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In vitro characterization of a novel polymeric-based pH-sensitive liposome system

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Abstract

This study demonstrates rapid and pH-sensitive release of a highly water-soluble fluorescent aqueous content marker, pyranine, from egg phosphatidylcholine liposomes following incorporation of *N*-isopropylacrylamide (NIPA) copolymers in liposomal membranes. The pH-sensitivity of this system correlates with the precipitation of the copolymers at acidic pH. In vitro release can be significantly improved by increasing the percentage of anchor in the copolymer and thus favoring its binding to the liposomal bilayer. In the case of liposomes containing a poly(ethylene glycol)–phospholipid conjugate, the insertion of the pH-sensitive copolymer in the liposomal membrane appears to be sterically inhibited. Dye release from these formulations at acidic pH can still be achieved by varying the anchor molar ratio and/or molecular mass of the polymers or by including the latter during the liposome preparation procedure. Removal of unbound polymer results in decreased leakage only when the copolymer is inserted by incubation with preformed liposomes, but can be overcome by preparing liposomes in the presence of polymer. Aqueous content and lipid mixing assays suggest contents release can occur without membrane fusion. The results of this study indicate that the addition of pH-sensitive copolymers of NIPA represents promising strategy for improving liposomal drug delivery.

Keywords: pH-sensitive liposome; Poly(*n*-isopropylacrylamide); Sterically stabilized liposome; Fusion; In vitro release

Abbreviations: ACCN, 1,1′-azobis(cyclohexane carbonitrile); AIBN, 2,2′-azobisisobutyronitrile; Chol, cholesterol; DDAB, dimethyldioctadecylammonium bromide; DMPC, dimyristoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DPX, *p*-xylene-bis-pyrimidium bromide; DSPC, distearoyl phosphatidylcholine; EPC, egg phosphatidylcholine; Hepes, *N*-(-2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine); LCST, lower critical solution temperature; MAA, methylacrylic acid; MES, 2-*N*-(morpholino)ethanesulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine; NIPA, *N*-isopropylacrylamide; OA, oleic acid; ODA, octadecyl acrylate; PEAA, poly(2-ethyl-acrylic acid); PEG-DSPE, *N*-(ϖ-methoxypoly(oxyethylene)-α-oxycarbonyl)-1,2-distearoyl-*sn*-3-phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; SSL, sterically stabilized liposomes; Suc-DOPE, *N*-succinyl-dioleoyl phosphatidylethanolamine

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1. Introduction

Liposomes are being increasingly employed to specifically deliver chemotherapeutic agents, antisense oligonucleotides, and genes to various therapeutic targets [1–3]. While significant progress has been made in overcoming many of the original obstacles to effective delivery of these agents via liposomes, the ability to carefully regulate their bioavailability has, for the most part, eluded the field. One approach for controlling bioavailability has been to design pH-sensitive liposomes, which are stable at neutral pH, but become destabilized and release their contents upon acidification of the endosomal and lysosomal lumens [4–6]. Thus, drugs are delivered specifically and with greater efficiency to intracellular sites where they can elicit their pharmacological responses after endocytosis.

The most studied pH-sensitive liposomes are composed of mildly acidic amphiphiles and unsaturated phosphatidylethanolamines [7–9]. While effective in delivering small molecules, such as fluorophores and antisense oligonucleotides to the cytosol of cells in culture, they are severely hampered in their present form under in vivo conditions. Plasma or serum has been shown to both destabilize some formulations, by causing premature contents leakage, and reduce the pH-sensitivity of other formulations [10–13]. In addition, the presence of unsaturated lipid components, together with an overall negative surface charge results in physical characteristics typically associated with rapid removal of the liposomes from the circulation by macrophages of the mononuclear phagocytic system (MPS) [14,15]. The presence of poly(ethylene glycol)–distearoyl phosphatidylethanolamine (PEG-DSPE) in liposome formulations has been shown to sterically stabilize liposomes, resulting in significantly increased circulation lifetimes, even in the presence of unsaturated or anionic lipid components [16–18]. However, steric stabilization also prevents aggregation and dehydration of membrane surfaces, and thus reduces liposome fusion and contents release [19–21]. These results suggest significant improvements need to be made to this variety of pH-sensitive liposomes for in vivo drug delivery to become a reality.

Recent studies have demonstrated polymer coatings may not only play a protective role, but may participate in the drug release process by responding to external stimuli such as temperature [22–24] and pH [25,26]. Several recent studies have shown that liposomes coated with hydrophobically modified copolymers of *N*-isopropylacrylamide (NIPA) acquire thermoresponsive properties [22–24]. This temperature sensitivity triggers destabilization of the lipid bilayers of liposomes, and consequently the release of their contents in response to an increase in the external temperature. The homopolymer of NIPA is physically characterized by its lower critical solution temperature (LCST), which is approximately 32°C in water [27]. The polymer is soluble below its LCST, but undergoes a phase transition and collapses when heated above it. A novel polymeric-based pH sensitive liposome system has recently been described by Meyer et al. [25]. By randomly introducing a small proportion of titratable methacrylic acid (MAA) into NIPA copolymers, the LCST rises above 37°C and causes the polymer to be sensitive to pH. Insertion of the copolymer in the phospholipid bilayer resulted in a rapid and pH-sensitive release of a water-soluble fluorescent marker from the liposomes. Release was also shown to be dependent on the presence of a hydrophobic anchor (octadecylacrylate, ODA), demonstrating the importance of polymer binding in the destabilization process.

