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Mechanism of the interaction of hydrophobically-modified poly-(N-isopropylacrylamides) with liposomes

Alla Polozova, Françoise M. Winnik *

Department of Chemistry, McMaster University, 1280 Main St. W., Hamilton, Ontario, Canada, L8S 4M1

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Abstract

Interactions of hydrophobically-modified poly-(N-isopropylacrylamides) (HM PNIPAM) with phospholipid liposomes were studied as a function of the lipid type, the lipid bilayer fluidity, and the polymer conformation. Fluorescence experiments monitoring non-radiative energy transfer (NRET), between naphthalene attached to the HM PNIPAM and 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into the lipid bilayer, confirmed the direct penetration of hydrophobic anchor groups linked to the polymer into the liposome hydrophobic core. Contraction of the polymer backbone above the lower critical solution temperature (LCST) resulted in a partial withdrawal of the anchor groups from the lipid bilayer. Analysis of polymer/lipid mixtures by centrifugation and quasi-elastic light scattering (QELS) revealed the polymer-induced fission of liposomes in the liquid-crystalline state, resulting in the formation of vesicles 150–230 nm in diameter. The process is reversible and upon transition of the bilayer into the gel state these vesicles are converted into larger aggregates. According to the results of gel-filtration experiments the HM PNIPAM is in dynamic exchange between the liquid-crystalline lipid bilayer and the water solution, while the binding to the bilayer in the gel state is more static in nature. The binding constant for mixture of HM PNIPAM with DMPC liposomes, evaluated from the centrifugation experiments, was found to be 120 M^{-1}.

Keywords: Amphiphilic polymer; Liposome-polymer interaction; Drug delivery system; Non-radiative energy transfer; Quasi-elastic light scattering; Gel-filtration

1. Introduction

The modification of liposomes with synthetic polymers is a promising approach towards the development of new highly efficient drug carriers. By coating the surface of liposomes with specially-designed water-soluble polymers, several major improvements may be achieved, such as increase in the liposome circulation time, specific targeting, and responsiveness to external stimuli [1]. The performance of polymer-modified liposomes as drug carriers is directly related to the reliable anchoring of the polymer within the lipid bilayer. One aspect of particular importance is the lifespan of the lipid/polymer complexes. Some macromolecules with great affinity to the bilayer may in fact be in a fast dynamic equilibrium between the bound and unbound states. If this is the case, the liposomes will lose their polymer coating very rapidly after injection in the blood stream.

The most common approach to achieving anchoring of the polymer to liposomes is to use hydrophobi-

* Corresponding author. Fax: +1 (905) 5401310.
E-mail: winnikf@mcmaster.ca

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cally modified water-soluble polymers (HM polymers). These are polymers carrying along their backbone a small number of long alkyl chains which are believed to act as anchors into the lipid bilayer. We and others have explored the use of hydrophobically-modified poly-(N-isopropylacrylamides) (HM PNIPAM) as liposome coating [2–8]. The polymers consist of a neutral hydrophilic chain to which a few octadecyl groups are attached at random. A distinctive property of the aqueous solutions of HM PNIPAM is that, like poly-(N-isopropylacrylamide) itself, they undergo phase separation when heated above a critical solution temperature (LCST). Detailed investigations of this phase transition have established that, at the LCST, the PNIPAM chains undergo a collapse from hydrated extended chains to hydrophobic globules which aggregate and form a separate phase [9–13]. The contraction of the polymer chain offers the possibility of controlling the structure of the liposome through the polymer coating.

In a previous study [2,3] we have used fluorescence spectroscopy together with differential scanning calorimetry and quasi-elastic light scattering to establish that HM-PNIPAM's are anchored into the lipid bilayer and that they remain incorporated during the temperature-induced collapse of the PNIPAM chains. The process was thermo-reversible and the liposomes kept their integrity. Several issues, however, needed to be clarified. Do polymer-coated liposomes and unbound polymers coexist in solution? If so, what is the binding constant of the polymer in water to the lipid bilayer? How does the lipid bilayer phase state affect the anchoring process? The objectives of this report are to address these specific issues through a combination of fluorescence measurements, centrifugation studies, and gel filtration chromatography.

2. Materials and methods

2.1. Materials

Water was deionized with a Barnstead NANOpure water purification system. Dimyristoylphosphatidylcholine (DMPC) and egg phosphatidylcholine (EPC) were obtained from Avanti Polar Lipids (Birmigham, AL). Sodium azide (NaN_3), sodium chloride (NaCl), ethylenediamine tetracetate (EDTA), and Tris were purchased from BDH Chemicals (VWR Scientific, Ontario, Canada). The composition of the buffer used throughout this work was as follows: 10 mM Tris, 1 mM EDTA, 0.02% NaN_3, pH = 7.4. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemicals (Sigma-Aldrich Canada Ltd, Ontario, Canada). The polymers were prepared by free radical polymerization in dioxane and the corresponding chromophore-substituted acrylamides, as described in detail elsewhere [22]. They are PNIPAM-Na, a random copolymer of PNIPAM and N-[2-(1-naphthyl)ethyl]-N-n-octadecylacrylamide ($M_v = 279\,000$, $[Na] = 4.05 \times 10^{-5}$ mol g^{-1}), and PNIPAM-Py, a random copolymer of PNIPAM and N-[4-(1-pyrenyl)butyl]-N-n-octadecylacrylamide ($M_v = 390\,000$, $[Py] = 4.4 \times 10^{-5}$ mol g^{-1}). The chemical structures of the polymers and the molar ratios of hydrophobic substituents to PNIPAM are shown in Fig. 1.

