Biochimica et Biophysica Acta, 1153 (1993) 335–344

BBAMEM 76170

Interactions of liposomes and hydrophobically-modified poly-(*N*-isopropylacrylamides): an attempt to model the cytoskeleton

Helmut Ringsdorf [a], Erich Sackmann [b], Joachim Simon [a] and Françoise M. Winnik [c,1,*]

[a] *Institut für Organische Chemie, Johannes Gutenberg-Universität Mainz, J.-J. Becher Weg 18–20, 55099 Mainz (Germany),* [b] *Physik Department, Technische Universität München, Abteilung Biophysik E22, D-8046 Garching (Germany) and* [c] *Xerox Research Center of Canada, 2660 Speakman Drive, Mississauga, Ont. L5K 2L1 (Canada)*

(Received 3 June 1993)

Key words: Non-radiative energy transfer; Liposome–polymer Interaction; Cytoskeleton; Model membrane; Fluorescence; DSC; Quasi-elastic light scattering

The interactions of small unilamellar vesicles (SUV) and water-soluble copolymers were studied by fluorescence spectroscopy, differential scanning calorimetry (DSC) and quasi-elastic light scattering (QELS). The anchoring onto liposomal bilayer membranes of copolymers of *N*-isopropylacrylamide, *N*-(2-(1-naphthyl)ethyl)-*N*-n-octadecylacrylamide and or *N*-[4-(1-pyrenyl)butyl]-*N*-n-octadecylacrylamide (0.5 mol% of the octadecylacrylamide comonomer) was monitored by non-radiative energy transfer between excited naphthalene and pyrene. The anchoring process occurred on zwitterionic lecithin liposomes and on negatively charged phosphatidic acid liposomes, whether the bilayer was in the crystalline or the liquid-crystalline phase. Insertion of the copolymer octadecyl groups within crystalline bilayers was attributed to the presence of packing defects. Aqueous solutions of poly-(*N*-isopropylacrylamide) and of its hydrophobically-modified copolymers exhibit a lower critical solution temperature (LCST). The coil to globule collapse of the polymer chains which is known to occur as the aqueous solution is heated through the LCST, also took place when the copolymers were anchored onto vesicular bilayers. The copolymers remained anchored during this collapse and the liposomes were not destroyed. The process was thermo-reversible. Detailed aspects of the reversibility of the phenomenon depended on the relative values of the phase transition temperatures of the liposomes and of the polymer LCST.

Introduction

The formation of self-organized plasma membranes marked an important step in the evolution of life by allowing within a well-separated cytoplasm the formation and replication of proteins and nucleotides. In higher organisms this compartmentalization role is only one of the many functions of the cell membranes. They participate actively in a number of cellular processes controlling the energy balance, the metabolism, and the reproduction of the cell. Cell membranes are complex organized systems of lipids, proteins, and carbohydrates. In many cells and especially in mammalian red blood cells (Fig. 1a) the cell membrane is supported by the cytoskeleton, a two-dimensional protein network attached to the cytoplasmic side of the cell membrane [1–3]. Its primary function is to stabilize the lipid bilayer and to maintain the membrane integrity as the red blood cell undergoes large elastic deformations during blood circulation [4]. The dynamics of this network enable local motions within the cell membrane, facilitating the processes of endo- and exocytosis, as well as the transport of metabolites through the membrane.

The ultimate objective of the work described here is to prepare organized structures of liposomes and functional synthetic polymers which can mimic the dynamic motions of a two-dimensional cellular cytoskeleton attached to a lipid bilayer. Numerous approaches have been pursued towards modeling the lipid double layer of biomembranes [5]. There have been much fewer attempts to design model membranes which would include a two-dimensional network woven within the lipid bilayer, thus getting closer to the multilayered structure of plasma membranes. To model the dynam-

* Corresponding author. Fax (present address): +1 (905) 5222509.
[1] Present address: Department of Chemistry, McMaster University, 1280 Main Street W, Hamilton, Ontario L8S 4M1, Canada.

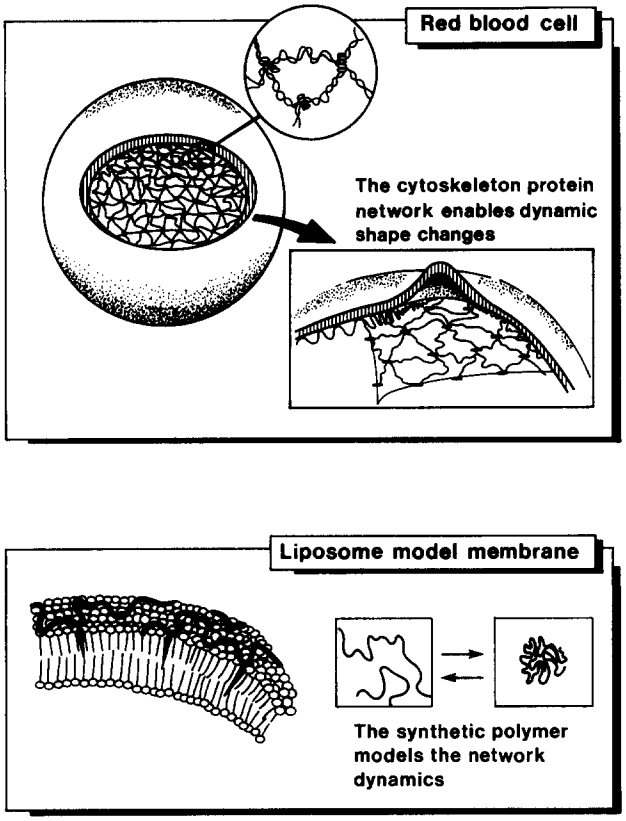

Fig. 1. The concept of red blood cell model cytoskeleton explored in this study.

ics of cell membranes brings a new dimension to the applications of specially designed and synthesized polymer–liposome systems. First attempts were studied by Gaub et al. using polymerizable lipids [6]. Artificial cell wall-like structures have been constructed by building polymeric lipids into the lipid bilayer to increase the structural stability of liposomes [7]. The strengthening of lipid membranes by adsorption of polymers has been demonstrated by the pioneering work of Sunamoto and co-workers, who exploited the affinity for liposomes of naturally occurring polysaccharides and their hydrophobically-modified derivatives [8–11]. Liposomes coated with polyethylene glycols have been shown to have prolonged lifetimes in vitro, compared to untreated samples [12,13].