The present study is aimed at more carefully characterizing the interaction of the copolymer with liposomes and optimizing both the copolymer and liposome composition for maximum release and potential in vivo compatibility.

2. Materials and methods

2.1. Synthesis, molecular mass and phase transition determination of (non) pyrene-labeled copolymers

Polymers of different molecular masses were prepared by random radical copolymerization in distilled 1,4-dioxane of NIPA, MAA and ODA (Aldrich, Milwaukee, WI) with 2,2′-azobisisobutyronitrile (AIBN) (0.06 mol%) (Eastman Kodak, Rochester, NY) or 1,1′-azobis(cyclohexane carbonitrile) (Aldrich) (ACCN) (0.12 mol%), used as the initiator. Prior to polymerization, NIPA was purified by re-

crystallization from heptane, solubilized by addition of acetone, and then allowed to crystallize at 4°C. MAA was purified from its polymerization inhibitor using an inhibitor remover disposable column for hydroquinone and monomethylether hydroquinone (Aldrich). AIBN and ACCN were purified as previously described [25].

The solution containing the monomers and the initiator was degassed by bubbling N_2 for 15 min and then heated under stirring to 65°C for 15 h. Polymers were recovered by several reprecipitations from diethylether [25], after solubilization in tetrahydrofuran (THF). Finally, in order to completely remove unreacted monomers and residual solvents, polymers were dissolved in water, dialyzed (MWCO 6000–8000, Spectrum Laboratories, Laguna Hills, CA) for 3 days and subsequently freeze-dried. The percentage of MAA (pH-sensitive moiety) was fixed at 5 mol% and determined by acid–base titration [28]. The initial proportion of ODA (anchor) was varied from 2–4 mol% (Table 1) and was measured by ^{1}H-NMR analysis, by comparing the proton signals of NIPA and ODA, respectively [28]. Labeled copolymer was synthesized by introducing 1-pyreneacrylic acid (0.3 mol%) during polymerization. 1-Pyreneacrylic acid was obtained from 1-pyrenecarboxyaldehyde and malonic acid and crystallized from ethyl malonate [29,30]. Removal of free labeled monomer was verified by thin-layer chromatography (TLC) on aluminium sheets of silica gel 60 without fluorescent indicator, eluted with diethylether/THF 80:20. The emission spectra of the labeled copolymer and of 1-pyreneacrylic acid were determined to confirm the actual binding this latter to the polymer (Fig. 1A,B). The weight- and number-average molecular masses of polymers were determined by gel chromatography in THF, using polystyrene standards. The number of anchors per polymer chain (N_{ODA}) was calculated using the following equation [25]:

$$N_{ODA} = (M_{nPolymer} f_{ODA})/(M_{wNIPA} f_{NIPA} + M_{wMAA} f_{MAA} + M_{wODA} f_{ODA}) \quad (1)$$

where $M_{w\alpha}$ and f_α are the molecular masses and the molar fractions of comonomer α, respectively, and $M_{nPolymer}$ the number-average molecular mass of the copolymer.

The pH at which the polymer precipitates (25 μg/ml) was determined by 90° light scattering ($\lambda_{ex} = \lambda_{em} = 480$ nm) after incubation at 37°C for 5 min in MES-buffered saline (20 mM MES, 144 mM NaCl) of pH values ranging from 4.9 to 7.2 [25].

2.2. Liposome preparation and characterization

Unilamellar liposomes (20 mM total lipid) composed of either EPC, EPC/Chol (3:2 molar ratio), or EPC/Chol/PEG_{2000}-DSPE (3:2:0.3 molar ratio) (Avanti Polar Lipids, Alabaster, AL) were prepared by the reverse-phase evaporation procedure [31], followed by repeated extrusion through 0.1 μm pore-size membranes, at room temperature [32]. Particle size distributions were determined by dynamic laser light scattering (N4 Plus, Coulter Electronics, Hialeah, FL, USA). All liposome preparations had an average size of 140–160 nm, with a coefficient of variation of $<20\%$ and an unimodal size distribution.

2.3. Free polymer separation and determination of polymer binding to liposomes

Free polymer was removed by passage over a Sepharose 2B column (Pharmacia, Uppsala, Sweden, i.d. 1 cm, length 23 cm), equilibrated with Hepes buffer, pH 7.2 (20 mM Hepes, 144 mM NaCl) at 20°C. To determine the polymer binding to liposomal vesicles, pyrene-labeled copolymer was mixed with liposomes at different mass ratios, and either incubated overnight at 4°C or incorporated during the hydration step of liposome preparation. Phospholipid concentrations were determined using the Bartlett assay for total phosphate [33]. The amount of polymer bound to liposomes was determined by measuring the fluorescence ($\lambda_{ex} = 346$, $\lambda_{em} = 382$ nm) of fractions (1 ml) containing free pyrene-labeled polymer following elution of the liposome-copolymer complexes (100 μl). This was compared to a calculated amount of labeled free copolymer eluted under identical conditions.

