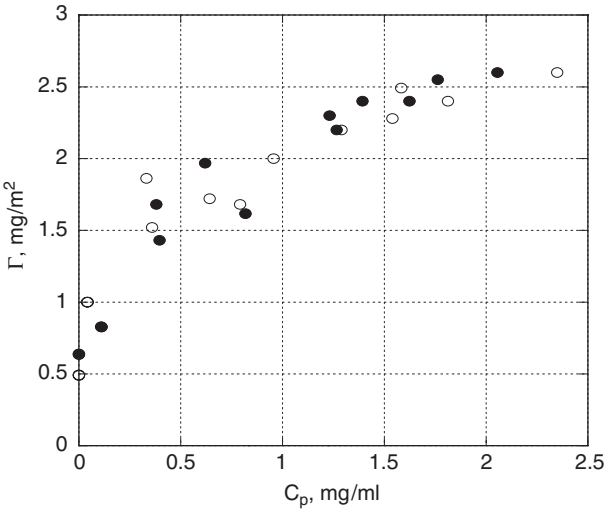

Fig. 8. Adsorption isotherms for the electrostatically modified PEG polymer EMPEG113–DMAEMA31 on polystyrene latex spheres in either 1 mM TES buffer (pH 7.4, ○) or 1 mM sodium citrate buffer (pH 5.5, ●). EMPEG113–DMAEMA31 is a block copolymer with 113 monomers of PEG bound to 31 cationic anchors of DMAEMA. The graph depicts surface coverage, Γ, versus the free polymer in solution, C_p. Each sample contained 1.0 mM lipid and 0.1–3.5 mg/ml polymer. Samples were equilibrated for 24 h before separating the polymer-coated latex spheres from free polymer in solution.

2.4 times that of binding to a latex particle ($\Gamma = 1.3\,\mathrm{mg/m^2}$ and $\Gamma = 3.1\,\mathrm{mg/m^2}$ for adsorption of EMPEG113–DMAEMA31 on latex spheres and liposomes, respectively). The higher binding constant cannot result from higher charge density since latex spheres have an order of magnitude greater charge density than liposomes. Further, stronger binding constants do not result from curvature effects, since greater curvature should play a minor role in initial adsorption and should allow higher levels of polymer adsorption at saturation. The most likely explanation is that the mobility of the individual charges on a liposome surface can adapt to maximize charge–charge interactions [29].

We compared the controlled release of block copolymers and a strictly alternating PEG–amine copolymer to assess architectural attributes for a reversible polymer protection layer. We have recently reviewed the association of hydrophobically modified comb-graft copolymers with liposomes [7]. This study showed that the binding affinity of these copolymers increased with increasing numbers of hydrophobic anchors. Binding also decreased with increasing PEG molecular weight. Adsorption was dependent on the ability of the polymers to pack efficiently on the surface [7]. We draw similar conclusions for the electrostatically modified PEG polymers. The following general rules apply: (1) increasing the number of polycations increases the binding affinity of the polymer with liposomes; (2) increasing the hydrophilicity of the PEG chain decreases the amount of polymer adsorbed; and (3) the PEG:CA ratio, at constant PEG length, dictates the overall association constant where lower ratios have higher binding affinities and higher ratios have lower binding affinities.

Unlike the hydrophobically modified PEGs studied previously, full surface coverage of electrostatically modified PEG polymers may be controlled by (1) neutralization of the liposome's charge and (2) repulsive polymer–polymer interactions. If we assume one-to-one binding of each polycation to each phosphate group, the amount of polymer required to neutralize the liposome surface is given in Table 1. High levels of adsorption that exceed the amount of polymer required for neutralization demonstrate that not all of the cationic anchors are bound. Thus, the polycation anchors do not exhibit one-to-one binding. The ability of the strictly alternating PEG–amine copolymer to bind moderately to liposomes may be inherent to its charge distribution and packing efficiency. The binding of polycation anchors is strong enough to balance the osmotic forces of having overlapping polymer segments. In our study of hydrophobically modified PEG polymers, we determined that the amount of adsorbed polymer needed to inhibit protein interactions was twice that achieved at full coverage. We are able to meet this criteria with electrostatically modified PEGs.

Based on scaling models for the free energies of polymer brushes [21,30,31], we have estimated the minimum anchoring energy needed for the EMPEG polymers to bind the liposome surface at full coverage, where the mushroom (unconstrained polymer) to brush (constrained polymer) transition occurs. The electrostatic anchoring energy must balance the sum of changes in the osmotic and elastic energy ($f = f_{el} + f_{os}$) [30]. Tirrell derives the following equations for the elastic free energy per square meter and the osmotic free energy per square meter, such that k_B is the Boltzmann constant (J/K), T is the temperature (K), a is the monomer length (3.5 Å for PEG [21]) (m), b is given as $a = \sqrt{6}b$ (m), σ is the polymer density on the surface (m^{-2}), L is the height of the polymer brush (m), N is the number of monomers where $R_f = aN^{\upsilon}$, c is the mass density of polymer (kg/m^3), m is the monomer molecular weight (kg/mol), N_{AV} is Avogadro's number (mol^{-1}), υ is the exponent that describes the molecular weight dependence on the viscosity, and K_Π is a constant from fitting data (= 2/3) [30]:

$$f_{el} = \left(\frac{k_B T}{4b^2}\right)\left(\frac{\sigma L^2}{N}\right)\left(\frac{c}{\beta}\right)^{(2\upsilon-1/(3\upsilon-1))} \times \left(\frac{m}{N_{AV}}\right)^{-(2\upsilon-1/(3\upsilon-1))}\left(\frac{4\pi b^3}{3}\right)^{(2\upsilon-1/(3\upsilon-1))} \quad (3)$$

and

$$f_{os} = ([3\upsilon - 1]K_\Pi k_B T)(N\sigma)\left(\frac{c}{\beta}\right)^{(1/(3\upsilon-1))} \times \left(\frac{m}{N_{AV}}\right)^{-(1/(3\upsilon-1))}\left(\frac{4\pi b^3}{3}\right)^{(1/(3\upsilon-1))}. \quad (4)$$

For EMPEG113–DMAEMA31 and EMPEG113–DMAEMA62, the minimum anchoring energy is 3.0 and 5.0 J/m^2, respectively. The osmotic energy dominates by three orders of magnitude. More energy is necessary for EMPEG113–DMAEMA62 to remain bound because the molecular weight of the polymer is larger (9.9 vs. 14.7 kg/mol) which results in a higher polymer mass per unit area. Thus, there is an increase in osmotic pressure. A similar result is observed for EMPEG45–DMAEMA5 (2.8 kg/mol) and EMPEG45–DMAEMA20 (5.1 kg/mol), where the minimum free energy of anchoring is 3.5 and 7.5 J/m^2, respectively. EMPEG122–K21 (8.6 kg/mol) has a minimum anchoring energy of 2.3 J/m^2. The small anchoring energy is a result of the lower molecular weight per charge of the polylysine chain in comparison with the DMAEMA chain. These calculations provide a framework for understanding the minimum anchoring energy for adsorption, whereby the osmotic energy dominates the free energy change and is similar in magnitude for all polymers.

4. Conclusions

We have established the framework for a controlled release polymer layer for liposomal gene delivery. We have demonstrated the triggered release of PEG-b-polycation copolymers from pH-dependent liposomes. The mechanism for triggered release is based on using tunable, pH-sensitive liposomes that incorporate DAP, which has a *p*Ka of ~6.7. The liposomes are neutral at pH 7.4 and become positively charged on lowering the pH to 5.5. This allows adsorption of a protective polymer layer (at pH 7.4) and its desorption (at pH 5.5). We observe both adsorption and desorption dependence on the PEG:CA ratio and PEG molecular weight. The results show adsorption at PEG:CA ratios ranging from 1.8 to 9 for block copolymers. PEG polymer chains with 45 or 113 monomers and a PEG:CA ratio of ~2 were strongly adsorbed, even in the presence of 150 mM NaCl. In addition, deprotection at pH 5.5 is sensitive to the PEG molecular weight. Increasing the PEG block length improves the ability of the polymer to desorb from the liposome surface. The type and number of polycationic anchors is important in allowing for flexibility and rearrangement on the liposome surface as well as efficient packing. For EMPEG113–DMAEMA62 and EMPEG45–DMAEMA20, the adsorption is in the brush regime, as demonstrated by an analysis of the surface area required for polymer adsorption at the transition between the mushroom and brush regimes. For evasion of the immune system, the mass of polymer adsorbed must exceed both full surface coverage and neutralization of the liposome. Future work will focus on in vitro experiments to identify the architecture of electrostatically modified PEG copolymers that will optimize the strength of adsorption in the bloodstream and the kinetics of desorption.

Acknowledgments

The authors would like to thank James Whitacre and Samuel Kahn who worked on the preliminary electrophoretic mobility measurements of charged liposomes. The authors would also like to thank Dr. Das Bolikal for synthesis of the electrostatically modified comb-graft copolymer (EMPEG45–N-DP15) and Dr. Maria Vamvakaki for synthesis of the EMPEG–DMAEMA diblock copolymers. In addition, the authors acknowledge funding from Elan Pharmaceuticals (formerly The Liposome Company) and the New Jersey Center for Biomaterials. D. Auguste acknowledges the 1930 Wallace Memorial fellowship from the Princeton University Graduate School.