We have chosen derivatives of the temperature-sensitive polymer, poly-(*N*-isopropylacrylamide) (PNIPAM), as the active polymers in model membranes. The polymers consist of a neutral hydrophilic chain made up of *N*-isopropylacrylamide units to which are attached at random a few long alkyl chains. Such long alkyl chains are known to interact strongly with lipid bilayers [8,14] and thus can serve as anchors for the polymer onto the liposomes. PNIPAM was chosen because it is very soluble in cold water, however its solution undergoes phase separation as it is warmed-up past a critical temperature (32°C, the lower critical solution temperature or LCST) [15,16]. The phe-

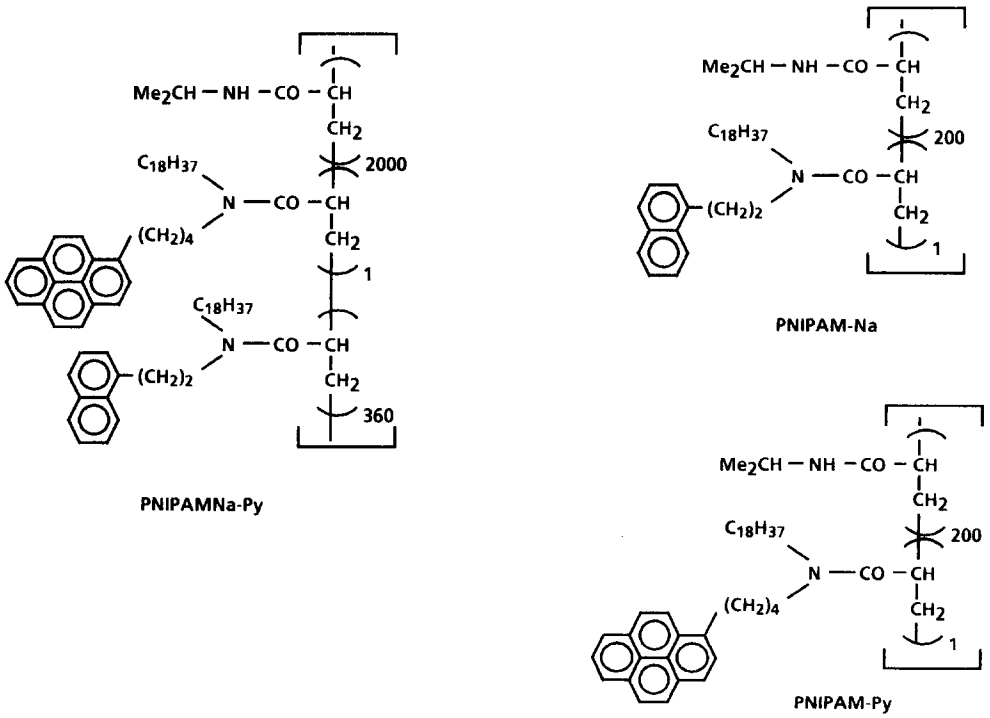

Fig. 2. Structures of the polymers used in this study.

nomenon is reversible: cooling the milky suspension below 32°C results in immediate redissolution of the polymer. On a molecular level, the process involves the collapse of hydrated expanded flexible PNIPAM coils into contracted globules, with subsequent aggregation of these individual globules into a separated polymer-rich phase [17–19]. Hydrophobically-modified poly(*N*-isopropylacrylamides) (HM-PNIPAM) still form clear solutions in cold water, with an LCST at 32°C or slightly below this temperature, if the level of hydrophobic group incorporation is kept below 1 molar% [20,21]. As in the case of the homopolymer, the LCST phenomenon in solutions of HM-PNIPAM is driven by the contraction of the PNIPAM backbone.

We have used this temperature-sensitivity of the polymer chain to simulate the dynamics of cell membranes [22], by anchoring the polymer onto liposomes and subjecting the systems to repeated heating-cooling cycles in temperature domains including the polymer LCST. During this treatment the polymer chains collapse and re-expand. The polymer conformation changes are accompanied by motions of the lipidic anchor groups within the membrane: collapse of the polymer forces them to come into closer proximity, re-expansion of the chains moves them apart from each other. The active role of the anchored polymer was demonstrated by monitoring changes in photophysical properties of chromophores attached to the anchored polymer. The polymer was a specially designed hydrophobically-modified PNIPAM, PNIPAM-Py (see Fig. 2), in which a fluorescent dye, pyrene (Py), was linked to the polymer next to the octadecyl chain. In water this polymer exhibits emission from isolated excited pyrenes, Py* ('monomer' emission, 380 to 410 nm range) and from excited pyrene dimers (Py..Py)*, ('excimer' emission, 400–600 nm range). The variations in the ratio I_E/I_M of pyrene excimer emission intensity to pyrene monomer emission intensity give a qualitative indication of the changes in the average distance between pyrene groups, since a pyrene excimer can form only if two chromophores are in close enough proximity during the lifetime of Py* [23]. The distance (4 to 5 Å) probed by this spectroscopic technique is small on the scale of the lipid bilayer. Thus only limited information was gained from this type of experiment. It was thus necessary to devise a spectroscopic tool enabling one to scan larger distances between groups anchored within the lipids of the external liposome bilayer.