2.2. Measurement techniques

The mean diameter of liposomes was evaluated by quasi-elastic light-scattering (QELS) using a Brookhaven Instrument Corporation Particle Sizer Model BI-90 (Holtsville, NY). The data acquisition

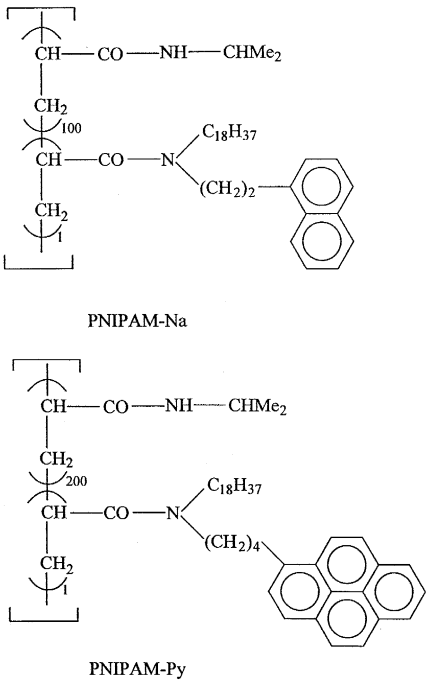

Fig. 1. Structure of the polymers used in this study.

time in a typical experiment was 1000 s and the average value of three independent measurements was taken as a mean diameter. Transmission electron microscopy (TEM) was performed with a JEOL (Japan) transmission electron microscope operating at an accelerating voltage of 80 kV. Samples were prepared as follows: 5 μl of sample containing 10–25 μg of lipid was deposited on a carbon-Formwar-coated copper grid (400 mesh size, Marviak Ltd., Canada) and allowed to adsorb for 30 s, then excess of the sample was removed by a filter paper. The sample was stained for 60 s with a 5 μl drop of 2% (w/v) phosphotungstic acid and blotted dry. Fluorescence spectra were recorded on a SPEX Fluorolog 212 spectrometer (Edison, NJ) equipped with a DM3000F data system. The temperature of the water-jacketed cell holder was controlled with a Neslab (Portsmouth, NH) circulating bath. The temperature of the sample fluid was measured with a thermocouple immersed in the sample. Emission spectra were recorded with an excitation wavelength of 330 nm (Pyrene) and 290 nm (Naphthalene). The extent of non-radiative energy transfer (NRET) between naphthalene and DPH is reported in terms of the ratio $E = 100 \times (I_0\text{-}I_1)/I_0$, where I_0 is the intensity of naphthalene emission, taken as the intensity at 341 nm, in liposome-PNIPAM-Na mixtures in the absence of DPH, and I_1 is the intensity of naphthalene emission at 341 nm in mixtures of PNIPAM-Na and liposomes containing DPH (1% mol/mol, relative to the lipid content). Gel filtration experiments were carried out on a GradiFrac system (Pharmacia Biotech, Uppsala, Sweden) equipped with a XK 16X70 column. The column was packed with Sephacryl S-1000 SF (Pharmacia Biotech, Uppsala, Sweden) and equilibrated with the same buffer as the one used for the sample preparations (10 mM Tris, 1 mM EDTA, 0.02% NaN_3, pH = 7.4). Typically 0.65 ml of a sample containing 15 mg of lipid and 1.5 mg of polymer was injected into the column. The flow rate was 0.5 ml/min and the effluent was monitored at 280 nm. The fractions were collected by volume (2 ml each).

2.3. Spectroscopy

Non-radiative energy transfer (NRET) between chromophores originates in dipole-dipole interactions between an energy donor in its excited state and an energy acceptor in its ground state. The probability of energy transfer between two chromophores depends sensitively on their separation distance and on their relative orientation. The DPH-naphthalene pair of chromophores is known to interact as energy donor (Na) and energy acceptor (DPH) by NRET with a characteristic distance $R_0 = 35$ Å [14], R_0 being defined as the interchromophoric distance at which half of the excited donor population decays by energy transfer. The fluorescence of PNIPAM-Py in water consists of two contributions: a broad signal centred at 482 nm due to pyrene excimer (intensity I_e) and a contribution of isolated excited pyrenes (monomer emission, intensity I_m). The ratio of excimer to monomer intensity was taken as the ratio of the intensity at 482 nm to the half sum of the intensities at 378 and 398 nm.

2.4. Sample preparation

2.4.1. Large multilamellar vesicles (LMV)

A solution of lipid in chloroform was evaporated under a stream of nitrogen in a glass test tube. (In the case of DPH-containing liposomes, the DPH was dissolved in THF and added to the lipid solution in chloroform at a level of 1 mol%.) The resultant thin film of lipid was dried under the high vacuum of the oil pump for at least 2 h. The dry lipid film was dispersed in buffer (10 mM Tris, 1 mM EDTA, 0.02% NaN_3, pH = 7.4) at a concentration 20 g/l.

2.4.2. Giant unilamellar vesicles (GUV)

GUV were prepared by the reverse phase evaporation technique [15]. The lipid (20 mg) was placed in a round-bottomed flask and dissolved in 10 ml of a diethyl ether-chloroform (1:1) mixture. Buffer (1 ml) was rapidly injected into the lipid solution using a 5-ml syringe fitted with a 23-gauge needle. The system was sealed with a glass stopper, placed in a bath sonicator and sonicated for 3 min. Then the flask was quickly transferred to a rotary evaporator and the solvent was removed under a low vacuum at 37°C until a gel was formed. The gel was converted into a fluid suspension of GUV through vigorous mechanical agitation on a vortex mixer. Then evaporation was continued for 5 min, and the last trace of solvent was removed by dialysis against 1 l of buffer overnight.

