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An assessment by fluorescence spectroscopy of the stability of polyanions/positively charged liposome systems in the presence of polycations

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Abstract

The interactions between cationic non-phospholipid liposomes and pyrene-labeled hydrophobically modified sodium poly-2-(acrylamido)-2-methylpropane sulfonates (HM-PAMPS) have been examined by fluorescence spectroscopy and dynamic light scattering. Evidence from changes in the relative intensity of pyrene excimer and monomer emissions indicates that the polyanions bind to cationic liposomes and that, for systems in aqueous 0.2 M NaCl, the binding is only mildly affected by the charge density of the liposomes. The reversibility of the polyanion binding to the liposomes was assessed by addition of the polycation (*N*-isopropylacrylamide)–*N*-dimethylethyl(2-acrylamidoethyl)ammonium bromide copolymer (PNIPAM–DMEAB). Based on the level of pyrene fluorescence recovery upon addition of the polycation to complexes between PAMPS and liposomes containing a quencher of fluorescence in their bilayer, we demonstrate reversibility of the binding of sodium poly-2-[acrylamido-2-methylsulfonate–*N*-(1-pyrenylmethyl)]acrylamide (PAMPS–Py1). In contrast, under the same conditions, a HM-PAMPS sample carrying along its backbone 5 mol% of *n*-octadecyl groups is not desorbed from the surface of the liposome.

Keywords: Non-phospholipid liposome; Interpolyelectrolyte complex; Pyrene

1. Introduction

Several theoretical treatments have been developed to model the adsorption of polyelectrolytes to oppositely charged colloids, such as surfactant micelles [1–4]. Their results, consistent with experimental observations, predict that the interaction energy increases with the surface charge density of the colloid, the polymer linear charge density, and the debye length, which itself is inversely proportional to $I^{1/2}$, where I is the ionic strength of the medium. These results strongly suggest that electrostatic forces predominantly control the interaction. Convincing evidence of this conclusion is provided by the work of Dubin and coworkers [5,6], who have investigated in a systematic fashion the interactions between polyelectrolytes and mixed micelles of ionic and non-ionic surfactants. The modulation of the strong electrostatic attraction between oppositely charged surfactant and polyelectrolyte, via the addition of a non-ionic surfactant, is such that it is possible to study systems with surfactant concentrations well above the critical micelle concentration (cmc), without complications due to macroscopic phase separation [7].

Attaching a low level of hydrophobic groups on a polyelectrolyte often affects dramatically its solution properties and its interactions with oppositely charged colloids. The effects have been documented for a wide range of polyelectrolytes and hydrophobic modifiers [1]. The polymers of interest here are hydrophobically modified sodium poly-2-(acrylamido)-2-methylpropanesulfonates (HM-PAMPS). Morishima and co-workers have demonstrated that the interactions of HM-PAMPS with mixed micelles of cationic and non-ionic surfactants are controlled not only by electrostatic forces, but also by hydrophobic forces triggered by the association of polymeric hydrophobic groups and surfactants [8]. The study was carried out with surfactant micelles composed of cetyltrimethylammonium chloride (CTAC) and *n*-dodecylhexaoxyethylene glycol monoether ($C_{12}E_6$) in varying molar ratios. The initial work was performed with polyelectrolytes only lightly modified with hydrophobic substituents. More recent work by the same group, but using HM-PAMPS with a higher fraction of hydrophobic

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monomeric units, further illustrates the role of hydrophobic forces [9].

It is known that, under certain conditions, mixtures of non-ionic surfactants such as $C_{12}E_6$ and cationic surfactants, such as CTAC, can form vesicles, known as non-phospholipid liposomes (NPL) [10]. Polozova and Winnik have reported that these vesicles interact strongly with hydrophobically modified weak polyelectrolytes, such as copolymers of *N*-isopropylacrylamide, *N*-*n*-alkylacrylamide, and *N*-glycine acrylamide [11]. We report here a study of systems consisting of cationic NPL and HM-PAMPS samples of varying levels of hydrophobic modification. This study will be related to reports from other groups on the interactions of polyelectrolytes and liposomes, a field of intense activity in view of its relevance to the design of novel delivery systems and to the fundamental understanding of the interactions of proteins and cell membranes [12]. Because the surface of cells usually carries a net negative charge, interactions of cationic polyelectrolytes with negatively charged phospholipid liposomes have attracted the attention of most researchers in this area. Important contributions include the work of Campos and coworkers on the system poly(4-vinylpyridine)/dimyristoylphosphatidic acid [13], and studies originating from the group of Kabanov, on systems consisting of anionic phospholipid liposomes and either poly(*N*-ethyl-4-vinylpyridinium bromide) [14] or hydrophobically modified derivatives of this polymer, where fractions of the *N*-ethyl substituents are replaced with long *N*-alkyl chains [15]. The latter work demonstrates the importance of hydrophobic interactions between the polycation and the liposome bilayer in increasing the stability of the polymer/liposome complexes, especially when the complexes are placed in contact with negatively charged polymeric competitors.

The work of Kabanov and coworkers [15] on ternary systems consisting of anionic liposomes brought in contact with mixtures of oppositely charged polyelectrolytes addresses the fascinating issue of multilayers formed by the alternated adsorption of anionic and cationic polyelectrolytes [16]. A number of studies have been devoted to the understanding of multilayer buildup on solid surfaces [17]. Others focussed on the properties of interpolyelectrolyte complexes (PECs) in solution [18], and in the presence of colloidal particles, such as silica [19]. These macromolecular assemblies are stabilized primarily by interpolymeric salt bonds, but other interactions, such as hydrogen bonding, hydrophobic interactions, charge transfer complexation and van der Waals forces can participate significantly in the formation and stability of PECs [20]. PECs are of considerable interest because of their potential use as polymeric reagents or additives in biotechnology and as flocculants in various industrial and environmental processes [21,22]. There are only few reports on the formation and stability of PECs built onto liposome surfaces. It is usually observed that linear polyelectrolytes remove from liposome surfaces electrostatically adsorbed oppositely charged linear polyelectrolytes [23–25]. Yaroslavov et al. however, have demonstrated recently that it is possible to control the desorption of polyelectrolytes by oppositely charged polymers, via control of the liposome surface charge [14,15].