This concern prompted us to initiate the work described here. We set as our objectives to monitor the heat-induced changes incurred by the lipid bilayer as well as by its polymeric partner. We synthesized new Naphthalene-labeled polymers (PNIPAM-Na and PNIPAM-Na-Py, see Fig. 2) and we expanded the battery of measurement techniques to include differential scanning calorimetry (DSC) and quasi-elastic light scattering (QELS), in addition to fluorescence spectroscopy. The spectroscopic experiments relied primarily on the phenomenon of non-radiative energy transfer (NRET) between two different chromophores. This technique, which permits one to probe distance scales in the 20–50 Å range for the pair of chromophores used here, is an excellent complement to experiments based on the photophysics of the pyrene excimer.

Materials and Methods

Materials. Water was deionized with a Millipore Milli-Q water purification system (specific conductance: 0.056 $\mu\Omega\,cm^{-1}$, pH 6.5). Dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC) and egg phosphatidic acid (PA) were purchased from Sigma Chemicals. Single unilamellar vesicles (SUV) were prepared by the sonication technique [24] as described in detail elsewhere [25]. The polymers were prepared by free-radical copolymerization in dioxane of NIPAM and the corresponding chromophore-substituted acrylamides [20,21]. They are PNIPAM-Py, a random copolymer of *N*-isopropylacrylamide (NIPAM) and *N*-[4-(1-pyrenyl)butyl]-*N*-n-octadecylacrylamide; PNIPAM-Na, a random copolymer of *N*-isopropylacrylamide and *N*-[2-(1-naphthyl)ethyl]-*N*-n-octadecylacrylamide; and a doubly labeled copolymer PNIPAM-Na-Py, a random copolymer of *N*-isopropylacrylamide, *N*-[4-(1-pyrenyl)butyl]-*N*-n-octadecylacrylamide, and *N*-[2-(1-naphthyl)ethyl]-*N*-n-octadecylacrylamide. The chemical structures of the polymers and the molar ratios of hydrophobic substituents to NIPAM units are shown in Fig. 1. Physical properties and compositions of the polymers are listed in Table I.

Measurement techniques. Liposome sizes were determined at 25°C by quasi-elastic light scattering (QELS) with a fixed 90° scattering angle using a Brookhaven Instrument Corporation Particle Sizer Model BI-90 equipped with a He-Ne laser. In a typical measurement values were determined over 2000 cycles with a count rate of 50 kcps. The software provided by the manufac-

TABLE I

Molecular and physical properties of the polymers

Polymer	M_v [a]	M_w/M_n [b]	Cloud point (°C)	[Na] (mol g^{-1})	[Py] (mol g^{-1})	Ref.
PNIPAM	380000	1.75	31.8			10
PNIPAM-Na	279000	1.92	30.4	$4.0\cdot10^{-5}$		11
PNIPAM-Py	390000	1.66	30.6		$4.4\cdot10^{-5}$	10
PNIPAM-Na-Py	260000	2.18	30.7	$1.3\cdot10^{-5}$	$0.5\cdot10^{-5}$	11

[a] From intrinsic viscosity mesurements.
[b] From GPC measurements in THF.

turer was employed to determine the liposome sizes. An average value over 10 consecutive measurements is reported. Differential scanning calorimetry was performed with a Microcal MC 2 DSC microcalorimeter. The samples were analyzed at a heating rate of 0.75C° min^{-1} in the temperature range of 10–40°C for DMPC liposomes and 25–65°C for DSPC liposomes. Fluorescence spectra were recorded on a SPEX Fluorolog 212 spectrometer equipped with a DM3000F data system. The temperature of the water-jacketed cell holder was controlled with a Neslab 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) and were not corrected. The pyrene excimer to monomer ratios I_E/I_M were calculated by taking the ratio of the intensity (peak height) at 480 nm to the half-sum of the intensities at 379 nm and 399 nm. The monomer emission was determined in each case by subtracting a normalized (at 343 nm) spectrum. The extent of pyrene emission due to non-radiative energy transfer (NRET) from naphthalene is reported in terms of the ratio I_{Na}/I_{Py} of the intensity (peak height) at 340 nm to the intensity at 378 nm of the emission spectra from excitation at 290 nm.

Sample preparation. Liposomes were prepared by the sonication technique, as described in detail previously [25]. The liposome suspensions in deionized water (approx. 1.0 gl^{-1}) were filtered through a 0.22 μm membrane. Under the conditions used, predominantly single unilamellar vesicles (SUV) were formed. The absence of multilamellar vesicles in the preparations was confirmed by the absence of signals due to pretransitions in the DSC scans of the liposome suspensions in water (see below) and by electron microscopy observation of freeze-fractured samples which showed predominantly typical unilamellar vesicles. The diameters of the vesicles were determined by QELS to range between 90 and 120 nm. Samples were used only if they retained constant diameter for a period of at least 6 h. Stock solutions (1 gl^{-1}) of the polymers were prepared at room temperature and kept at 5°C for at least 24 h. Solutions of polymer mixtures were prepared at room temperature from the aqueous stock solutions and diluted with deionized water in the desired ratios and amounts. They were allowed to stand at room temperature for 2 h prior to use, unless otherwise specified. They were added to freshly prepared liposome suspensions. The resulting suspensions were allowed to stand at room temperature for 3 h, unless otherwise indicated. The relative amounts of polymers to lipids are presented in Table II. They were such that there was a large excess of liposomes relative to the number of polymer chains (approx. 1 liposome per macromolecule). This value was estimated with the following assumptions: (1) all the liposomes are unilamellar and each liposome (diameter: 100 nm) contains about 83000 lipids [26]; (2) the molecular weight and compositions (Table I) of the polymers are those calculated from viscosity measurements and ^{1}H-NMR and UV absorption spectroscopy, respectively. The same protocol was followed to prepare samples for microcalorimetry (polymer concentration: 0.13 gl^{-1}, lipid concentration: 3 gl^{-1}).