2.4. In vitro release kinetics

Liposomes containing the highly water-soluble flu-

orophore, HPTS, and the collisional quencher, DPX, were prepared by including them in the lipid hydration buffer (20 mM Hepes, 35 mM HPTS, 50 mM DPX, pH 7.2) prior to liposome formation. Untrapped dye was removed by gel filtration using a Sephadex G-100 resin (Pharmacia). Liposome–polymer complexes were prepared as previously described (see Section 3.1). The polymer–liposome complexes (10 μl, 0.2 μg of total lipid) were added to the desired buffer (2 ml; 20 mM MES, 144 mM NaCl, pH 4.9–7.0) and the release of liposome contents was monitored using a fluorescence dequenching assay [34]. The extent of contents release was calculated from the fluorescence of HPTS (λ_{ex} = 413 nm, λ_{em} = 512 nm) following a 3-min incubation in buffers at varying pH and 37°C, and made relative to the same measurement following sample lysis in 0.1% (w/v) Triton X-100 to give complete release of encapsulated HPTS and DPX.

2.5. Lipid and aqueous content fusion assays

For lipid mixing experiments, liposomes composed of EPC/Chol/N-NBD-PE/N-Rh-PE (3:2:0.05:0.05 molar ratio) were combined with unlabeled liposomes composed of EPC/Chol (3:2 molar ratio) in a 1:10 molar ratio. All liposomes were incubated overnight at 4°C, at a copolymer/lipid mass ratio of 0.3. The mixed liposomes were injected into pH 7.2 and 4.5 buffers, and fluorescence (λ_{ex} = 470 nm, λ_{em} = 520 nm) was measured continuously over time (4.5 min). A 100% control liposome formulation of EPC/Chol/N-NBD-PE/N-Rh-PE (3:2:0.005:0.005 molar ratio) was prepared and used as the standard for 100% lipid mixing. Fluorescence resonance energy transfer experiments with N-NBD-PE and N-Rh-PE demonstrate lipid mixing, which is necessary for membrane fusion, by measuring the increase in NBD fluorescence upon dilution of the lipid probes in unlabeled membranes and caused by the reduced amount of fluorescence-energy transfer from NBD to rhodamine [35].

Mixing of vesicle contents was measured by a modified method of Ellens et al. [36], encapsulating 17.5 mM HPTS in one population of EPC liposomes incubated with copolymer and 96 mM DPX in the other. Mixing of aqueous contents leads to a decrease in HPTS fluorescence as it becomes quenched by DPX. These assays have been performed at various pH ranging from 4.9 to 7.4. The fluorescence of HPTS was continuously measured at the pH-independent isosbestic point (λ_{ex} = 413 nm, λ_{em} = 512 nm).

3. Results

3.1. Binding studies

The binding of poly(NIPA-co-MAA-co-ODA) to EPC liposomes was studied with a pyrene-labeled copolymer containing approximately 2 mol% ODA (Table 1). The content in ODA was twice that used in our previous study [25] since it was expected that the binding stability, as well as the extent of pH-mediated release, could be improved by increasing the amount of hydrophobic anchor in the copolymer. The labeled copolymer was synthesized by adding 1-pyreneacrylic acid during the copolymerization reaction. Once grafted to the polymer, the probe shows

Table 1
Characterization of (NIPA/MAA/ODA) copolymers

Initiator (mol%)	Initial ODA content (mol%)	Final ODA content (mol%)	M_w	M_n	M_w/M_n	N_{ODA}
AIBN (0.06)	1[a]	n.a.	9756	4161	2.34	0.4[b]
AIBN (0.06)	2 (labeled)	2.5	31 066	11 370	2.73	2.5
AIBN (0.06)	2	1.6	44 633	19 189	2.24	2.7
ACCN (0.12)	2	2.9	16 779	5141	3.36	1.3
AIBN (0.06)	4	6.2	45 255	20 183	2.33	10.4

n.a., not available.
[a]From [25], different purification procedures.
[b]Calculated from using the initial ODA content.

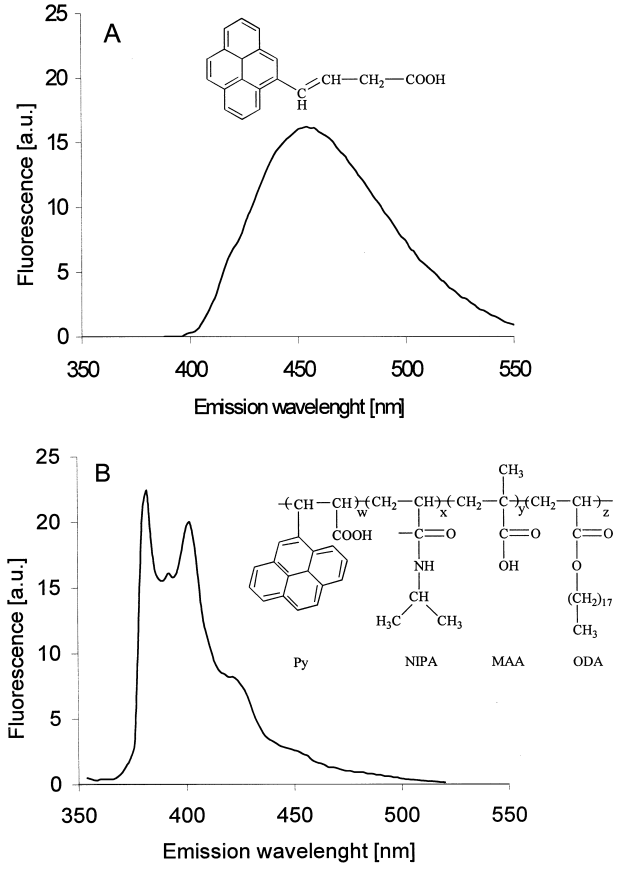

Fig. 1. Emission wavelength spectrum of (A) 1-pyreneacrylic acid (λ_{ex} = 365 nm) and of (B) pyrene-labeled copolymer (NIPA/MAA/ODA 93:5:2 mol%, M_w = 31 066) (λ_{ex} = 346 nm).