The liposomes were examined by negative staining TEM electron microcopy. 95% of liposomes were found unilamellar, the diameters were evaluated as 500 nm and higher.

2.4.3. Large unilamellar vesicles (LUV)

For preparation of extruded liposomes the suspension of LMV was forced through polycarbonate filter membranes (Nucleopore) of 100 nm pore diameter using a Lipofast extruder (Avestin, Canada). To prepare LUV of 400 nm in diameter the giant unilamellar liposomes made by the reverse-phase evaporation technique were extruded through 400-nm pore size polycarbonate filters.

2.4.4. Lipid-polymer mixtures

Polymer stock solutions (6 g l^{-1}) were prepared in buffer. Liposomes suspensions and polymer solutions were mixed in the desired proportion and they were allowed to equilibrate before measurement, first at a temperature above the lipid main phase transition (1 h) and, second, at the temperature of the experiment (1 h).

2.5. Centrifugation experiments

A temperature-controlled Eppendorf 5403 (Germany) centrifuge was used for centrifugation experiments. All centrifugation studies were carried out using GUV. In a control experiment with pure GUV it was found that 98% of the lipid are recovered in the pellet formed during centrifugation. Liposomes were mixed with various amounts of the polymer in plastic microcentrifuge tubes. The volume of each sample was adjusted to 1.5 ml with buffer. After the appropriate temperature equilibration (as described in Section 2.4) an aliquot (0.1 ml) from each test tube was taken as a reference sample of original concentration and then the liposomes were pelleted at 4000 $\times g$ for 30 min. Immediately after the centrifugation, samples were separated into two fractions: the top fraction containing the supernatant (1.2 ml) and the bottom fraction containing the pellet (0.2 ml). The supernatant fractions were analyzed for lipid concentration, and, to test the analysis accuracy, both supernatant, pellet fractions and original reference aliquots in all samples were analyzed for polymer concentration. (The results of the analysis were considered to be accurate if the sum of the polymer concentrations in the supernatant and the pellet coincided within 5% deviation with the original concentration.)

2.6. Quantitative analysis

Analysis of the lipid concentration was done by the method described by B.N. Ames [16]. For analysis of HM PNIPAM concentration we employed the fluorescence intensity of Py attached to the polymer. To avoid self-quenching of chromophores due to intra- or interpolymer interactions, all samples were standardized by solubilization in an excess of detergent sodium dodecyl sulfate (SDS). Typically, a 100-μl aliquot of each fraction containing up to 100 μg of polymer was mixed with 1 ml of a 50 mM solution of SDS. The fluorescence intensity of PNIPAM-Py was calibrated at different known concentrations of polymer dissolved in a 50-mM SDS solution. For all polymer concentrations studied ranging up to 500 μg/ml no excimer (emission maximum at 482 nm) was detected, and the dependence of the monomer emission intensity at 378 nm (Py monomer) on the polymer concentration was linear in all the concentration ranges studied.

3. Results

3.1. Fluorescence measurements

The interactions of hydrophobically modified PNIPAM with phospholipid liposomes were monitored by non-radiative energy transfer (NRET) using the naphthalene/DPH donor acceptor pair. The naphthalene chromophore (Na) was attached to the polymer, HM PNIPAM-Na (see Fig. 1), while DPH was incorporated into the hydrophobic core of the lipid bilayer. As a measure of energy transfer efficiency we monitored the decrease in the fluorescence emission intensity at 341 nm (maximum for Na) in polymer-liposome mixtures in the presence of DPH (curve 1, Fig. 2) and compared it to the emission of polymer-liposome mixtures at the same conditions, but without DPH (curve 2, Fig. 2) (see Section 2 for details). The extent of NRET was monitored at different temperatures to study the effect of the HM polymer LCST (32°C, [3,17]) and for different lipids to assess the

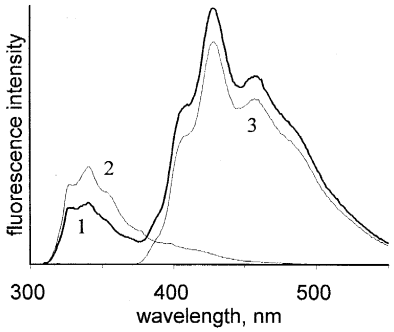

Fig. 2. Emission spectra of: (1) mixture of 0.1 g/l PNIPAM-Na with 0.5 g/l DMPC liposomes containing 1% DPH; (2) mixture of 0.1 g/l PNIPAM-Na with 0.5 g/l blank DMPC liposomes (no DPH); (3) 0.5 g/l DMPC liposomes containing 1% DPH. The excitation was set at 290 nm and the spectra were monitored at 27°C. Liposomes were prepared by extrusion through 100 nm pore diameter polycarbonate filter.