Spectroscopy. The occurrence of non-radiative energy transfer (NRET) between chromophores [27,28] has been employed extensively in biochemistry, for example in studies of the tertiary structure of proteins [29], or to monitor the fusion of bilayer vesicles [30]. The process 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 pyrene-naphthalene pair of chromophores is known to interact as energy donor (naphthalene) and energy acceptor (pyrene) by nonradiative energy transfer with a characteristic distance, $R_o = 29$ Å [19], R_o being defined as the interchromophoric distance at which half of the excited donor population decays by energy transfer. Under circumstances where the requirements of NRET from Na* to Py are satisfied, excitation at 290 nm results in an

TABLE II

Compositions of the samples examined in the fluorescence measurements

Liposome (1 gl^{-1})	Size (nm)	Polymer	Concentration	
			gl^{-1}	Na:Py ratio
DMPC	110–130	PNIPAM-Na + PNIPAM-Py	$4 \cdot 10^{-3}$	5:1
DSPC	110–120	PNIPAM-Na + PNIPAM-Py	$4 \cdot 10^{-3}$	5:1
PA	not measured	PNIPAM-Na + PNIPAM-Py	5:1	
DMPC	110–130	PNIPAM-Na-Py	$4 \cdot 10^{-3}$	2.5:1
DSPC	110–120	PNIPAM-Na-Py	$4 \cdot 10^{-3}$	2.5:1

emission consisting of a contribution from Na* (310 to 400 nm) and a contribution from Py* excited by transfer of energy from Na* ($\lambda > 380$ nm).

The fluorescence spectra of a mixture of PNIPAM-Na and PNIPAM-Py in water and in a DMPC suspension are shown in Fig. 3 as an illustration of the NRET technique. In water the polymers form micellar structures [21,22], which bring the hydrophobic chromophores into close proximity. Hence in this situation the process of NRET is very efficient, as shown by the strong contribution from Py* (378–550 nm) to the emission of the polymer mixture ($\lambda_{exc} = 290$ nm). The weaker emission in the 300 to 350 nm range is attributed to Na* chromophores unable to undergo NRET with Py during their lifetime. As the polymer mixture is brought in contact with liposomes (Fig. 3, right) the Py* emission intensity undergoes a substantial decrease and the emission from Na* increases. It is this effect that will be exploited in this study. The extent of NRET will be reported here either in terms of the pyrene emission intensity (I_{Py}, peak height at 378 nm) or of the ratio, I_{Na}/I_{Py}, of the intensity (peak height) at 340 nm to the intensity at 378 nm of the emission spectra ($\lambda_{exc} = 290$ nm). In both calculations, the pyrene emission was corrected to account for the small contribution of the emission from directly excited pyrenes.

Results and Discussion

Experimental design

Experimental conditions were chosen to allow one to monitor the properties of the polymers during the adsorption process and the heating–cooling cycles. In all cases there was a large excess of liposomes in relation to polymer, to ensure the absence of free polymeric micelles. Hence measurements aimed at detecting intrinsic properties of liposomes, such as phase transition temperatures and size, yield values averaged over the entire population of coated and virgin vesicles.

One consideration in the choice of the phospholipids was related to the temperature (T_c) of their respective phase transitions. At temperatures below T_c a liposome membrane exists in the solid analogous, crystalline phase. The alkyl chains in an all-*trans* conformation are closely packed. Thus the lateral diffusion coefficient of the lipids is low (10^{-11} cm^2 s^{-1}) [31]. As the temperature is raised above T_c, the bilayer undergoes a transition into a fluid liquid crystalline phase state (L_a). Void volumes are created within the mem-