The present report describes a fluorescence spectroscopy and dynamic light scattering (DLS) study of the interactions of a HM-PAMPS with cationic NPL composed of cholesterol, *n*-octadecyldiethylene oxide ($C_{18}E_2$), and dimethyldioctadecylammonium bromide (DDAB). These experiments will allow us to draw qualitative conclusions on the relative importance of electrostatic and hydrophobic forces in the formation of HM-PAMPS/liposome complexes. In a second part, we explore the possibility of building polyelectrolyte complexes on the surface of NPL. The stability of the HM-PAMPS/liposome complexes in the presence of competitor cationic polymers is assessed via a fluorescence assay based on the quenching of pyrene emission by cetylpyridinium chloride (CPC) incorporated within the liposome bilayer.

2. Experimental section

2.1. Materials

Water was purified with a Barnstead NANOPure water purification system. DDAB, and dimyristoyl phosphatidyl choline (DMPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Cholesterol, *n*-octadecyldiethylene oxide ($C_{18}E_2$), acryloyl chloride, azobisisobutyronitrile (AIBN), *N*,*N*-dimethylethylenediamine, bromoethane, CPC, *N*-isopropylacrylamine and 1-pyrenylmethylamine hydrochloride were purchased from Aldrich Chemicals. *N*-Acryloxysuccinimide (NASI) was obtained from Eastman Kodak Chemicals. The preparation of the modified sodium poly-(2-acrylamido-2-methylpropane sulfonates) PAMPS–PyODA5 and PAMPS–Py1 was described previously [26]. The polymer PAMPS–Py1–ODA5 was prepared following the same conditions [27].

2.2. Sample preparation

Synthesis of N–[(dimethylamino)ethyl]acrylamide (DMEAA). A solution of acryloyl chloride (10.07 g, 0.111 mol) in dichloromethane (20 ml) was added drop wise at 5°C and under nitrogen to a stirred suspension of dichloromethane (83 ml), *N*,*N*-dimethylethylenediamine (8.86 g, 0.10 mol) and aqueous sodium hydroxide (6 N, 27 ml). At the end of the addition, the reaction mixture was brought to room temperature and stirred for 15 h. The organic layer was separated, washed twice with water, and once with brine. It was dried over magnesium sulfate. Evaporation of the solvent under vacuum yielded DMEAA (7.7 g, yield: 54%) as a slightly yellow oil. The crude product was purified by vacuum distillation. ^{1}H NMR ($CDCl_3$, δ) 2.23 (s, 6H, $(CH_3)_2N-$), 2.46 (t, 2H, $-CH_2-N(CH_3)_2$), 3.42 (m, 2H,

Table 1
Composition of the liposomes prepared

Liposome	Lipid structure	Lipid weight ratio	Lipid molar ratio
Cationic liposomes			
NPL (0.7)	E_2C_{18}/cholesterol/DDAB	79/20/1	80.1/19.2/0.7
NPL (1.2)	E_2C_{18}/cholesterol/DDAB	78/20/2	79.6/19.2/1.2
NPL (3.0)	E_2C_{18}/cholesterol/DDAB	75/20/5	77.6/19.4/3.0
NPL (9.3)	E_2C_{18}/cholesterol/DDAB	65/20/15	70.6/20.1/9.3
CPC–NPL (3.8)	E_2C_{18}/cholesterol/DDAB/CPC	75/20/4/1	77.2/19.0/2.3/1.5
CPC–NPL (10.6)	E_2C_{18}/cholesterol/DDAB/CPC	64/21/14/1	68.4/21.0/8.8/1.8
Amphoteric liposomes			
DMPC	DMPC	100	100

–CH_2–NH–CO), 5.55–5.60 (m, 2H, vinyl protons), 6.25–6.27 (m, 4H, vinyl protons), 7.53 (br, 1H, –N*H*–CO); *m/z* (DCI NH_3) 143 ($M + H^+$), 72 $[(CH_2)_2N(CH_3)_2^+$.

Copolymer of N-isopropylacrylamide (NIPAM) and N-dimethylethyl(2-acrylamidoethyl)ammonium bromide (DMEAB) (PNIPAM–DMEAB15). A solution of NIPAM (3.81 g, 33.65 mmol) and DMEAA (0.94 g, 6.63 mmol) in *t*-butanol (20 ml) was degassed by vigorous bubbling of nitrogen for 15 min. A solution of AIBN (0.10 g, 0.61 mmol) in *t*-butanol (5 ml) was added to the solution. It was heated at 65°C for 24 h. The reaction mixture was cooled to room temperature. The polymer was recovered by precipitation into diethyl ether (4.0 g, 84%). A solution of the polymer (1.0 g) in methanol (10 ml) was treated with bromoethane (0.64 g) at 35°C for 10 h. The quaternized polymer (0.92 g) was recovered by precipitation into diethyl ether. It was purified by repeated precipitations from methanol into diethylether. ^{1}H NMR (D_2O, δ) 3.78 (br s, –NH–C*H*$(CH_3)_2$), 3.2–3.4 (m, –NH–CH_2CH_2–N^+(CH_2–CH_3)$(CH_3)_2$), 2.96 (s, $N^+(CH_2CH_3)(CH_3)_2$), 1.24 (s, $N^+(CH_2CH_3)(CH_3)_2$), 1.04 (br s, NH–CH$(CH_3)_2$).