role of the lipid bilayer fluidity. In the case of DMPC liposomes significant energy transfer was detected at 27°C, a temperature below the polymer LCST but above the lipid phase transition temperature (23.5°C, according to Ref. [18]). For polymer/liposome samples prepared at 18°C, a temperature lower than the lipid phase transition, the extent of NRET was slightly lower. When this mixture was heated to 40°C, a temperature above the polymer LCST, a decrease in NRET efficiency was noted. Polymer/EPC liposomes samples displayed an overall similar behaviour, but the NRET efficiency was lower at all temperatures, compared to the case of DMPC liposomes (Table 1). The critical distance R_0 for NRET in the case of the naphthalene/DPH pair is 35 A [14]. Therefore the occurrence of NRET transfer between the two chromophores, one linked to the polymer next to the octadecyl substituent, and the other

Table 1
Non-radiative energy transfer efficiency depending on the lipid type and temperature

Lipid type	Temperature (°C)	NRET [a] efficiency (%)
DMPC	18	31
	27	37
	40	17
Egg PC	27	14
	40	10

[a] The NRET was monitored in mixtures of 0.1 g/l PNIPAM-Na with 0.5 g/l liposomes containing 1 mol% DPH.

located within the lipid bilayer, is a direct indication of the insertion of the hydrophobic anchoring group into the bilayer. This result confirms data of previous NRET data carried out between fluorescent labels of HM-PNIPAM [2].

3.2. Centrifugation studies

A centrifugation assay was used to study the distribution of HM PNIPAM-Py (see Fig. 1) between the aqueous and the lipid phases in HM PNIPAM-Py/liposome suspensions. The polymer was incubated with giant unilamellar vesicles (GUV) under various conditions described in detail in Section 2. The resulting suspensions were subjected to centrifugation at $4000 \times g$ for 30 min. The polymer content of the supernatant and the pellet was determined by a fluorescence assay. The lipid concentration of the supernatant was measured as well. We first monitored HM PNIPAM/EPC liposome suspensions. To our surprise the polymer concentration was nearly the same in the supernatant and in the pellet. Contrary to our expectations it was even slightly *higher* in the supernatant than in the pellet (Fig. 3a). The absence of preferential incorporation of the polymer in the pellet may be taken as an indication that the polymer did not bind to the liposome at all. Such an interpretation contradicts the conclusions drawn from the NRET experiments described here and in previous studies [2,3]. Therefore we favour another interpretation of these results, namely that the interaction of HM PNIPAM-Py with GUV leads to a partial extraction of lipids from the large liposomes, with subsequent formation of small lipid/polymer aggregates. These would remain in the supernatant under the centrifugation conditions used in our experiments. The increased lipid concentration in the supernatant supports the proposed mechanism (Fig. 4, curve 1).

Analysis of the supernatant by QELS and TEM lent further support to our interpretation. The presence of aggregates in the supernatant of polymer/EPC samples was confirmed by QELS experiments. Particles approx. 225 nm in diameter were detected in the supernatant obtained upon centrifugation of HM PNIPAM-Py/EPC liposomes (Table 2). These are larger than the micelles formed by HM PNIPAM-Py in solution which range in size from 50 to 110 nm [19]. Control experiments with a sample

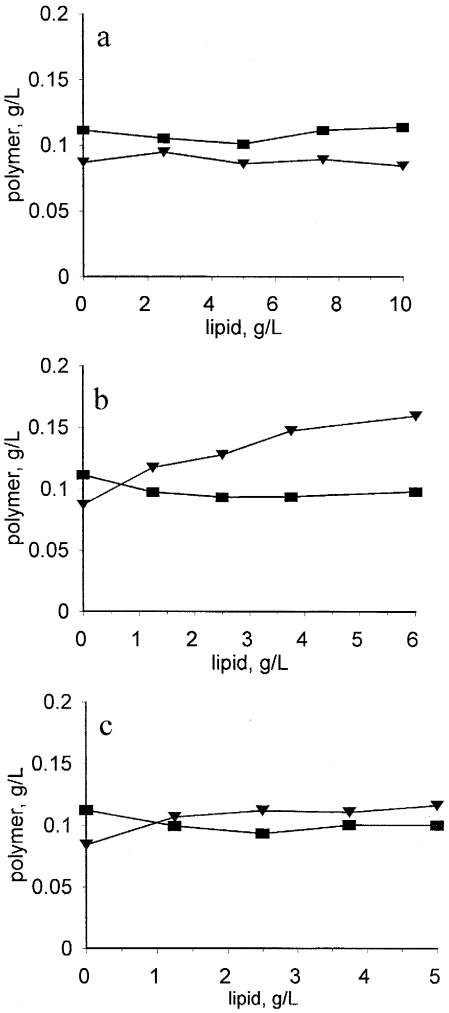

Fig. 3. Distribution of PNIPAM-Py between supernatant and pellet fractions after centrifugation of mixtures of GUV with polymer at 4000× *g*. The polymer concentration in all samples was 0.1 g/l, the lipid concentration was ranging from 0 to 10 g/l. ■, polymer concentration in supernatant fractions; ▼, polymer concentration in pellet fractions in mixtures with (a) EPC liposomes at 25°C; (b) DMPC liposomes at 18°C; (c) DMPC liposomes at 27°C.

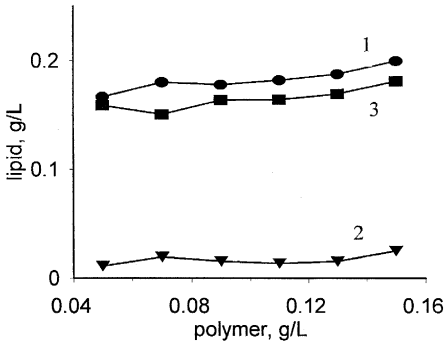

Fig. 4. Lipid concentration in supernatant after centrifugation at 4000× *g* of PNIPAM-Py mixtures with GUV depending on lipid type and temperature: The original lipid concentration in all samples was 2.5 g/l, the polymer concentration ranged from 0.05 g/l to 0.15 g/l. Lipid type and temperature: (1) ●, EPC, 25°C; (b) ▼, DMPC, 18°C; (3) ■, DMPC, 27°C.

containing only liposomes confirmed that there were no detectable particles in the supernatant. TEM observation of the supernatant revealed the presence of liposomes, approx. 150 to 250 nm in diameter. No liposomes were observed in the supernatant isolated from a control sample of liposomes alone.