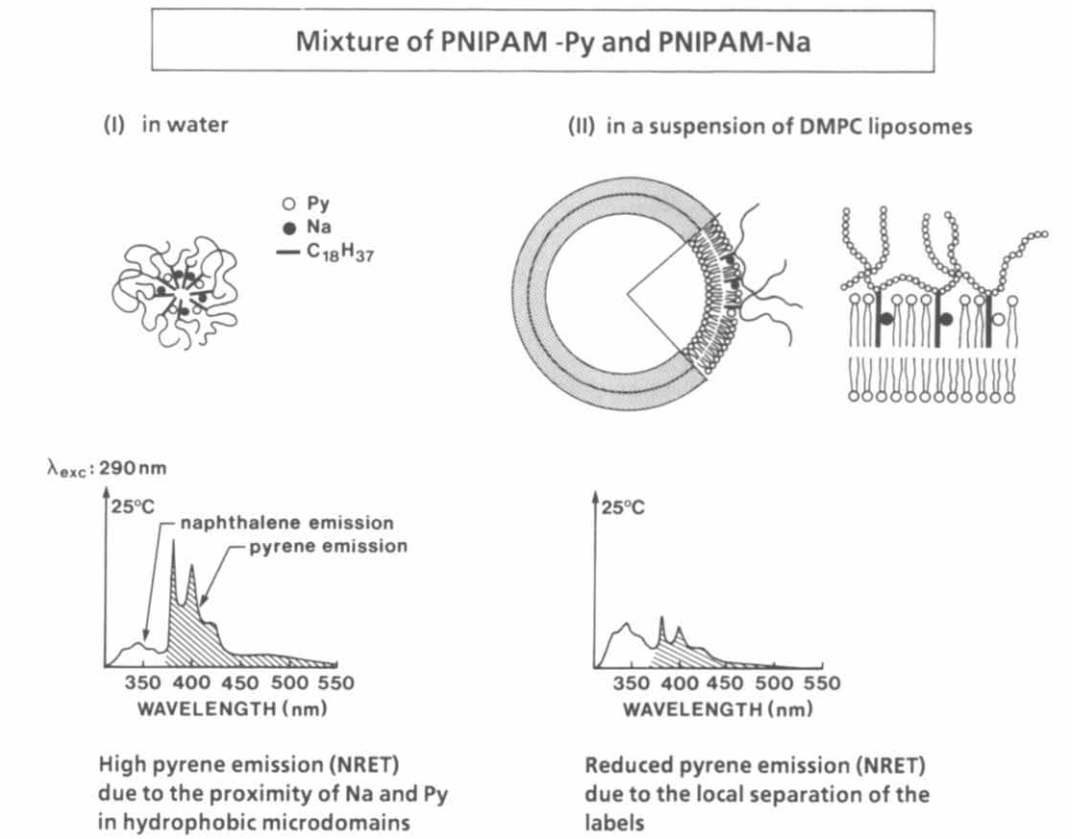

Fig. 3. Fluorescence spectra of a mixture of PNIPAM-Na and PNIPAM-Py in water (left) and with liposomes of DMPC (right) and schematic representations of the interactions; 25°C, $\lambda_{exc} = 290$ nm.

TABLE III

Non-radiative energy transfer efficiency between Na and Py, expressed in terms of the ratio I_{Na}/I_{Py}, for polymers in solution and in the presence of liposomes*

Sample		I_{Na}/I_{Py} (25°C)	I_{Na}/I_{Py} (35°C)	I_{Na}/I_{Py} (56°C)	I_{Na}/I_{Py} (25°C, cooling)
NIPAM-Na/PNIPAM-Py	water	0.20	0.09	–	0.20
NIPAM-Na/PNIPAM-Py	DMPC	0.67	0.20	–	0.64
NIPAM-Na/PNIPAM-Py	DSPC	0.90	0.88	0.25	0.39
NIPAM-Na/PNIPAM-Py	PA	0.57	0.28	–	0.36
PNIPAM-Na-Py	DMPC	0.32	0.09	–	0.30
PNIPAM-Na-Py	water	0.02	0.09	–	0.02
PNIPAM-Na-Py	DSPC	0.51	0.54	0.17	0.23

brane allowing enhanced rotational and lateral diffusion within the plane of the membrane. The lateral diffusion coefficient of the lipids increases by four orders of magnitude (10^{-7} cm^2 s^{-1}) [31]. The insertion of hydrophobic molecules within the lipid chains is a facile process when the membrane exists in the fluid phase [32]. Most of the experiments described here were performed with DMPC and DSPC liposomes, which have phase transitions at 23°C and 54°C, respectively [33]. This temperature range encompasses the LCST of HM-PNIPAM (32°C). DMPC and DSPC exhibit similar surface charge properties dictated by the zwitterionic choline head group. Some measurements were carried out with negatively charged PA liposomes to assess the influence of surface charge of the liposomes on their interactions with HM-PNIPAM.

In the next two sections we present first the spectral response of the labeled polymers as they interact with liposome suspensions at room temperature. Then we focus on the effects of changes in temperature, using information obtained from spectroscopic data and from DSC scans.

Liposome–polymer interactions at room temperature

In previous publications [20,25] are reported detailed spectroscopic studies of the room temperature adsorption process of HM-PNIPAM onto liposomes. They indicate that: (1) the polymeric micellar structures (average diameter: 40–50 nm) are disrupted irreversibly upon contact with liposomes; (2) the polymer chains reorganize on the surface of the liposomes in a process which results in an increase of the separation distance between the hydrophobic substituents. Complementary measurements described next were carried out to assess the importance of the phase state of the liposomes and of their surface charge.

Effect of the liposome phase state. The ratios I_{Na}/I_{Py} calculated for a 5:1 PNIPAM-Py/PNIPAM-Na mixture and for PNIPAM-Py-Na in suspensions of DMPC and DSPC liposomes at room temperature are listed in Table III. It is useful to remember that a *high* value of I_{Na}/I_{Py} corresponds to a situation of *low* efficiency of NRET. Values recorded for either the polymer mixture or the doubly labeled polymer in the presence of DSPC or DMPC liposomes show much less energy transfer compared to solutions of the polymers in water at 25°C. The decrease in energy transfer efficiency indicates that the hydrophobic substituents are pulled apart when the polymers interact with the liposomes. Nevertheless, it can be expected that the polymer chains remain entangled. A significantly smaller decrease was observed in the case of the terpolymer. This is an indication of the occurrence of intrapolymeric energy transfer when the polymer interacts with the liposomes. Intrapolymeric energy transfer takes place also in aqueous solutions of the polymer [21]. The insertion of these groups within an organized, but fluid, lipid bilayer as in the case of DMPC liposomes can be expected to be a facile process [32]. However, their insertion within a bilayer in its solid state may surprise. We attribute this phenomenon to a high density of defects in the crystalline DSPC membrane. These may arise from the strain imposed on the membrane by the high curvature of small unilamellar liposomes, or from a mismatch between the packing lattices of the hydrocarbon chains and of the polar head groups [34,35]. Defect sites provide favorable loci for the insertion of hydrophobic groups within the lipid layer. The unexpectedly high solubility of pyrene in solid lipid bilayers has been attributed by Galla and Sackman to similar effects [36].

Effect of the liposome surface charge. A solution of a 5:1 mixture of PNIPAM-Py and PNIPAM-Na (see Table II) was added at room temperature to PA and DMPC liposome suspensions. These two lipids were selected since they both exist in the fluid state at room temperature, but they differ in their surface charge [37] *. The relative intensity of the naphthyl and pyrenyl emissions were determined in both cases (Table III).

* Note that it has been reported that liposomes of amphoteric lipids often bear net negative charges because they usually contain a small amount of acidic lipid; see for example: Matsumura, H., Obata, C., Kawahara, K. and Furusawa, K.J. (1993) Colloid Interf. Sci. 156, 269–273. The zeta potentials of the liposomes employed in our measurements were not measured.