the fluorescence emission spectrum of monomeric pyrene when excited at 346 nm (Fig. 1B), with a slight bathochromic shift. According to the emission spectrum, the pyrene-labeled polymer can be treated as a single fluorophore, without regard to the formation of excimers commonly seen with higher concentrations of free pyrene [30,37]. In addition, as illustrated in Fig. 2, the presence of this hydrophobic label (0.3 mol%) only slightly modifies the phase transition of the copolymer, which occurs between pH 5.1 and 5.7. This is likely due to the low amount of pyrene present in the label introduced into the polymer, which limits its hydrophobic contribution to the phase transition [37].

Bound and free copolymer can be effectively separated on a Sepharose 2B column (Fig. 2). The liposomes eluted in the void volume (5 ml) whereas the free copolymer appeared mainly in the 15-ml fraction. The coelution of labeled copolymer and liposomes indicates that the copolymer remains anchored to liposomes as it moves through the column. Because the interaction of pyrene with the liposome bilayer modifies its fluorescence spectrum [37], the percentage of bound copolymer was calculated by comparing the amount of free copolymer both before (Fig. 2A) and after (Fig. 2B) incubation with liposomes. It can be seen from Fig. 3 (inset) that increasing concentrations of copolymer progressively saturate the EPC liposome surface. Maximum binding occurs at an initial copolymer/lipid mass ratio of 0.12 (binding efficiency 30%). The data were fitted to a Langmuir isotherm (Fig. 3). The calculated affinity constant and maximum binding capacity were 15 l/g and 0.038 (g copolymer/g lipid), respectively. At this ratio, the number of molecules of polymer per EPC liposome was estimated to be 460, assuming that 150-nm liposomes contain 182 000 lipid molecules/vesicle [38]. Comparatively, Ringsdorf et al. [39] found that each 100-nm dimyristoyl phosphatidylcholine (DMPC) liposome can accommodate 35 molecules of poly(NIPA-co-ODA) (M_v = 360 000, N_{ODA} = 16). This number is similar to the value calculated here if the difference in molecular mass between the two polymers is taken into account. Accordingly, an initial mass ratio not less than 0.12 was used for all release studies to ensure surface satura-

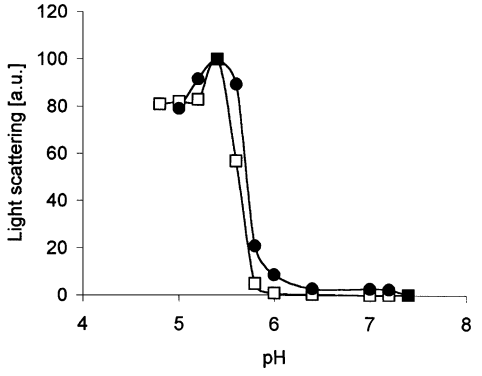

Fig. 2. pH-Dependent phase transition of both pyrene-labeled (solid) and non-labeled (empty) copolymers (NIPA/MAA/ODA 93:5:2 mol%, M_w = 31 066 and 44 633, respectively). Copolymers were incubated at 37°C for 5 min in MES buffers of varying pH (4.9–7.2). Polymer phase transition was measured as an increase in light scattering at 480 nm.

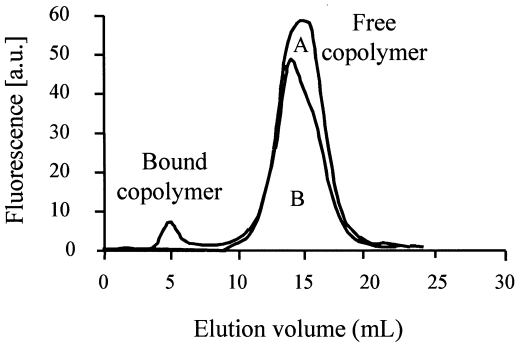

Fig. 3. Separation of bound and free copolymer by size-exclusion chromatography on a Sepharose 2B column, equilibrated with Hepes buffer (pH 7.2) at 20°C. Pyrene-labeled copolymers (NIPA/MAA/ODA 93:5:2 mol%, $M_w = 31\,066$) were eluted either before (A) or after (B) incubation with EPC liposomes for 16 h at 4°C.

tion in all preparations. The release studies were performed at 0.3 and 0.6 copolymer/lipid mass ratio. Since no significant differences were observed at these two concentrations, only the experiments performed at the 0.3 ratio are presented in Section 3.2.

The binding of the same copolymer ($\approx$2 mol% ODA) to liposomes of differing lipid composition was investigated, after overnight incubation (16 h) at 4°C (Fig. 4). The addition of Chol (40 mol%) to EPC liposomes had no influence on copolymer binding, with equivalent binding ratios, in both formulations. In contrast, the presence of PEG-DSPE (6 mol%) on the liposome surface strongly inhibited copolymer fixation, with less than 1% of the copolymer being liposome-associated. To overcome this reduced fixation efficacy, the copolymer was dissolved in the hydration buffer and added to the lipid mixture prior to liposome formation. This way, the copolymer is incorporated during the vesicle formation and has access to both sides of the bilayer, rather than just one in the case where copolymer is added to preformed liposomes. The result is a significant increase in binding (0.077 g copolymer/g lipid) of copolymer (Fig. 4), even in presence of PEG-DSPE (6 mol%), suggesting this procedure allows a more efficient incorporation of the polymer into the liposomal formulation.