Next we performed the centrifugation assay on mixtures of HM PNIPAM-Py and DMPC liposomes. The DMPC bilayer is known to undergo a phase transition at 23.5°C between the gel and the liquid crystalline phase [18]. Thus, we conducted two sets of experiments at 18°C and 27°C, temperatures lower than the LCST of the polymer, but, respectively, below and above the lipid phase transition. Centrifugation assays performed at 18°C led to a non-uniform distribution of the polymer between the supernatant and the pellet. The polymer concentration was significantly higher in the pellet, compared to the supernatant (Fig. 3b). The lipid concentration was very low in the supernatant, independently of initial polymer concentration. When the centrifugation was performed at 27°C polymer was present almost equally in both the pellet and the supernatant, but unlike the case of polymer/EPC liposomes described previously, the polymer concentration was slightly higher in the pellet, compared to the supernatant (Fig. 3c). The lipid concentration in the supernatant correlated well with that found in the case of EPC liposomes experiments (Fig. 4, curve 3). Centrifugation experi-

Table 2
Mean diameter of particles in supernatant evaluated by QELS

Lipid	Temperature (°C)	Mean diameter (nm)
Egg PC [a]	25	225
DMPC [b]	27	174
DMPC [b]	18	243

Sample composition: [a] 5 g/l EPC and 0.1 g/l HM PNIPAM-Py, [b] 2.5 g/l DMPC and 0.1 g/l HM PNIPAM-Py.

ments with HM polymer/DMPC mixtures clearly indicated that the polymer-induced formation of small mixed aggregates is a reversible process depending on the lipid phase state. We monitored particle size in the supernatant of PNIPAM-Py/DMPC mixture centrifuged at 27°C at two different temperatures. Above the lipid T_C the average particle diameter was 174 nm. In the same sample equilibrated at 18°C a significant increase in particle size was detected, and the mean diameter was evaluated as 243 nm (Table 2).

In summary, the centrifugation assay confirmed the incorporation of HM PNIPAM-Py within the liposome bilayer also observed by NRET experiments. It also points to the key influence of the phase of the lipid bilayer in directing the polymer/liposome interactions.

3.3. Gel-filtration studies

Gel filtration of HM-PNIPAM-Py/liposome mixtures was performed on Sephacryl S1000. This medium is suitable for the size analysis and fractionation of lipid vesicles with diameters up to 300 nm [20,21]. The experiments were carried out with DMPC liposomes prepared by extrusion through polycarbonate filters of either 400 nm or 100 nm pore sizes. Polymer/liposomes mixtures prepared under various conditions were eluted through the column. The eluted fractions were analysed for polymer content by a fluorescence assay and for lipid content by chemical analysis (see Section 2). Thus each experiment yielded two elution profiles: one for the polymer and one for the liposomes. A superposition of the two profiles, indicating co-elution of polymer and liposomes, can be taken as a strong indication that the polymer remains anchored into the liposomes as it moves through the column. In contrast, the occurrence of two separate profiles implies the existence of a dynamic exchange of the polymer from the aqueous phase to the lipid phase, as the mixture elutes through the column.

For polymer/liposome samples prepared with liposomes extruded through the 400-nm pore-size filters and fractionated at 18°C, the lipid distribution corresponds exactly to the lipid profile of pure 400 nm DMPC liposomes, confirming that the liposomes remain intact upon interaction with polymer. In contrast, the polymer profile displays a significant redistribution of the polymer from the elution volume corresponding to pure polymer (approx. 80 ml) to that corresponding to the elution of liposomes (approx. 40 ml) (Fig. 5a). The same tendency was detected in the case of the 100-nm liposome samples fractionated at 18°C (Fig. 5b). The effect is less pronounced, probably due to the poorer separation of the unbound polymer and the small liposomes. We note, however, that the lipid elution profile of 100 nm liposomes features a shoulder on the left side of the lipid band, indicating an increase in the amount of larger liposomes.

Next, the two samples were fractionated at 27°C, a temperature above the phase transition of the lipid bilayer, but still lower than the polymer LCST. In this case the elution profile of the polymer in the presence of liposomes, 400 nm or 100 nm in size, corresponded very nearly to the profile of the pure