No significant difference was observed. This result emphasizes the paramount importance of hydrophobic forces in controlling the polymer–liposome interactions.

Temperature studies of the liposome–polymer interactions

The experiments described in this section address the following issues. Does the PNIPAM chain collapse still take place when HM-PNIPAM are anchored on a lipid membrane? Does the polymer remain anchored onto the vesicle membrane during its phase transition and during the phase transitions of the membranes? Are the phenomena truly reversible? These questions are addressed in the following sections which describe results obtained by microcalorimetry and fluorescence spectroscopy.

Microcalorimetry. Differential scanning calorimetry (DSC), a well established technique in the study of the phase transitions of liposomes [38], has proven extremely useful also in characterizing the interactions between liposomes and proteins [39] and the polymer-induced structural reorganizations of vesicle membranes [40]. It can be employed to determine the thermodynamic parameters associated with phase separations of polymer solutions [41]. The microcalorimetry scans described here were performed on systems as similar as possible to those observed by fluorimetry, namely systems with a large excess of liposomes relative to polymers. Thus the membrane transition signals will represent a response averaged over coated and virgin liposomes. Therefore, our discussion will focus on the polymer transition.

Microcalorimetric traces of the DMPC-polymer systems are shown in Fig. 4. The trace corresponding to the HM-PNIPAM-DMPC system exhibits an endotherm at 32.3°C attributed to the polymer LCST transition. The LCST endotherm is smaller and broader, compared to HM-PNIPAM in water (not shown). The main signal, 24°C, is assigned to the P/L transition of the lipid membrane. It is not affected by the presence of the polymer. The transition temperature and enthalpies associated with the two transitions were unchanged in repeated heating-cooling cycles. Control experiments were run also with a system consisting of DMPC and of poly(*N*-isopropylacrylamide). Signals corresponding to the polymer and to the liposome transition were observed while heating this sample as well. Nevertheless, the enthalpy associated to the transition of this unmodified polymer is larger and the peak width is narrower, than in the case of the HM-PNIPAM-DMPC system. The characteristics of the LCST endotherm are much closer to those of this polymer in water.

To our surprise the first heating scan performed with the DSPC-HM-PNIPAM system (Fig. 5) did not

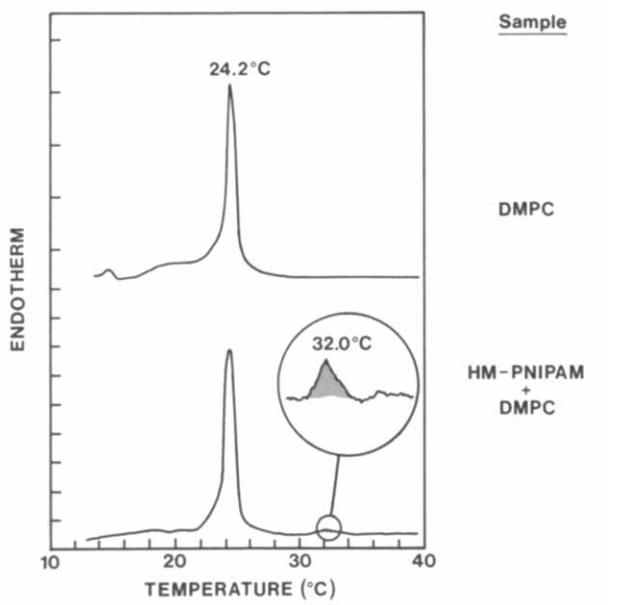

Fig. 4. Microcalorimetric endotherms for aqueous DMPC/polymer samples. Top: polymer-free liposomes; bottom: PNIPAM-Na and PNIPAM-Py mixture.

show a clear signal corresponding to the polymer LCST. However, this signal was recovered in traces obtained upon reheating the sample. The LCST transition of the homopolymer was not affected by the presence of the liposomes, as reported by Schild in a study of mixtures of PNIPAM and liposomes of dipalmitoyl L-α-phosphatidylcholine (DPPC), phosphatidic acid, or phosphatidyl glycerol [16] and of a mixture of DPPC liposomes and a hexadecyl substituted HM-PNIPAM [42]. In

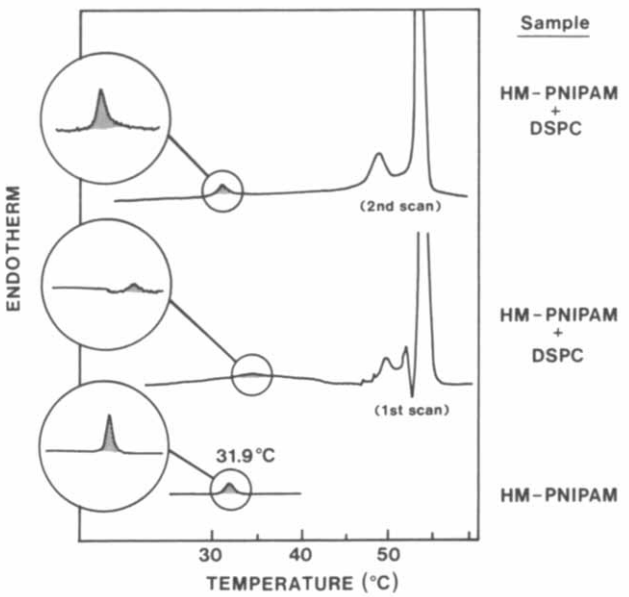

Fig. 5. Microcalorimetric endotherms for aqueous DSPC/polymer samples. Bottom: liposome-free mixture of PNIPAM-Na and PNIPAM-Py; middle and top: DSPC liposomes and the same polymer mixture, first and second heat scan.