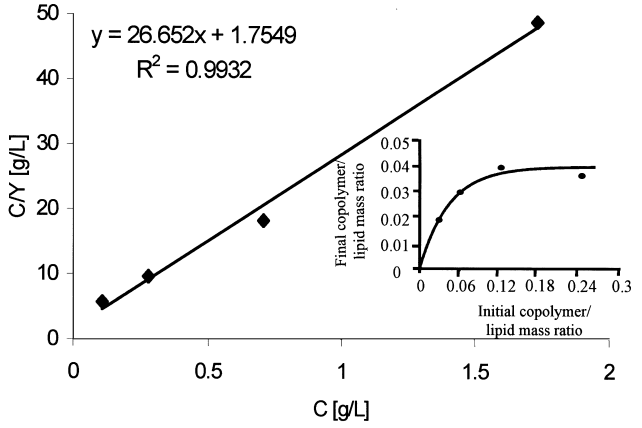

Fig. 4. Adsorption of labeled copolymer (NIPA/MAA/ODA 93:5:2 mol%, $M_w = 31\,066$) on EPC liposomes according to a Langmuir isotherm. C, equilibrium concentration of copolymer (g/l); Y, amount (g) of copolymer adsorbed per gram of lipids. Inset: binding of copolymer to EPC liposomes, as a function of copolymer/lipid mass ratio, after 16 h incubation at 4°C, after size-exclusion chromatography on a Sepharose 2B column, equilibrated with Hepes buffer (pH 7.2) at 20°C.

3.2. In vitro release kinetics

In a previous study conducted with EPC liposomes and SSL [25], we showed that a low molecular mass pH-sensitive copolymer of NIPA (M_w 9756) bearing 1 mol% ODA could trigger the release of approximately 25% and 15% of liposomal contents at pH 4.9 and 5.5, respectively, without removing free polymer. It can be seen from Fig. 6 that when the molecular mass (M_w 44 633) and the ODA content (1.6 mol%) are increased, a substantial improvement in maximal release from EPC liposomes is obtained; indeed, release of up to 42% at pH 4.9 and 16% at pH 5.5, after free polymer removal. In the presence of free

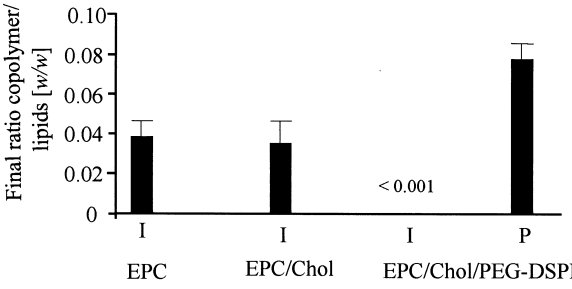

Fig. 5. Effect of lipid composition on copolymer binding to liposomes. The pyrene-labeled copolymer (NIPA/MAA/ODA 93:5:2 mol%, $M_w = 31\,066$) was either incubated 16 h at 4°C with the liposomes (I) or incorporated during liposome preparation (P) at an initial copolymer/lipid mass ratio of 0.12. Mean ± S.D. ($n = 3$).

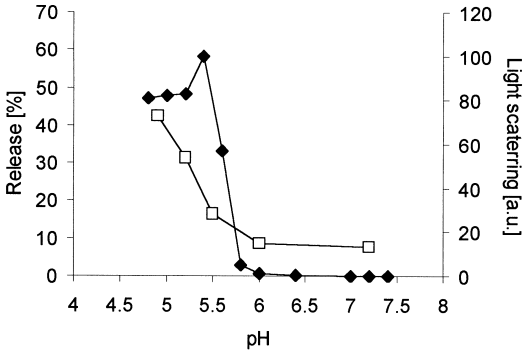

Fig. 6. A comparison of the phase transition of the copolymer (NIPA/MAA/ODA 93:5:2 mol%, $M_w = 44\,633$) (□) and the release of HPTS from copolymer-bound EPC liposomes (♦), as a function of pH, after 16 h incubation at 4°C at a copolymer/lipid mass ratio of 0.3. All measurements were taken after free polymer removal on a Sepharose 2B column. HPTS release was measured as an increase in HPTS fluorescence ($\lambda_{ex} = 413$ nm, $\lambda_{em} = 512$ nm) following release from the liposomes. 100% contents release was obtained by adding Triton X-100 and the data are expressed as percentage of total HPTS released. Polymer phase transition was measured as an increase in light scattering at 480 nm.

copolymer the release was 72% and 23% at pH 4.9 and 5.5 (not shown). This suggests either a slight desorption of the copolymer during separation, or the implication of the unbound polymer in the destabilization process when not removed. Although less likely, this latter hypothesis cannot be excluded since polyelectrolytes such as poly(2-ethyl-acrylic acid) (PEAA) in solution have been shown to bind to liposomes upon a decrease in pH and induce the formation of pores in the lipid bilayer [26,40]. Accordingly, unless otherwise specified, all subsequent release studies were performed after removal of free copolymer on a Sepharose 2B column.

According to Fig. 6, the coil–globule transition (pH 5.1–5.7) of the polymer can be correlated to the pH-triggered release, suggesting that anchoring of the copolymer to liposomes does not modify its phase-transition pH.