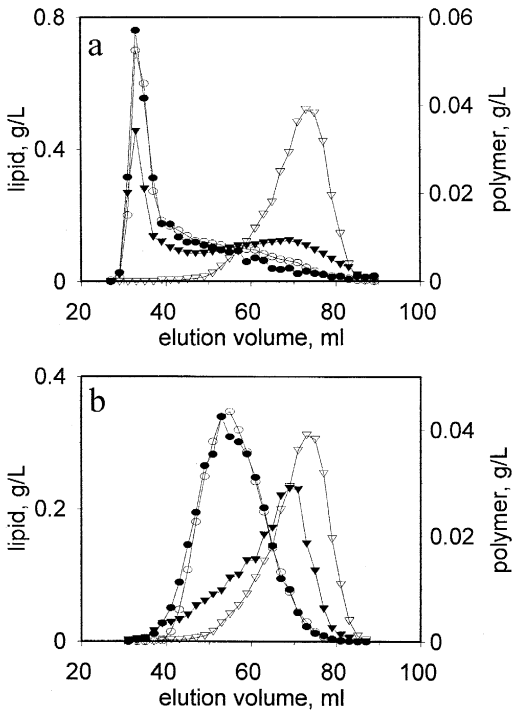

Fig. 5. Elution profiles from Sephacryl S1000 at 18°C. Volume of all samples applied to the column was 0.65 ml, lipid concentration: 15 g/l, polymer concentration: 1.5 g/l. Lipid concentration in fractions collected for: ○, DMPC liposomes; ●, PNIPAM-Py/DMPC liposomes mixture. Polymer concentration in fractions collected for: ▽, PNIPAM-Py; ▼, PNIPAM-Py/DMPC liposomes mixture. Liposomes were prepared by extrusion through (a) 400 nm or (b) 100 nm pore size polycarbonate filter.

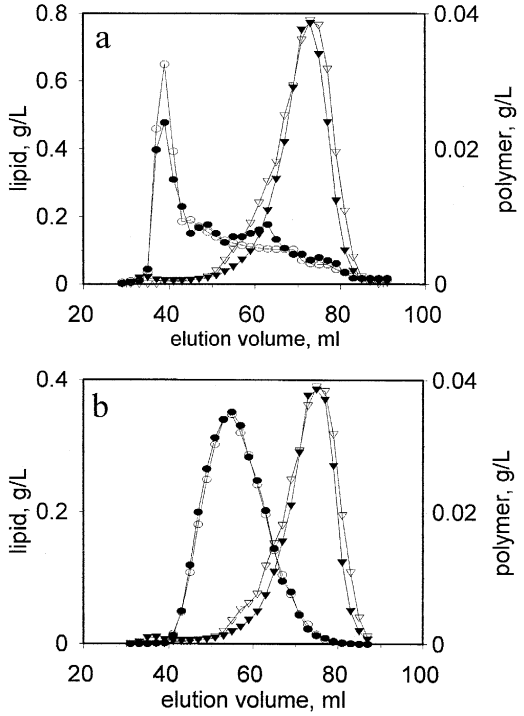

Fig. 6. Elution profiles from Sephacryl S1000 at 27°C. Volume of all samples applied to the column was 0.65 ml, lipid concentration: 15 g/l, polymer concentration, 1.5 g/l. Lipid concentration in fractions collected for: ○, DMPC liposomes; ●, PNIPAM-Py/DMPC liposomes mixture. Polymer concentration in fractions collected for: ▽, PNIPAM-Py; ▼, PNIPAM-Py/DMPC liposomes mixture. Liposomes were prepared by extrusion through (a) 400 nm or (b) 100 nm pore size polycarbonate filter.

polymer (Fig. 6a and Fig. 6b respectively). Hence in this case the gradual dilution of the samples by the eluting buffer results in a separation of the polymer from the liposomes. This result is extremely significant since it lends strong support to a dynamic exchange mechanism of the association of HM-PNIPAM's and a lipid bilayer in the liquid-crystalline state. A comparison of the lipid elution profiles in the case of the 400 nm liposomes fractionated at 27°C reveals an additional elution band at a volume corresponding to smaller vesicles. This redistribution of the lipid is consistent with a polymer-induced partial reduction of the liposome size, a phenomenon also observed by centrifugation assay (see above). As described in Section 2, all samples were incubated at 27°C prior to gel filtration studies. The absence of additional small phospholipid/polymer aggregates in samples fractionated at 18°C can be taken as further indication of the thermo-reversibility of the formation of polymer/lipid aggregates, at least when the temperature of incubation remains below the LCST of the polymer.

The fluorescence spectrum of HM PNIPAM-Py in water presents two contributions, a well resolved spectrum with maxima at 378 and 398 nm, corresponding to the emission of isolated pyrene chromophores, and a broad band centred at 482 nm, due to pyrene excimer emission. In aqueous solutions of HM PNIPAM-Py, the pyrene excimer emission intensity (I_e) is high, relative to the pyrene monomer emission intensity (I_m) [17]. In previous studies we ascertained that in water, the hydrophobic chromophores are brought in close proximity within interpolymeric micelles, thus enhancing the excimer contribution to the overall emission. However, in the presence of liposomes we have shown that the excimer emission all but vanishes, implying a disruption of the polymeric micelles upon interaction with liposomes [3]. Presumably the pyrene substituents are kept apart from each other as they become inserted

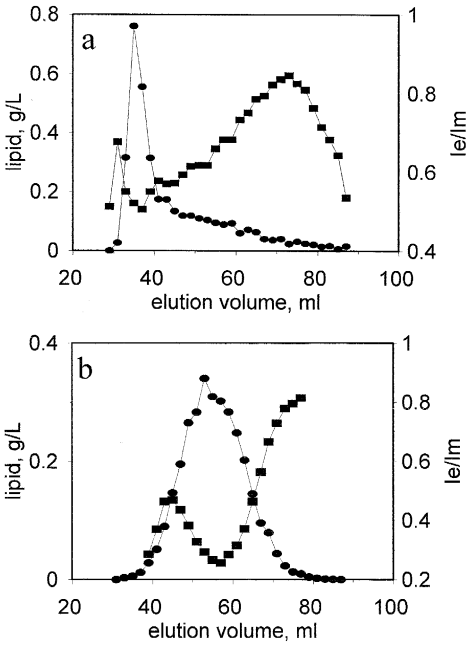

Fig. 7. Elution profiles from Sephacryl S1000 for PNIPAM-Py/DMPC liposome mixtures at 18°C. ●, lipid concentration, ■, Ie/Im. Volume of all samples applied to the column was 0.65 ml, lipid concentration: 15 g/l, polymer concentration: 1.5 g/l. Liposomes were prepared by extrusion through (a) 400 nm or (b) 100 nm pore size polycarbonate filter.