Copolymer of NIPAM and DMEAB (60 mol%) (PNIPAM–DMEAB60). The polymer (0.84 g) was obtained by the same process as PNIPAM–DMEAB15, starting with NIPAM (0.86 g, 7.59 mmol) and DMEAA (1.77 g, 12.44 mmol). ^{1}H NMR (D_2O, δ) 3.78 (br s, –NH–C*H*$(CH_3)_2$, 3.2–3.4 (m, –NH–CH_2CH_2–N^+(CH_2–CH_3)$(CH_3)_2$), 2.96 (s, $N^+(CH_2CH_3)(CH_3)_2$), 1.24 (s, $N^+(CH_2CH_3)(CH_3)_2$), 1.04 (br s, NH–CH$(CH_3)_2$).

Liposomes. A solution in chloroform of *n*-octadecyldiethylene oxide ($C_{18}E_2$), cholesterol, and DDAB in the amounts listed in Table 1 was poured into a 50-ml round-bottom flask. The solvent was evaporated under a stream of nitrogen. The resulting lipid film was dried under high vacuum for 2 h at room temperature. The dry lipid film was hydrated in an aqueous solution of NaCl (0.2 M, 10 ml). The lipid suspension was warmed to 60°C and subjected to extrusion through polycarbonate membranes (100 nm pore size, Nucleopore) using a Lipofast extruder (Avestin, Canada).

Liposome-polymer mixtures. Stock solutions of the polymers (0.5 g l^{-1}) in 0.2 M NaCl were prepared. The polyelectrolyte solutions were sonicated for 2 min. All solutions were allowed to equilibrate for 24 h. Suspensions of liposomes (lipid concentration: 2 g l^{-1}) were prepared by dilution of the suspensions obtained by extrusion. Liposome suspensions were added to polymer solutions in the desired proportions. The mixtures were allowed to equilibrate at room temperature for at least 5 h prior to measurement.

Liposome/polymer ternary mixtures. Stock solutions of the polymers in 0.2 M NaCl were prepared with the following concentrations: PAMPS–Py1: 0.05 g l^{-1}; PAMPS–Py1–ODA5: 0.05 g l^{-1}; PNIPAM–DMEAB15: 0.01 g l^{-1}; PNIPAM–DMEAB60: 0.01 g l^{-1}. The solutions were kept at room temperature for 24 h. Suspensions of liposomes CPC–NPL (10.6) (see Table 1) were added to solutions of PAMPS–Py1 or PAMPS–Py1–ODA5 such that the polymer concentration was 0.01 g l^{-1} and the lipid concentration 0.1 g l^{-1}. Aliquots of PNIPAM–DMEAB15 or PNIPAM–DMEAB60 stock solutions were added to the liposome/PAMPS–Py1 and liposome/PAMPS–Py1–ODA5 mixed solutions. The ternary mixtures were kept at room temperature for 6 h prior to measurements.

2.2. Instrumentation

^{1}H NMR spectra were recorded on Brucker 200 or 500 MHz spectrometers. Infrared spectra were recorded on a BioRad FTS-40 spectrometer. UV-spectra were measured with a Hewlett Packard 8452A photodiode array spectrometer. Gel permeation chromatography (GPC) was performed with a Waters 590 programmable HPLC system (eluent: 0.5 M acetic acid, flow rate of 0.7 ml min^{-1}, Ultrahydrogel columns (Waters)) equipped with a Waters 486 UV detector and a Waters 410 Differential Refractometer. Standard pullulan samples, obtained from Showa Denko K.K, were used for calibration The mean diameters of polymer micelles and liposomes were evaluated by DLS using a Brookhaven Instrument Corporation Particle Sizer Model BI-90 (Holtsville, NY). The data acquisition time in a typical experiment was 1000 s and the average size obtained from three independent measurements was taken as the mean diameter.

Fluorescence measurements. Fluorescence spectra were recorded on a SPEX Fluorolog 212 spectrometer operated

by a GRAMS/32 data system. Temperature control of the samples was achieved using a water-jacketed cell holder connected to a Neslab circulating bath. The temperature of the sample fluid was measured with a thermocouple immersed in a water-filled cuvette placed in one of the four cell holders. Excitation spectra were measured in the ratio mode. Emission spectra were not corrected. They were recorded with an excitation wavelength of 344 nm. Emission and excitation slit widths were set at 1.0 mm. Solutions in water were not degassed. In all solutions the polymer concentration was kept low, in order to ensure that the maximum absorbance of a solution at the excitation wavelength remains below 0.07, thus avoiding undesirable inner filter effects and self quenching. The fluorescence of PAMPS–PyODA5 in salt solutions consists of two contributions: a broad featureless band centered at 482 nm attributed to pyrene excimer emission (intensity I_E) and a well-resolved spectrum with the [0,0] band at 378 nm, attributed to the emission of spatially isolated pyrene groups (pyrene monomer emission, intensity I_M). The ratio of excimer to monomer intensity is taken as the ratio of the intensity at 482 nm to the half sum of the intensities at 378 nm and 398 nm. Excimer emission requires that an excited pyrene (Py^*) and a pyrene in the ground state come in close proximity during the Py^* lifetime. Excimer formation occurs in concentrated Py solution or under circumstances where microdomains of high local pyrene concentration form, even though the macroscopic pyrene concentration is low. In salt solutions of PAMPS–PyODA5 the excimer emission is strong, indicating the formation of polymer micelles [26,27].