Fig. 7 compares the release of HPTS at pH 4.9 and 7.2 from liposomes differing in lipid composition after removal of free polymer. Despite the similar binding of the two formulations (Fig. 5), a significant increase of contents release (82%) was observed after inclusion of Chol (40 mol%) (Fig. 7) at pH 4.9. However, some aggregation was observed with EPC/Chol liposomes coated with the polymer, which cannot be explained at the present time. In contrast, the presence of PEG-DSPE in the liposome formulation resulted in minimal release of the tracer (Fig. 7). This can be correlated to the low binding of the copoly-

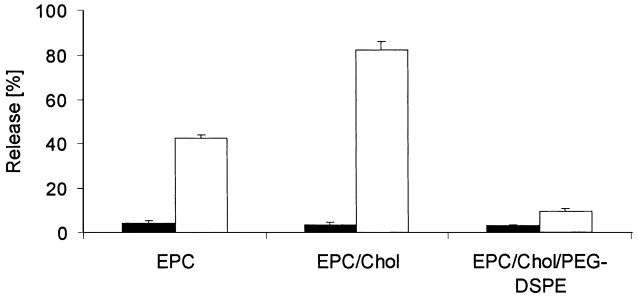

Fig. 7. Effect of pH and lipid composition on HPTS release from copolymer-bound liposomes, after free polymer removal on a Sepharose 2B column, except for the formulation containing PEG-DSPE. The copolymer (NIPA/MAA/ODA 93:5:2 mol%, $M_w = 44\,633$) was incubated overnight at 4°C with liposomes at an initial copolymer/lipid mass ratio of 0.3. Copolymer-bound liposomes were incubated for 3 min at 37°C at either pH 7.2 (solid) or 4.9 (empty). HPTS release was measured as an increase in HPTS fluorescence ($\lambda_{ex} = 413$ nm, $\lambda_{em} = 512$ nm) following release from the liposomes. 100% contents release was obtained by adding Triton X-100 and the data are expressed as percentage of total HPTS released. Mean ± S.D. ($n = 3$).

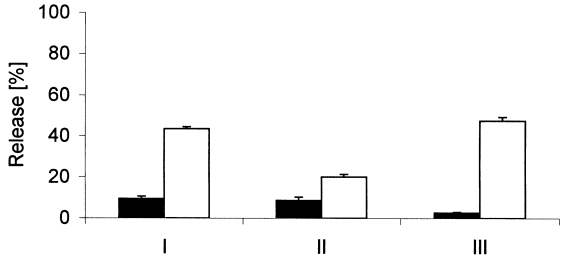

Fig. 8. Effect of polymer composition and molecular weight on HPTS release from EPC/Chol/PEG-DSPE liposomes. Liposome–copolymer system was prepared by incubation 16 h at 4°C with copolymer **I** (2% ODA, $M_w = 16\,778$), copolymer **II** (4% ODA, $M_w = 45\,255$), or after incorporation of copolymer **III** (2% ODA, $M_w = 44\,633$) during the liposome preparation, at an initial copolymer/lipid mass ratio of 0.3. All measurements were taken after free polymer removal on a Sepharose 2B column. Copolymer bound liposomes were incubated for 3 min at 37°C at either pH 7.2 (solid) or 4.9 (empty). HPTS release was measured as an increase in HPTS fluorescence ($\lambda_{ex} = 413$ nm, $\lambda_{em} = 512$ nm) following release from the liposomes. 100% contents release was obtained by adding Triton X-100 and the data are expressed as percentage of total HPTS released. Mean ± S.D. ($n = 3$).

mer to SSL (Fig. 5). However, the release was even lower than that obtained by Meyer et al. [25] after incubation of the liposomes with a copolymer bearing only 1 mol% ODA. We conjectured that the difference seen in release resulted from the difference between the molecular mass of the two copolymers. In order to prove this hypothesis, a pH-sensitive copolymer containing approximately 2 mol% ODA and with a M_w of 16 779 was synthesized (Table 1). This resulted in a substantial increase in pH-triggered release (40%) (Fig. 8), which indirectly confirmed the importance of the polymer weight in binding to SSL. Moreover, we tested whether the steric repulsion provided by the PEG coating could be overcome by further increasing the ODA content to 6.2 mol%, while keeping a relatively high M_w (Table 1). As shown in Fig. 8, some contents leakage (20%) could be achieved with this copolymer. Another way of circumventing the steric repulsion of PEG was to incorporate the copolymer containing 1.6 mol% ODA (M_w 44 633) during the hydration step of the liposome preparation procedure. In this case, a 40% release was observed (Fig. 8). With this method, no difference was observed in the release capacity of SSL, before and after free polymer removal, which can be attributed to the better fixation of the copolymer with this incorporation method (data not shown).