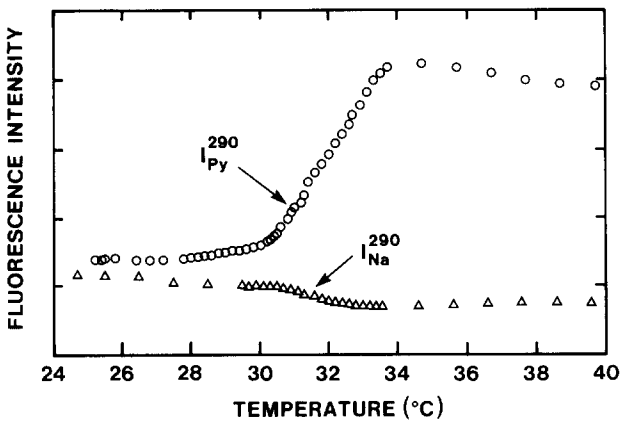

Fig. 6. Changes in pyrene fluorescence intensity as a function of temperature for a mixture of PNIPAM-Py and PNIPAM-Na on DSPC liposomes during a heating–cooling cycle; $\lambda_{exc} = 290$ nm.

summary, the DSC measurements confirm that even in the presence of liposomes the HM-PNIPAM copolymers undergo a phase transition at the temperature corresponding to their LCST. In the case of DMPC liposomes ($T_c <$ LCST) this transition is reversible and its thermodynamics are similar to those of the polymers in water. In the case of DSPC liposomes ($T_c >$ LCST) the situation is more complex. The polymer transition is undetectable in the first heating scan and it seems that the transition is not reversible. These clear differences between the two systems may be attributed to the phase state of the two lipid membranes.

Fluorescence experiments. Mixtures of several labeled polymers and DMPC, DSPC, or PA liposomes prepared and equilibrated at room temperature were subjected to heating–cooling cycles in temperature domains encompassing the phase transitions of the bilayers and of the polymers. Temperature-induced changes in energy transfer efficiency from Na* to Py in the various systems are reported in the following sections.

(i) The interactions of DMPC liposomes with a mixture of PNIPAM-Py and PNIPAM-Na. Fig. 6 displays the changes with temperature of the pyrene and naphthalene emission intensities in a suspension of DMPC liposomes incubated with a mixture of PNIPAM-Py and PNIPAM-Na (see Table II for the composition of the samples). Three trends can be observed:(1) There is a 5-fold increase of the pyrene emission intensity due to NRET, $[I_{Py}]^{290}$, as the sample temperature reaches 30.5°C; (2) Concomitant with the increase in $[I_{Py}]^{290}$ a modest decrease in I_{Na} is detected; (3) The pyrene emission intensity upon direct excitation $[I_{Py}]^{330}$ is not affected in this temperature range. Taken together these results indicate an increase in the efficiency of NRET between Na* and Py, hence the two chromophores are brought into closer proximity when the polymer-treated liposomes are warmed-up above the LCST. The value of the ratio I_{Na}/I_{Py} in the mixed polymer–liposome system is comparable to that recorded for micelles formed by the polymers in cold water in the absence of liposomes (see Table III). In control experiments we ascertained that the results were independent of the heating rate from 0.1°C min^{-1} to 1°C min^{-1}, that the naphthalene and pyrene emissions at 35°C remained constant with time (for at least 3 h), and that upon cooling and a 2-h equilibration at 25°C the original I_{Na}/I_{Py} values were almost recovered for systems cooled at the same rate as that used in the heating scan. Approximately 80% of the original I_{Na}/I_{Py} value was recovered immediately upon cooling. The I_{Na}/I_{Py} data (Table III) indicate a similarity in the behavior of the singly and doubly-labeled polymers on DMPC liposomes.

Given the well-known distance dependence of NRET efficiency [28], the increase in $[I_{Py}]^{290}$ reveals a shortening of the average separation distance between the chromophores anchored in the bilayer. The most important implications of this experiment are related to the temperature, 30.5°C, and the sharpness of the transition in the plot of the NRET efficiency versus temperature plot. The coincidence of this transition temperature with the LCST of the polymers permits us to conclude that it is the contraction of the polymer chain which forces the membrane anchored substituents into closer proximity by diffusion within the fluid lipidic bilayer. The relative magnitude of the ratios I_{Na}/I_{Py} of equiconcentrated PNIPAM-Na/PNIPAM-Py mixtures below and above the LCST in water and in DMPC suspensions (see Table III) argues against the expulsion of the anchored groups from the lipid bilayer into surrounding water. A much higher I_{Na}/I_{Py} value would be expected if a separated polymeric phase would form. Visual inspection of the warm specimens confirmed this hypothesis: the suspensions remained optically clear after several hours at 35°C. The spectroscopic data confirm the conclusion drawn from the DSC experiments with the DMPC/HM-PNIPAM system that a membrane in its fluid state, such as that of DMPC or PA liposomes at 25°C allows the polymers to undergo their characteristic thermoreversible conformational transition. Since we do not observe any significant differences in the spectroscopy of the mixture of singly-labeled polymers and of the doubly-labeled polymer, it must be assumed that the contraction of the polymer chain induces changes in the separation distance of anchor groups attached to a single polymer chain as well as to different polymers.