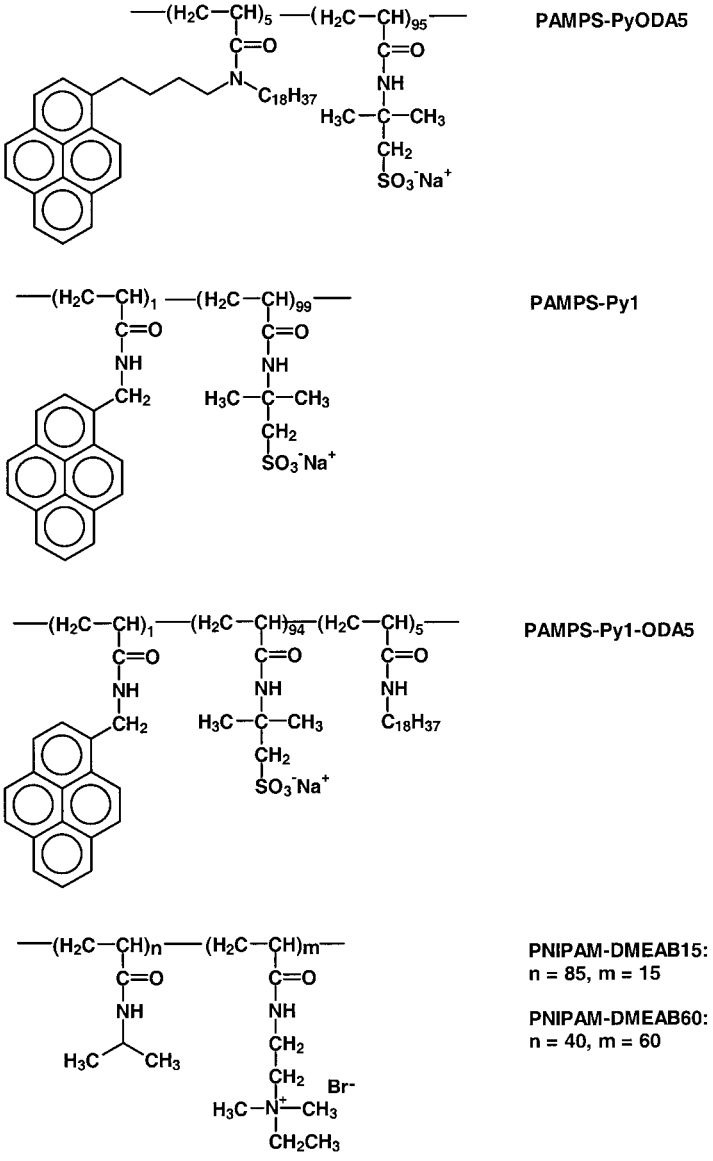

Fig. 1. Structure of the polymers used in this study.

Table 2
Chemical characteristics of the polymers used in this study

Polymer	Composition (mol%)	M_n[a]	M_w (M_w/M_n)	Reference
PAMPS–PyODA5	Py: 4.5 ± 0.1[b,c]	25,000	65,000 (2.4)	26
PAMPS–Py1–ODA	Py: 1.1 ± 0.1[b] ODA: 5.2 ± 0.1[c]	28,000	72,000 (2.1)	27
PAMPS–Py1	Py: 1.2 ± 0.2[b]	66,000	156,000 (2.3)	26
PNIPAM–DEAAB15	DEAAB: 14 ± 1[c]			This work
PNIPAM–DEAAB60	DEAAB: 58 ± 1[c]			This work

[a] From GPC data calibrated against pullulan standards.
[b] From UV absorbance spectra.
[c] From ^{1}H NMR spectrum (solvent: $CDCl_3$).

3. Results and discussion

3.1. The polymers

The HM-PAMPS samples were prepared by free-radical polymerization in dimethylformamide, following procedures reported previously [26,27]. Their chemical structures are shown in Fig. 1 and their chemical compositions and molecular weights are listed in Table 2. The three AMPS copolymers carry a low level of pyrene, used as a fluorescent label. PAMPS–PyODA5 and PAMPS–Py1–ODA5 also contain *n*-octadecyl substituents linked either separately along the polymer chain (PAMPS–Py1–ODA5) or attached to a tertiary amide nitrogen linked to a 4-(1-pyrenyl)-butyl moiety (Fig. 1).[1] In the latter polymer, as the pyrene and *n*-octadecyl groups are linked to the same monomer unit, the emission of pyrene captures directly changes in the environment of the alkyl moieties. The third AMPS copolymer, PAMPS–Py1, contains no hydrophobic substituent, save for a small amount of pyrene groups. This polymer was shown to possess little amphiphilic characteristics and to behave in aqueous solution as a typical strong polyelectrolyte [28]. In contrast, both PAMPS–PyODA5 and PAMPS–Py1–ODA5 form polymeric micelles in water and in salt solutions [26,27].

Two cationic copolymers, PNIPAM–DMEAB15 and PNIPAM–DMEAB60 (Fig. 1) were prepared to act as competitive polymers in mixed HM-PAMPS/liposome systems (vide infra). We opted for copolymers consisting of varying relative amounts of a neutral water soluble monomer (*N*-isopropylacrylamide, NIPAM) and a positively charged monomer (dimethylethyl-(2-acrylamidoethyl)-ammonium bromide, DMEAB). The selection of the synthetic procedure (Fig. 2) was dictated by the need to obtain a random distribution of the two monomers. Thus, we performed first a free radical copolymerization of NIPAM and dimethylethyl-2-acrylamide in organic medium, and second, quaternization of the tertiary amine groups with ethyl bromide. Evidence that the quaternization occurred is provided by the occurrence, in the ^{1}H NMR spectra of PNIPAM–DMEAB15 and PNIPAM–DMEAB60, of a sharp singlet at 1.05 ppm, attributed to the resonance of the methyl protons of the CH_3–$CH_2N(CH_3)^+$– residues. This singlet, together with the broad singlet at 3.8 ppm assigned to the methyne protons of the NIPAM units, was used to calculate the degree of quaternization of the polymers.