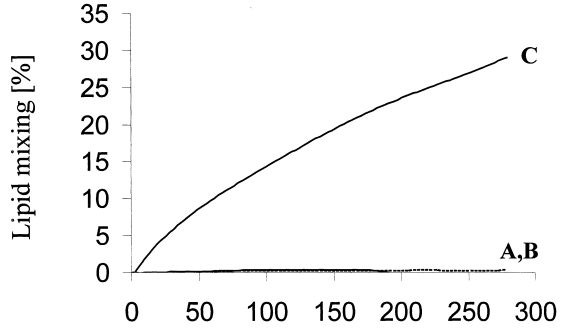

Fig. 9. Mixing of membrane lipid components in copolymer bound or suc-DOPE/DOPE (1:9) liposomes. EPC/Chol (3:2) liposomes were incubated overnight at 4°C with copolymer (NIPA/MAA/ODA 93:5:2 mol%, M_w = 44 633). Labeled liposomes (1% N-NBD-PE, 1% N-Rh-PE) were incubated at pH 4.5 (trace A) with unlabeled liposomes in a 1:9 ratio. An identical experiment was carried out with suc-DOPE/DOPE (1:9) liposomes at pH 7.2 (trace B) or pH 4.5 (trace C) in the presence of 1 mM $CaCl_2$. The mixed liposomes were injected into pH 7.2 and 4.5 buffers and fluorescence (λ_{ex} = 470 nm, λ_{em} = 520 nm) was measured continuously over time (4.5 min). A 100% control liposome formulation of EPC/Chol/N-NBD-PE/N-Rh-PE (3:2:0.005:0.005 molar ratio) was prepared and used as the standard for 100% lipid mixing.

3.3. Fusion assays

To test whether pH-induced liposome destabilization was due in part to membrane fusion, a lipid mixing assay was utilized as described by Struck et al. [35]. Mixing of membrane lipids between labeled and unlabeled liposomes results in dilution of the fluorescent probes and a decrease in fluorescence energy transfer between N-NBD-PE and N-Rh-PE. If copolymer-bound liposomes are similar to other membranes that undergo fusion, we would expect to see an increase in the mixing of the lipid components. This was compared to another liposome formulation (suc-DOPE/DOPE, 1:9) previously shown to fuse under conditions of low pH in the presence of Ca^{2+} (1 mM, Fig. 9). Suc-DOPE/DOPE liposomes were shown to fuse and release water soluble contents markers (HPTS and calcein) in response to decreases in pH and increases in Ca^{2+} concentrations (Drummond and Daleke, unpublished observations). A modified version of this experiment shows rapid lipid mixing as the pH is lowered from pH 7.4 to 4.5, indicating a pH-dependent fusion of these liposomes. Alternatively, the liposomes used for incorporation of the copolymer contain high concentrations of EPC and Chol, a combination usually refractory towards fusion. However, as demonstrated above, significant drug leakage did occur at low pH in the presence of bound copolymer. Lipid mixing experiments with these liposomes, showed no lipid mixing at pH 4.5 (Fig. 9). Lack of mixing of aqueous content confirmed the absence of fusion (data not shown). These results suggest a transient destabilization being responsible for contents leakage, rather than membrane fusion, as shown for other pH-sensitive liposome formulations.

4. Discussion

This study has shown an effective binding of the copolymer on liposomes, which can be fitted to a Langmuir isotherm. The release under weakly acidic conditions can be correlated to the amount of bound

copolymer and to the number of anchors per polymer. Thus, when this number increases, the binding efficacy improves, as well as its ability to destabilize the lipid bilayer upon acidification. Previous studies have demonstrated that hydrophobically modified NIPA copolymers could efficiently bind liposomes whether the latter were in the liquid-crystalline or gel phase [22,24].

In the case of SSL, the molecular mass of the copolymer is relevant, since the presence of PEG-DSPE chains could sterically inhibit the insertion of the anchor in the liposomal membrane. Indeed, it is well known that the steric interference of surface-associated PEG can significantly affect the ability of macromolecules such as plasma opsonins to bind to the membrane surface [41–43]. The incorporation of the copolymer during liposome preparation could trigger a significant binding of this latter to the liposomal bilayer. However, this method may not be compatible with other liposomal technologies such as drug-loading using pH [44], ammonium sulfate gradients [45], or the hydration of high phase transition lipid compositions, because in both cases the copolymer could precipitate. When combined with these preparation procedures it would certainly be preferable to bind the copolymer following lipid hydration and drug loading to prevent premature destabilization.

In our study, the coil–globule transition pH of the polymer appears between pH 5.1 and 5.7 and is not modified after its anchoring to liposomes. This has been previously evidenced by Ringsdorf et al. [46] for the LCST of hydrophobically modified poly(NIPA) anchored to DMPC or distearoyl phosphatidylcholine (DSPC) liposomes using differential scanning calorimetry. However, a recent study by Winnik et al. [47] showed that in presence of dimethyldioctadecylammonium bromide (DDAB) the LCST of modified poly(NIPA) could be decreased, depending on lipid concentration. This phenomenon was attributed to a gradual neutralization of the carboxylic acid groups of the polymer by the cationic groups on the liposome surface. This charge neutralization mechanism competes with the pH-sensitive protonation of the polymer and thus controls the cloud point.

Once anchored to liposomes, the copolymer triggers significant contents release below pH 5.5, which corresponds to endosomal pH [48]. Comparatively, pH-sensitive liposomes of DOPE/CHEMS show a leakage of an aqueous marker below pH 5.5 [9,36], whereas DOPE/OA liposomes are more sensitive and release their contents below pH 6.5 [8].

The significant increase of contents release after incorporating 40 mol% of Chol, compared to plain EPC formulations, cannot be explained at the present time. In fact, Liu and Huang [49] showed that the addition of increasing concentrations of Chol to pH-sensitive liposomes containing DOPE and OA triggered a significant decrease in calcein release in vitro. In their study, the percentage of leakage content decreased linearly from 85% to 40% at pH 5, when the concentration of Chol increased from 10 mol% to 40 mol%. Similarly, the presence of Chol reduces the efficiency of the peptide GALA [50] and PEAA [51] to induce leakage at acidic pH. While the addition of Chol has been reported to decrease the membrane permeability of phospholipid bilayers by effecting tighter-packing lipids, it can also promote membrane fusion by inducing the formation of non-bilayer lipid phases [52].