(ii) The interactions of DMPC liposomes with PNIPAM-Py. Experiments carried out with a dilabeled polymer will report on interpolymeric and intrapolymeric interactions. Aware of this added complication we limited our study to the DMPC/PNIPAM-Na-Py system. As in the case of the polymer mixture, we observed a decrease in the extent of NRET upon

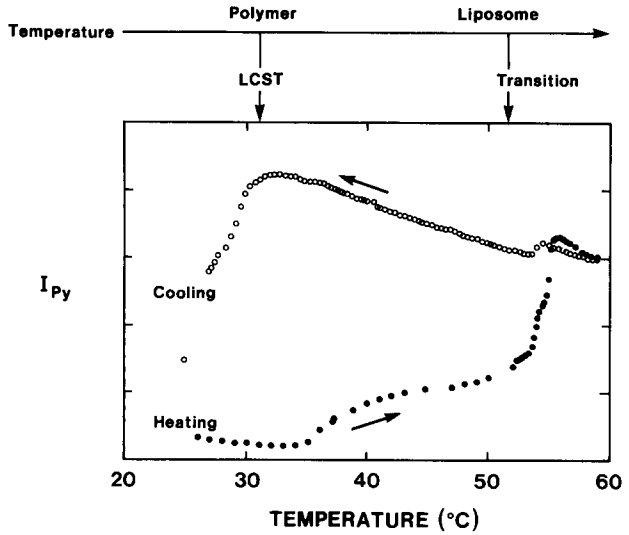

Fig. 7. Changes in pyrene fluorescence intensity as a function of temperature for a mixture of PNIPAM-Py and PNIPAM-Na on DSPC liposomes during a heating–cooling cycle; $\lambda_{exc} = 290$ nm.

anchoring of the polymer within the DMPC bilayer (room temperature adsorption) and a sharp increase in NRET efficiency at the LCST of the polymer. Cooling the system to room temperature and subsequent equilibration led to a recovery of the initial situation.

(iii) The interactions of DSPC liposomes with a mixture of PNIPAM-Na and PNIPAM-Py. Next we performed heating-cooling cycles on suspensions of DSPC liposomes treated with the same mixture of labeled polymers (see Table III). Here we scanned a larger temperature range (25–55°C) to monitor changes triggered by the collapse of the polymer backbone at 32°C and by the phase transition of the lipid bilayer (54°C). The results are visualized in terms of the changes in the pyrene emission intensity $[I_{Py}]^{290}$ with temperature (Fig. 7). The lower curve (heating scan) exhibits a sharp increase in $[I_{Py}]^{290}$ at approx. 53–55°C, the temperatures of the pre-transition and the rigid-fluid transition of a DSPC bilayer. No change in emission intensity was noticed at 32°C, neither as this temperature was first reached upon heating nor after incubation of a specimen at 32°C for up to 3 h. In the cooling scan (top curve, Fig. 7) starting from 56°C we observe first a small but reproducible decrease in $[I_{Py}]^{290}$ at approx. 54°C, then a region of slight increase in emission intensity, and as the system reaches 32°C, a sharp drop in emission intensity to a value larger by approx. 10% than the value recorded for the starting sample. This value remained constant over several hours in the cooled sample. The data collected in a second heating scan show an increase in NRET efficiency already at 32°C. Only minor changes were detected upon further heating up to 54°C. This disruption of the thermoreversibility of the spectroscopy of the polymer confirms the DSC observations discussed previously.

We propose the following interpretation of these fluorescence results. The room-temperature insertion of the hydrophobic anchor groups within the rigid DSPC bilayer is believed to occur mostly in defect sites. Contraction of the polymer backbone does not provide a strong enough driving force for the anchor groups to diffuse towards each other within a rigid DSPC bilayer. Thus the extent of NRET remains constant at the polymer LCST transition. As the membrane becomes fluid ($T > 54$°C), the barrier against diffusion of the hydrophobic groups within the bilayer is removed. Chromophores linked to collapsed polymer chains can move towards each other, thus the efficiency of NRET increases to a value comparable to that recorded in the mixed polymer-DMPC system at 35°C. Upon cooling below 54°C the membrane reverts to its rigid phase, the anchor groups are frozen in a fixed configuration, thus no changes in NRET take place. During the LCST-driven re-expansion of the polymer backbone, the organization of the external bilayer must undergo significant changes, as evidenced by the decrease in NRET efficiency, but the integrity of the liposomes is preserved, as vouched by electron micrographs and by QELS measurements. It seems unlikely that the spatial rearrangements indicated by the fluorescence data would result from a diffusion of the chromophores among the stearyl chains. Rather we suggest that the rehydration of the polymer backbone associated with the LCST phenomenon forces the chains to break intimate contact with the liposome surface, thereby pulling a fraction of the anchor groups out of the bilayer. That the polymer remains bound to the liposome is confirmed by the difference in I_{Na}/I_{Py} values recorded for the cooled DSPC liposome–polymer systems and a cooled solution of an equiconcentrated mixture of the two polymers (Table III). The reversibility of the fluorescence phenomena also provides strong support to the premise that the liposomes' integrity is preserved during the thermal cycles. This assumption was confirmed in a recent study of trans-bilayer lipid migration ('flip-flop') in surface-differentiated liposomes coated with HM-PNIPAM [43]. The experiments demonstrated that insertion of HM-PNIPAM into the membranes of surface-differentiated DDPC liposomes had little effect on the dynamics of the flip-flop reequilibration at temperatures below the polymer LCST. Above the LCST, but below the lipid bilayer phase transition temperature, the flip-flop rate was significantly enhanced. This catalytic effect was even more pronounced above T_c.

Conclusions

An artificial bilayer membrane consisting of hydrophobically-modified polymers anchored onto small unilamellar liposomes has been shown to respond re-

versibly to changes in temperature. The polymers not only strengthen the bilayer, but also trigger controlled diffusion motions within the lipid chains. Conversely the membrane phase state affects the organization of the polymers on the liposome surface. Major improvements must be achieved to reach a stage where this synthetic system truly models the numerous changes in shape of red blood cells. The response of the present system relies entirely on the coil-globule transition of a poly-(*N*-isopropylacrylamide) chain. The effects can be modulated via other stimuli, such as a pulse of light, pH jumps, or selective complexation, which may act in concert with the temperature-driven chain collapse.

Acknowledgements

Financial support for this work was provided in part (H.R. and J.S.) by the Bundesministerium für Forschung und Technologie (BMFT) and by the Deutches Forschungsgemeinshaft (DFG).

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