within the lipid bilayer. In the present set of experiments we recorded the fluorescence of all polymer containing fractions, prior to the treatment with SDS required for the quantitative assay. These measurements yielded elution profiles of the ratio I_e/I_m (Fig. 7b). For HM-PNIPAM-Py/liposomes fractionated at 18°C, the ratio presented a marked maximum for elution volumes corresponding to the first lipid fractions. It was much lower in the fractions with highest lipid content. It increased again in the fractions corresponding to the maximum polymer elution. Overall this behaviour is consistent with our previous observations. The fact that in the first lipid fractions the ratio is higher than in the majority of the liposome fractions may indicate a higher concentration of polymer bound to the liposome surface. The enhanced amount of large liposomes noted in the lipid elution profile could also be ascribed to a dense polymer coating of the liposomes. The same trends were observed for the mixtures of polymer with 400 nm liposomes, but they were less pronounced (Fig. 7a).

4. Discussion

The measurements of NRET between the Na and DPH pair allowed us to monitor directly the association of the polymer with the lipid bilayer depending on the polymer conformation, the lipid type, and the bilayer fluidity. The binding of the polymer to liposomes results in energy transfer between Na linked next to hydrophobic side chains of the polymer to DPH incorporated into the lipid bilayer. The NRET efficiency is significantly lower for polymer-liposome mixtures at temperatures above the LCST. We interpret the decrease in energy transfer as an indication of the partial withdrawal of the C_{18} chains from the lipid bilayer, as the polymer backbone contracts upon heating above the LCST.

Higher values of the NRET efficiency for DMPC liposomes, in comparison with EPC liposomes, probably indicate the higher affinity of the C_{18} chains for a bilayer consisting of saturated lipid. Surprisingly, the bilayer fluidity does not appear to affect the polymer binding significantly. The NRET efficiency in the polymer-liposome mixture at 18°C (below T_C = 23.5°C, [18]) is only slightly lower than at 27°C (above T_C).

The importance of the bilayer fluidity in defining the mode of HM PNIPAM action was revealed by centrifugation and gel-filtration experiments. For mixtures of HM polymers with liposomes in the liquid-crystalline state, we detected almost equal distribution of polymer between the supernatant and the liposome pellet. A significant amount of small liposomes was present in the supernatant. Moreover, in the gel-filtration experiments, the shape of the lipid profile was modified with a significantly contribution of smaller liposomes for the liposome-polymer mixture fractionated above the T_C of lipid. These results are consistent with an HM PNIPAM-induced fission of liposomes, resulting in the formation of small liposomes.

Previously, Simon et al. found that HM PNIPAM labeled by fluorescein tends to form polymer-rich domains on a bilayer surface [5]. They detected significant changes in the shape of giant liposomes, resulting in liposome budding as the polymer was undergoing the coil-globule transition at LCST. To explain this behavior, they proposed the mechanism involving phase separation of the contracted polymer on the liposomes surface. The formation of buds was explained by a change in the local curvature due to the accumulation in these areas of the polymer considered as an amphiphile with bulky hydrophilic part.

Our findings show that such behavior of HM PNIPAM is of a more general character. It is possible that the tendency of the polymer to segregate in the plane of the lipid bilayer gives rise to liposome budding even below the LCST. The size of the small liposomes formed during this process was evaluated by QELS and electron microscopy as 150–250 nm (which is beyond the resolution of light microscope used in Ref. [5]), while the size of buds described previously was about 5000 nm. As the PNIPAM undergoes conformational change at LCST, the originally hydrated and extended backbone of the polymer collapses into a compact contracted globule, even when the polymer is anchored into the lipid bilayer [3]. Below the LCST the dimensions of the hydrophilic part of the HM polymer are larger in comparison to the one above LCST. The accumulation in certain areas of the amphiphile with the larger hydrophilic part will result in higher local curvatures [22] and, correspondingly, in smaller vesicles formed after the liposome fission. The proposed mechanism

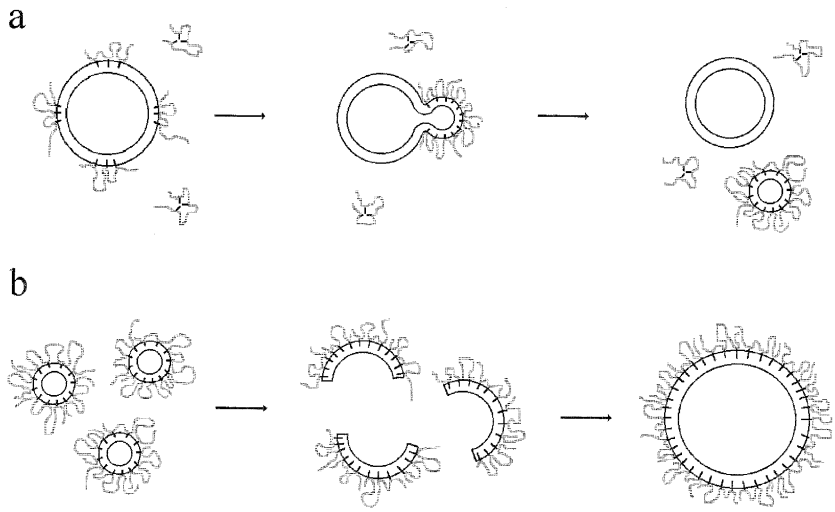

Fig. 8. Schematic representation of (a) liposome fission induced by polymer binding, (b) transformation and fusion of small polymer-coated liposomes into larger aggregates upon lipid liquid crystalline-gel transition.

of polymer-induced liposome fission is illustrated in Fig. 8a.