In contrast, the addition of PEG-DSPE (6 mol%) prevents the copolymer binding and thus triggered a minimal pH-sensitive release. Several approaches have been examined to improve contents release of PEG containing formulations. First, it has been shown that a decrease in the copolymer average molecular mass favors its insertion trough the PEG steric barrier. It has been described in depth that PEG prevents the adsorption of macromolecules to the liposome surface. To reach the liposome surface macromolecules have to penetrate the conformational 'cloud' formed by the PEG [41]. For this reason, it can be anticipated that lower molecular mass polymers may more readily reach the liposome surface.

A relative increase in the relative percentage of ODA also improved the release efficiency of the pegylated system. This can be attributed to the ability of the additional anchors to improve binding and to favor the collapsed copolymer within the liposome membrane.

The addition of copolymer during the liposome preparation also markedly improves the release efficiency compared to simple incubation with preformed liposomes. In this case, the polymer is located in both monolayers and could further

destabilize the liposomal membrane. This could be explained by a possible proton permeation triggered by the acidic destabilization of the copolymer located on the liposomal surface [53]. A similar improvement has been reported with a lipopeptide equally distributed into both membrane leaflets of EPC/Chol liposomes [54]. This preparation procedure would also encapsulate a certain amount free copolymer. The actual influence of the encapsulated polymer has not been investigated and is likely not relevant. However, the 40% release obtained with this method proves that at least part of the polymer is present at the surface of the liposomal bilayer and triggers the acidic destabilization.

This study showed that optimized formulations containing PEG-DSPE (6 mol%) have copolymer on their surface and thus maintain their pH-sensitivity. This is a considerable advantage in cancer therapy, considering the significant increase in circulation lifetime and accumulation in tumors provided by its inclusion which has been documented in several studies [17,18,55]. In fact, it has been described that the presence of the hydrophilic polymer on the liposomal surface prevents aggregation and membrane fusion, which is the main mechanism for destabilization of DOPE pH-sensitive liposomes, and it is generally recognized that these stabilized formulations lose their efficacy in the presence of PEG [20,21,56]. Recently, it was demonstrated that PEAA (M_w 30 000) in solution (1 mg/ml) was able to destabilize liposomes bearing PEG-DSPE under acidic conditions [51]. It was suggested that the cross-structural area of PEAA was small enough to allow the diffusion through the PEG layer and cause membrane disruption.

Lipid and aqueous content fusion assays have shown that fusion is not involved in the leakage of contents at acidic pH. However, since fusion may depend on polymer concentration and structure, and liposome composition, further studies are required to determine whether fusion can occur under different conditions. For instance, Chen et al. [57] showed that PEAA conjugated to EPC/Chol liposomes promoted vesicle fusion at pH 5. It was hypothesized that at this pH, the insertion of hydrophobic segments of PEAA into the membranes of neighboring liposomes lead to close vesicle–vesicle contact, facilitated local dehydration at the contact site and caused defects in the packing of the membrane lipids and eventually fusion. Hayashi et al. [23] showed that hydrophobically modified poly(NIPA) could trigger the fusion of dipalmitoyl phosphatidylcholine and DSPC liposomes at a temperature corresponding to the gel–liquid crystalline transition of the lipid membranes. Since our experiments were performed at a temperature exceeding the phase transition of EPC, this phenomenon could not be evidenced.

The mechanism involved in the acidic destabilization of the vesicles by pH-sensitive copolymers of NIPA remains to be elucidated. Transient reversible destabilization of the liposomal membrane structural integrity is a potential mechanism. This hypothesis is suggested because during the polymer coil to globule transition, the polymer remains anchored in the bilayer and the liposomes is not destroyed [37,46]. Transient membrane perturbation with or without membrane fusion may explain why 100% release was never achieved, although partial leakage could also be due to the fact that some populations of liposomes do not carry sufficient amounts of the polymer on their membranes to induce a complete release of their contents [22]. Moreover, it has been proposed that above the LCST, poly(NIPA) bearing C_{18} alkyl chains become more hydrophobic and contract, creating 'point defects' or spaces in the extraliposomal leaflet and stimulate lipid flip-flop [58]. By analogy, the same phenomenon may occur with pH-sensitive copolymers of NIPA at acidic pH. Also, it has been suggested that above the LCST, hydrophobically modified poly(NIPA) gives rise to liposome budding [59]. However, the formation of buds has been observed for giant liposomes and is unlikely for 150 nm liposomes.

Several points need to be verified, such as what effect the presence of the copolymer will have on liposomes plasma stability and circulation time. These experiments are presently being completed in our laboratory. Also, in vitro and in vivo studies are currently under way to determine the potential of this novel copolymer–liposome system for the intracellular delivery of drugs and to assess the toxicity of NIPA copolymers. We have recently shown that copolymers of NIPA were not toxic in vitro towards EMT-6 mouse mammary tumor cells at a concentration of 0.22 mg/ml [28] and do not trigger either local

or systemic inflammatory reactions at a concentration of 5 mg/ml, following subcutaneous injection to rats [60].

Because of their great versatility in either being compatible with a variety of lipid compositions or being responsive to both pH and temperature, the addition of pH-sensitive copolymers of NIPA represent a potential strategy for improving liposomal drug delivery.

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