We monitored the interactions between liposomes and the three AMPS copolymers by fluorescence spectroscopy using two approaches. To follow the adsorption and anchoring of polymers onto the liposome membrane, we used changes in the emission of pyrene, namely the relative intensity of pyrene excimer and pyrene monomer emission (see Section 2). To assess the competitive effect of polycations added to HM-PAMPS/liposome systems, we observed the degree of quenching of the pyrene emission by a quencher of fluorescence incorporated into the liposome bilayer. The two approaches are described in turn in the following sections.

3.2. PAMPS–PyODA5/Liposome systems

The fluorescence spectrum of PAMPS–PyODA5 in aqueous NaCl solution (0.2 M) (Fig. 3) presents a strong excimer emission centered at 482 nm in addition to the well-resolved emission due to the pyrene monomer contribution. The excimer emission is very strong in this case, since the hydrophobic pyrene chromophores are located in close proximity to each other within the core of polymeric micelles formed by this polymer in salt solutions [26]. Addition of liposomes to a solution of this polymer prompts significant changes in the emission of PAMPS–PyODA5. Most noticeable is the sharp increase in pyrene monomer emission intensity at the expense of pyrene excimer emission, indicating a severe disruption of the hydrophobic microdomains formed in aqueous solutions of PAMPS–PyODA5. The effect is illustrated in Fig. 3, which presents the spectrum of PAMPS–PyODA5 in the presence of cationic NPL. The decrease in pyrene excimer emission signals the incorporation of the hydrophobic groups into the liposome bilayer with concomitant spatial separation of the pyrene groups within the lipid bilayer. The ratio I_E/I_M of the intensities of pyrene excimer and monomer emissions is a convenient qualitative indicator of the degree of polymer binding to liposomes, as demonstrated in previous studies

[1] The digits in the designation of the polymers indicate the approximate levels of incorporation, in mol%, of the respective units.

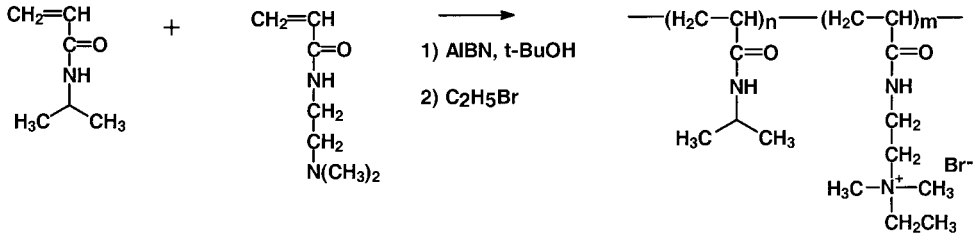

Fig. 2. Synthetic scheme employed to prepare the copolymers PNIPAM–DMEAB15 and PNIPAM–DMEAB60.

[11,29]. A high value of I_E/I_M reflects predominant occurrence of undisturbed polymeric micelles.

We examined the effects of the polymer/lipid molar ratio using two types of vesicles: (a) NPLs consisting of the non-ionic surfactant $C_{18}E_2$, cholesterol, and the cationic surfactant DDAB and (b) DMPC liposomes where the phospholipid bears an amphoteric head group and, consequently, these liposomes are electrically neutral (Table 1). The experiments were carried out in solutions of constant ionic strength (0.2 M NaCl) and PAMPS–PyODA5 concentration (0.005 g l^{-1}) using four batches of NPLs of identical properties, except for their DDAB content (Table 1) and one batch of neutral phospholipid liposomes. In all cases, addition of increasing amounts of liposomes to the polymer solution resulted in a decrease of the ratio I_E/I_M, from a value of 0.9 in the absence of liposomes to a value of 0.08 (Fig. 4). The sharpest decrease of I_E/I_M takes place upon addition of the first aliquots of liposomes. The ratio levels-off when the polymer/total lipid ratio reaches a value of ~1:12 (*w*/*w*) or 1:8 (AMPS/lipid molar ratio). We noted no effect of the liposome net charge on the changes in I_E/I_M, nor were there any differences related to the chemical composition of the liposomes. Curves of the ratio I_E/I_M vs lipid concentration are indistinguishable, within the experimental error. If the interactions between cationic NPLs and PAMPS–PyODA5 were principally controlled by the electrostatic attraction between the positive liposome surface and the polyanion, we would have detected significant differences among the five polymer/liposome systems, as reported in a previous study of systems consisting of the same liposomes interacting with a weak polyelectrolyte [11]. The uniformity in the patterns observed in the present study points to the overwhelming effect of hydrophobic forces in controlling the liposome/polymer interactions.

To gain a better understanding of the role played by electrostatic forces in our systems, we carried out a series of measurements with solutions of identical polymer and total lipid concentrations, but using liposome compositions with different levels of DDAB incorporation. The polymer and lipid concentrations were kept constant at values of 0.005 and 0.1 g l^{-1}, respectively, throughout the study. The DDAB content of the NPL was increased from 0.7 to 9.3 mol%. The ratio I_E/I_M rapidly reached the saturation value (0.08 for [DDAB] ~2 mol%), showing, as previously, a remarkably mild dependence on the liposome charge. It should be noted, though, that all the measurements were carried in 0.2 M NaCl. In solutions of such ionic strength the debye length is much shorter than 1 nm. Given also that the level of hydrophobic modification is rather high, it is not surprising that the hydrophobic forces prevail over the electrostatic contributions in the sets of conditions selected for