According to the results of the centrifugation studies such small liposomes carry the majority of the bound polymer, as the distribution of the polymer between the supernatant containing small liposomes and the pellet formed by large liposomes is equal. The proposed model of the liposome fission is also supported by the gel-filtration experiments showing an additional band corresponding to smaller particles in the lipid elution profile of HM PNIPAM/400 nm DMPC liposomes mixture. The other important finding is the complete separation of the polymer and liposomes during gel-filtration above T_C, indicating the relatively fast exchange between bound and unbound polymer molecules.

As a marked amount of polymer was found associated with the liposome pellet in centrifugation studies and with a lipid profile peak in gel-filtration experiments for the mixtures with liposomes below T_C, one may conclude that the liposome-polymer association in this case is more durable. We derived a binding curve for the DMPC and HM PNIPAM-Py system based on the results of the centrifugation study at 18°C (Fig. 9). All polymer left in the supernatant was considered as unbound. Since the amount of lipid in the supernatant was very low (Fig. 3) the amount of polymer bound to it should be insignificant. The linear character of the binding curve shows that the interaction of HM PNIPAM with the lipid bilayer is, probably, a simple partitioning between water and lipid phases, without apparent cooperativity of binding (otherwise a sigmoidal shape of the binding curve would be observed). The binding constant estimated from the binding curve is $K = 120\ M^{-1}$, a value which implies a relatively weak binding. For example, at a lipid concentration of 0.5 g/l and a polymer concentration of 0.1 g/l (typical experimental concentrations), only 30% of the total polymer amount will be bound to the liposome surface.

The fact that for the mixture of DMPC liposomes with HM PNIPAM at 27°C 8–12% of lipid was found in the supernatant, while less than 2% of lipid was detected there in the case of the same sample

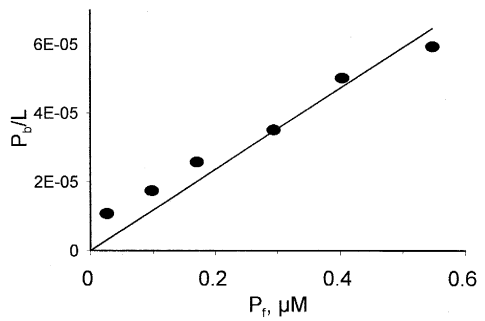

Fig. 9. Binding curve for mixtures of PNIPAM-Py with DMPC liposomes at 18°C. P_b: bound polymer, P_f: free polymer in solution, L: lipid.

cooled down to 18°C, indicates that the lightweight lipid polymer complexes (small polymer-coated liposomes) are converted to large and heavy associates during the lipid phase transition. We attribute the driving force of this association to the increased bilayer rigidity, causing the curvature strain in the relatively small (about 150–250 nm, according to QELS and TEM data) vesicles. As larger structures are preferable for stressed bilayers, the curvature strain probably forces liposomes to fuse with each other in order to release the stress (Fig. 8b).

It is not possible to directly evaluate the binding constant from the centrifugation studies for mixtures of HM PNIPAM with liposomes in the fluid state, because in this case the major part of the polymer-lipid complexes are small liposomes which do not form pellets. But we assume that the amount of polymer bound to the lipid bilayer when it is in a liquid-crystalline state is approximately the same as the amount of bound polymer in case of lipid bilayer converted to a gel state, according to the NRET data. When the polymer and liposomes were mixed below the lipid phase transition the recorded NRET efficiency was only slightly lower than one at 27°C. These results are consistent with previous reports, where this effect was attributed to the presence of defect sites in the lipid bilayer, where incorporation of the C_{18} side groups took place preferentially [2].

5. Conclusions

We examined the behavior of mixtures of hydrophobically-modified poly-(N-isopropylacrylamides) (HM PNIPAM) with phospholipid liposomes, as a function of the lipid type, the bilayer fluidity and the polymer conformation. The mechanism of the HM PNIPAM interaction with liposomes is affected dramatically by the lipid phase state. The binding of polymer to large (>400 nm in diameter) liposomes in the liquid-crystalline state results in the liposome fission and in the separation of smaller polymer-coated liposomes (150–250 nm in diameter). The separation is reversible, and upon transition of the bilayer to the gel state the small liposomes are converted into larger aggregates. The polymer bound to the fluid bilayer is in dynamic exchange with unbound polymer. Interaction of the polymer with the lipid bilayer in the gel state presumably occurs at bilayer defect sites and the binding is of a more static nature. The affinity of polymer carrying octadecyl chains depends on the lipid type. It is greater for the saturated lipids than the unsaturated ones. The binding constant for mixtures of HM PNIPAM with DMPC liposomes was evaluated as 120 M^{-1}, and it is approximately the same for bilayers in gel or liquid-crystalline state.

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