References

[1] Chonn A, Cullis PR. Recent advances in liposome technologies and their applications for systemic gene delivery. Adv Drug Deliver Rev 1998;30:73–83.
[2] Lasic DD, Needham D. The "stealth" liposome: a prototypical biomaterial. Chem Rev 1995;95(8):2601–28.
[3] Ceh B, Winterhalter M, Frederik PM, Vallner JJ, Lasic DD. Stealth liposomes: from theory to product. Adv Drug Deliver Rev 1997;24:165–77.
[4] Sunamoto J, Sato T, Taguchi T, Hamazaki H. Naturally occurring polysaccharide derivatives which behave as an artificial cell wall on an artificial cell liposome. Macromolecules 1992;25:5665–70.
[5] Maruyama K, Takizawa T, Takahashi N, Tagawa T, Nagaike K, Iwatsuru M. Targeting efficiency of PEG-immunoliposome-conjugated anitbodies at PEG terminals. Adv Drug Deliver Rev 1997;24:235–42.
[6] Blume G, Cevc G. Liposomes for the sustained drug release in vivo. Biochim Biophys Acta 1990;1029:91–7.
[7] Auguste DT, Prud'homme RK, Ahl PL, Meers P, Kohn J. Association of hydrophobically-modified PEG with liposomes. Biochim Biophys Acta 2003;1616(2):184–95.
[8] Ahl PL, Bhatia SK, Meers P, Roberts P, Stevens R, Dause R, et al. Enhancement of the in vivo circulation lifetime of L-a-distearoylphosphatidylcholine liposomes: importance of liposomal aggregation versus complement opsonization. Biochim Biophys Acta 1997;1329:370–82.
[9] Chonn A, Semple SC, Cullis PR. Association of blood proteins with large unilamellar liposomes in vivo. J Biol Chem 1992;267(26):18759–65.
[10] Savic R, Luo L, Eisenberg A, Maysinger D. Micellar nanocontainers distribute to defined cytoplasmic organelles. Science 2003;300:615–8.
[11] Stites DP, Rogers RPC. Basic and clinical immunology. Norwalk: Appleton and Lang; 1991.
[12] Avanti Polar Lipids I. Products Catalog, 4th ed. Alabaster; 2000.
[13] Deshpande MC, Garnett MC, Vamvakaki M, Bailey L, Armes SP, Stolnik S. Influence of polymer architecture on the structure of complexes formed by PEG-tertiary amine methacrylate copolymers and phosphorothioate oligonucleotide. J Controlled Release 2002;81:185–99.
[14] Brzezinska K, Curtin S, Deming T. Polypeptide end-capping using functionalized isocyanates: preparation of pentablock copolymers. Macromolecules 2002;35:2970–6.
[15] Shangguan T, Pak CC, Ali S, Janoff AS, Meers P. Cation-dependent fusogenicity of an N-acyl phosphatidylethanolamine. Biochim Biophys Acta 1997;1368:171–83.
[16] Chen PS, Toribara TY, Warner H. Anal Chem 1956;28:1756–8.
[17] Baleux B. Colorimetric determination of nonionic polyethylene oxide surfactants using an Iodine-Iodide solution. C R Acad Sci Ser C 1972;279:1617–20.

[18] Brandrup J, Immergut EH. Polymer handbook. 3rd ed. New York: Wiley and Sons; 1989.
[19] Satoh K. Determination of binding constants of Ca^{2+}, Na^{2+}, and Cl^{-} ions to liposomal membranes of dipalmitoylphosphatidylcholine at gel phase by particle electrophoresis. Biochim Biophys Acta 1995; 1239:2239–48.
[20] Auguste D, Prud'homme R, Ahl P, Meers P, Kohn J. Association of hydrophobically modified PEG with fusogenic liposomes. Biochim Biophys Acta 2003;1616(2):184–95.
[21] Hansen P, Cohen J, Podgornik R, Parsegian V. Osmotic properties of poly(ethylene glycols): quantitiative features of brush and bulk scaling laws. Biophys J 2003;84:350–5.
[22] Flory PJ. Principles of polymer chemistry. Ithaca: Cornell University Press; 1975.
[23] Kageshima M, Lantz M, Jarvis S, Tokumoto H, Takeda S, Ptak A, et al. Insight into conformational changes of a single α-helix peptide molecule through stiffness measurements. Chem Phys Lett 2001;343:77–82.
[24] Damaschun G, Damaschun H, Gast K, Zirwir D. Proteins can adopt totally different folded conformations. J Mol Biol 1999;291:715–25.
[25] Farinato R. In: Advanced polymer technologies lectures PSE 797R. University of Massachusetts; 1991.
[26] Kayitmazer A, Seyrek E, Dubin P, Staggemeier B. Influence of chain stiffness on the interaction of polyelectrolytes with oppositely charged micelles and proteins. J Phys Chem B 2003; 107:8158–65.
[27] Tam K, Farmer M, Jenkins R, Bassett D. Rheological properties of hydrophobically modified alkali-soluble polymers—effects of ethylene-oxide chain length. J Polym Sci 1998;36:2275–90.
[28] Seng W, Tam K, Jenkins R, Bassett D. Calorimetric studies of model hydrophobically modified alkali-soluble emulsion polymers with varying spacer chain length in ionic surfactant solutions. Macromolecules 2000;33:1727–33.
[29] Haleva E, Ben-Tal N, Diamant H. Increased concentration of polyvalent phospholipids in the adsorption domain of a charged protein. Biophys J 2004;86:2165–78.
[30] Kilbey S, Watanabe H, Tirrell M. Structure and scaling of polymer brushes near the Θ condition. Macromolecules 2001;34:5249–59.
[31] Watanabe H, Kilbey S, Tirrell M. A scaling model for osmotic energy of polymer brushes. Macromolecules 2000;33:9146–51.