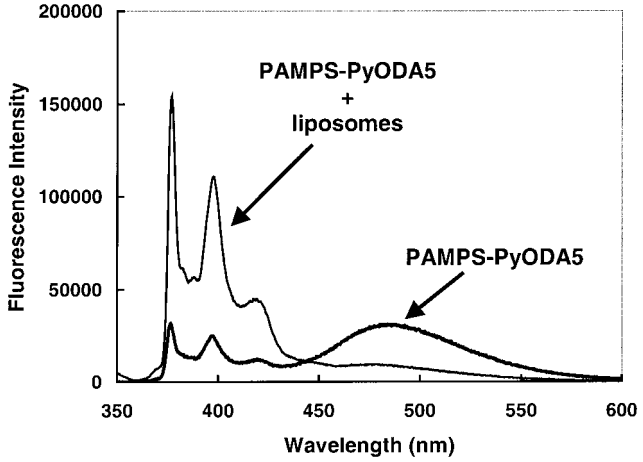

Fig. 3. Fluorescence spectra of PAMPS–PyODA5 in aqueous NaCl (0.2 M) and in the presence of cationic NPL; polymer concentration: 0.005 g l^{-1}; (λ_{exc} = 344 nm).

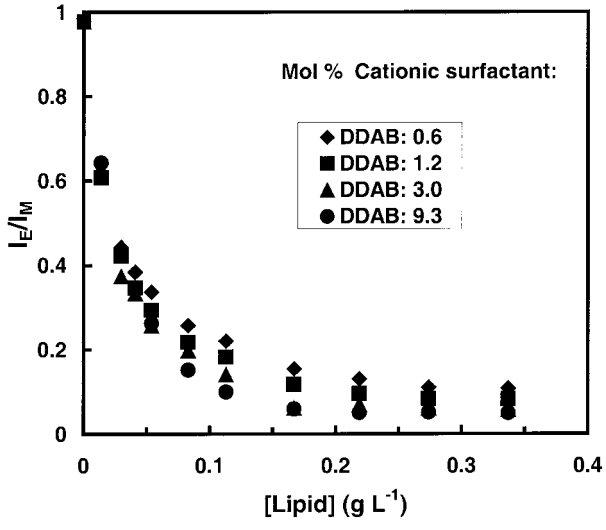

Fig. 4. Changes in the ratio of excimer to monomer emission intensities, I_E/I_M, for solutions of PAMPS–PyODA5 as a function of lipid concentration in the presence of liposomes of various compositions (see Table 1); polymer concentration: 0.005 g l^{-1}; [NaCl] = 0.2 M.

the measurements. Experiments using solutions of weaker ionic force are in progress to test this hypothesis.

To confirm that the liposomes maintain their integrity in the presence of PAMPS–PyODA5, we analyzed the samples by DLS, monitoring the size of the liposomes in solutions of increasing lipid concentration with and without added polymers. The hydrodynamic radius of the liposomes in the absence of polymer was 200 ± 20 nm for all the lipid concentrations (0.02 to 0.12 g l^{-1}). Histograms recorded with solutions of liposomes prepared under the same conditions and brought in contact with PAMPS–PyODA5 present the same overall characteristics as those recorded from suspension of naked liposomes, with an average hydrodynamic radius of 220 ± 20 nm.

3.3. Cationic polymers/HM-PAMPS/liposome ternary systems

The addition of a positively charged polymer to a solution of HM-PAMPS/cationic liposome complexes may result either: (1) in the desorption of the polyanion from the liposome surface and formation of PEC colloidal complexes or (2) in the adsorption of the polycation on the negatively charged surface of HM-PAMPS/liposome complexes, initiating the formation of multilayers (Fig. 5). The predominance of one mechanism over the other should depend on the relative charge densities of the polymers and the liposomes as well as on the relative importance of the hydrophobic interactions involved in the binding of HM-PAMPS to liposomes and, possibly, in PEC formation. To assess the influence of the electrostatic and hydrophobic forces, we carried out experiments with two cationic polymers of different charge density: PNIPAM–DMEAB15 and PNIPAM–DMEAB60 and three HM-PAMPS with different levels of hydrophobic modification: PAMPS–PyODA5, PAMPS–Py1–ODA5, and PAMPS–Py1 (Fig. 1).

An initial series of experiments was performed to ascertain that HM-PAMPS derivatives form PECs with the cationic PNIPAM derivatives selected. Previous work has taught us that the ratio I_E/I_M of pyrene excimer to monomer emission intensity of PAMPS–PyODA is a good indicator of the disruption of polymeric micelles. Thus, we monitored the changes in the spectrum of PAMPS–PyODA5 as a function of added PNIPAM–DMEAB15 and PNIPAM–DMEAB60. The ratio I_E/I_M decreased from an initial value of 0.9 in the absence of polycation to values of 0.5 and 0.4 in the presence of PNIPAM–DMEAB60 and PNIPAM–DMEAB15, respectively (Fig. 6). The decrease in I_E/I_M reflects an increase in pyrene monomer emission at the expense of pyrene excimer emission, suggesting disruption of the hydrophobic microdomains that exist in solutions of PAMPS–PyODA5 and concomitant formation of complexes between the oppositely charged polyelectrolytes. It is interesting to note that in neither case are the hydrophobic domains destroyed entirely and that the least charged polycation, PNIPAM–DMEAB15, seems to prompt the most severe reorganization of the PAMPS–PyODA5. These observations give important information on the PECs structure and stability, topics that are beyond the scope of this study. The results, however, tell us that indeed PECs form between HM-PAMPS and cationic PNIPAMs, and that the methodology is suitable to test the stability of HM-PAMPS/liposomes in the presence of linear polycations.

Next, we prepared solutions of cationic NPLs containing 9.3 mol% DDAB coated with PAMPS–PyODA5 under

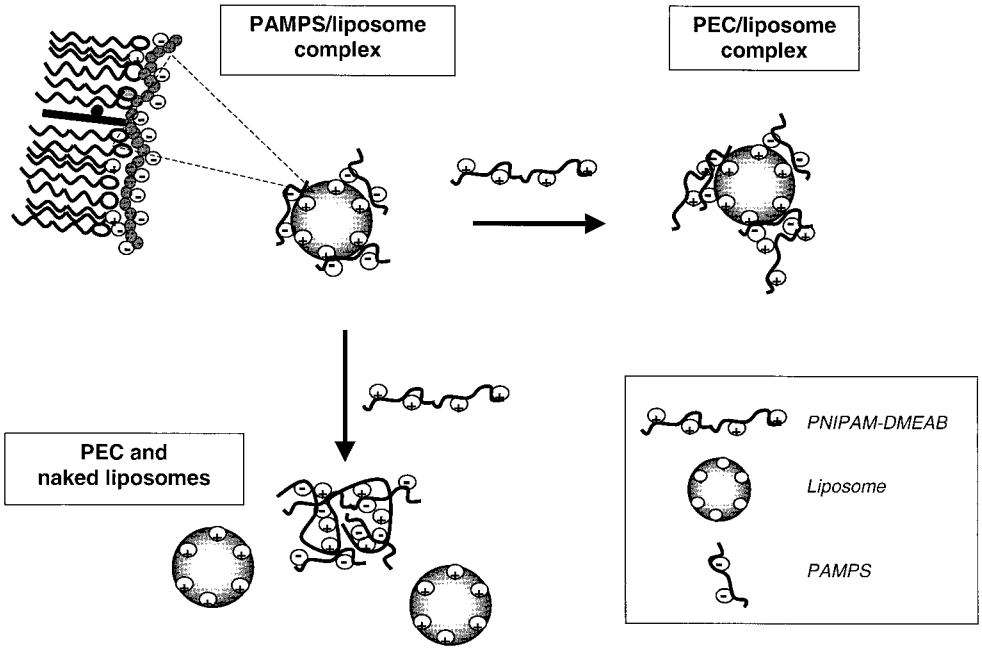

Fig. 5. Schematic representation of the interactions that may occur in a ternary system of liposomes, HM-PAMPS, and a linear polycation. For the sake of clarity the objects are not drawn to scale.

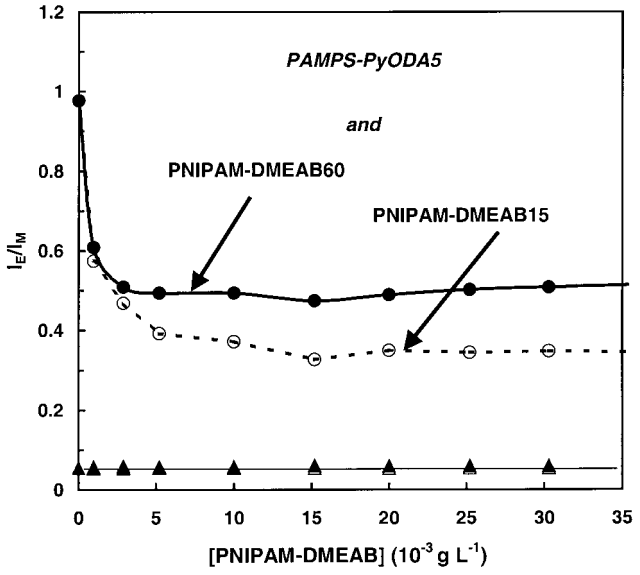

Fig. 6. Changes in the ratio of excimer to monomer emission intensities, I_E/I_M, as a function of the amount of added polycation for solution of PAMPS–PyODA5 in water (●: PEC of PAMPS–PyODA5 and PNIPAM–DMEAB60; ○: PEC of PAMPS–PyODA5 and PNIPAM–DMEAB15) and in the presence of cationic liposomes NPL (9.3), (Table1) (▲:PNIPAM–DMEAB60; △: PNIPAM–DMEAB15); PAMPS–PyODA5 concentration: 0.005 g l^{-1}; [NaCl] = 0.2 M.

conditions for which no free polymeric micelles remain in solution. Increasing amounts of either PNIPAM–DMEAB15 or PNIPAM–DMEAB60 were added to the NPL/PAMPS–PyODA5 solutions. The ratio I_E/I_M was monitored as a function of polycation concentration: it remained constant at all concentrations, retaining the low value (0.08) it assumes in the spectrum of polymer-coated liposomes in the absence of polycations (Fig. 4). This result is unfortunately quite ambiguous: it could be construed to hint that adsorption of polycations onto NPL/HM-PAMPS complexes took place without disturbing the liposome bilayer/polymer interactions. It could also, very simply, indicate that no interaction occurred between the two species or that a fraction of polyanion desorbed from the liposome surface forming PECs in too small an amount to be detected by the fluorescence method used.

To raise the ambiguity of the results of the fluorescence test based on the relative intensity of pyrene excimer and monomer emissions, we devised a different fluorescence assay that monitors the quenching of pyrene fluorescence by a quencher of fluorescence securely incorporated within the liposome bilayer. To this effect we prepared NPLs consisting of $C_{18}E_2$, cholesterol, and as ionogen, a mixture of DDAB and CPC (Table 1). The latter surfactant is known to incorporate readily in liposome bilayers and to act as an effective quencher of pyrene fluorescence. The experiments were carried out with PAMPS–Py1–ODA5 and PAMPS–Py1 (Fig. 1), rather than PAMPS–PyODA5 in order to avoid unnecessary complications related to the dual emission of pyrene from solutions of PAMPS–PyODA5. No excimer is detected in the spectra of solutions of PAMPS–Py1 [26] or PAMPS–Py1–ODA5 [27]. The former polymer does not undergo micellization in water due to the low level of modification, whereas polymeric micelles form in solutions of PAMPS–Py1–ODA5 via association of the octadecyl groups, but as the pyrene groups are linked to units far apart from the octadecyl chains, they do not participate in the micellization process [27].

In a typical quenching experiment a solution of PAMPS–Py1 (0.001 g l^{-1}) was placed in contact with a solution of liposomes doped with CPC (Table 1). The fluorescence emission of a solution of PAMPS–Py1 was measured before and after addition of the liposomes, taking care to keep the polymer concentration constant and ensuring that complete

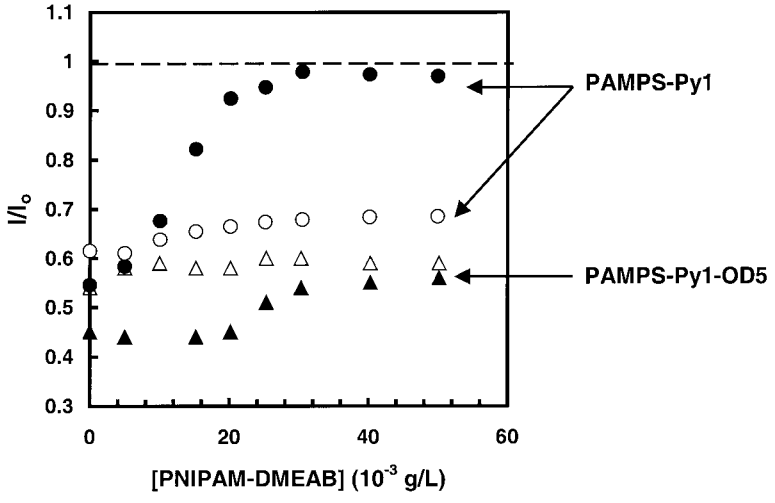

Fig. 7. Changes in the ratio I/I_0 in mixed solutions of CPC-containing liposomes NPL/10.6 (Table 1) for the polymers PAMPS–Py1 and PAMPS–Py1–ODA5 as a function of added polycation (●: PAMPS–Py1 and PNIPAM–DMEAB60; ○: PAMPS–Py1 and PNIPAM–DMEAB15; ▲:PAMPS–Py1–ODA5 and PNIPAM–DMEAB60; △: PAMPS–Py1–ODA5 and PNIPAM–DMEAB15); the dotted line represents the value of I/I_0 corresponding to complete fluorescence recovery; PAMPS–Py1–ODA5 and PAMPS–Py1 concentration: 0.01 g l^{-1}; liposome concentration: 0.1 g l^{-1}; [NaCl] = 0.2 M.

equilibration of the liposome/polymer solutions was achieved. From these measurements we obtain I_o, the fluorescence intensity of PAMPS–Py1 in the absence of quenching and I, the fluorescence intensity in the presence of CPC–NPL (10.6). The ratio I/I_0 (0.55) is less than 1, indicating that a fraction of the Py groups linked to the polymer are in close vicinity to the pyridinium head group of CPC incorporated in the NPL bilayer. Hence polymer adsorption takes place. Then, increasing amounts of PNIPAM–DMEAB60 were added to the mixed solution and the ratio I/I_0 was recorded as a function of cationic polymer concentration (Fig. 7). The ratio increased rapidly with polycation concentration, reaching unity for a PNIPAM–DMEAB60 concentration of $0.05\ g\,l^{-1}$. The recovery of fluorescence vouches for desorption of PAMPS–Py1 from the surface of the liposome. It is reasonable to propose that the polymer desorption is accompanied by the formation of PECs, but more experiments are needed to confirm this point. Also shown in Fig. 7 are the values of I/I_o obtained upon addition of PNIPAM–DMEAB15 to a PAMPS–Py1/NPL(10.6) mixed solution. The ratio retains its low value (0.65 ± 0.05) in the ternary mixtures. Hence this polycation, which carries a lower net charge than PNIPAM–DMEAB60, is unable to cause the polyanion to desorb. More importantly, when the quenching assay was performed in mixed systems consisting of CPC–NPL (10.6), the hydrophobically modified polyanion PAMPS–Py1–ODA5, and the polycation PNIPAM–DMEAB60, no recovery of fluorescence was detectable upon addition of polycation to polyanion-coated liposomes, even after adding polycation amounts in excess of the charge neutralization concentration. This result, although preliminary, brings further support to the importance of hydrophobic forces in the formation of NPL/polyelectrolyte complexes.

The validity of the experimental approach was confirmed by control experiments using PAMPS–Py1 and NPLs doped with different amounts of cationic species: 3.8 mol% and 10.6 mol% (Table 1). In mixtures of PAMPS–Py1 and the CPC–NPL (3.8) we detected no quenching of pyrene fluorescence, since the polymer does not adsorb to these lightly charged liposomes. If free CPC or CPC micelles existed in solution, one would have expected quenching to take place, even in mixed solutions of PAMPS–Py1 and CPC–NPL (3.8). Since no quenching was observed, we conclude with confidence that all the CPC used in preparing the liposomes was incorporated in the bilayer.

4. Conclusions

The interactions of positively charged NPL and hydrophobically modified polyanions are controlled by an interplay between electrostatic attraction and hydrophobic forces. In solutions of high ionic strength, hydrophobic interactions are predominant to the point of precluding desorption of the polyanion by added competing polycations. Several aspects of the systems need to be examined in more detail, in order to understand what happens to positively charged vesicles upon interacting with polyanions. Work is in progress to assess the effect of ionic strength on the vesicle/polyanion binding and its reversibility upon addition of polycation and to determine by calorimetry the thermodynamic parameters associated with the complexation.

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