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Polymer based pH-sensitive carriers as a means to improve the cytoplasmic delivery of drugs☆

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Abstract

pH-sensitive niosomal and liposomal formulations bearing alkylated *N*-isopropylacrylamide (NIPAM) copolymers were characterized with regard to vesicle–polymer interaction, pH-responsiveness and stability in human serum. The interactions between the pH-sensitive NIPAM copolymer and the vesicles were studied by spectrofluorimetry, using covalently-attached pyrene as a probe. In contrast to liposomes, where complexation of copolymer to the lipid bilayer is essentially mediated by hydrophobic interactions, the binding between niosomes and PNIPAM was mainly driven by hydrogen bonding. Both formulations were found to rapidly release their contents under mildly acidic conditions. However, the niosomes lost their pH-sensitivity after incubation in serum, whereas liposomes maintained their ability to respond to pH only when complexed with a copolymer containing a high proportion of hydrophobic anchor. The ability of pH-sensitive liposome/polymer complexes to enhance the cytotoxicity of cytosine arabinofuranoside (ara-C) was evaluated in vitro using macrophage-like J774 cells. Ara-C encapsulated in pH-sensitive liposomes exhibited a higher cytotoxicity than the control formulation. This study showed that both niosomes and liposomes can be rendered pH-sensitive by anchoring a randomly-alkylated NIPAM copolymer to their surface. The interactions that take place between the polymer and the vesicles strongly depend on the vesicle nature. pH-sensitive PNIPAM-based liposomes can improve the in vitro efficiency of ara-C. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: pH-sensitivity; Liposomes; Niosomes; Poly(*N*-isopropylacrylamide)

1. Introduction

Over the last decade, the design and synthesis of molecules that can influence the intracellular trafficking of drugs have increased, especially with the advent of gene therapy. While significant progress has been made in passive tis-

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sue targeting using colloidal carriers, which exploit the so-called 'enhanced permeation and retention effect (for a review, see Maeda et al., 2000), delivering drugs to specific cellular compartments remains a major challenge. As the pH of endosomes is lower than that of extracellular fluids, this feature has been exploited in the design of systems that facilitate the delivery of active compounds to the cytoplasm via a pH-dependent membrane-disruptive and/or fusogenic action. For example, certain viral infections implicate membrane disruption by amphiphilic peptides that undergo conformational transition upon reduction of the pH (Carrasco, 1994). Thus, peptides and conjugates, which mimic viral fusion peptides, have been investigated for the preparation of pH-responsive/fusogenic carriers (Hahn and Kim, 1991; da Costa and Chaimovich, 1997), as well as in DNA delivery (Wagner et al., 1992; Plank et al., 1994). However, these peptides are generally less efficient than viral vectors (Schatzlein, 2001), and their potential immunogenicity may somehow restrict their systemic administration. Accordingly, several synthetic macromolecules, which exhibit membrane-disruptive properties under mildly acidic conditions, have been proposed to increase the escape of membrane-impermeable compounds from endosomes/lysosomes. Synthetic polymers offer several advantages over peptides and proteins such as simple large-scale production, low cost and low immunogenicity.

Synthetic membrane–active polymers are generally polyelectrolytes that respond to acid titration by a change in their conformation and affinity for membranes. Poly(ethylenimine) (PEI), a polycation, has been extensively studied as a non-viral gene delivery vehicle (for a review, see Godbey et al., 1999). It has been postulated that, following endocytosis, PEI acts as a sponge for protons that are pumped into lysosomes. As the pumped protons are accompanied by an influx of chlorate ions, an increase in the osmolarity occurs, inducing lysosomal swelling and bursting (Boussif et al., 1995). This buffering capacity of PEI can also lead to inactivation of lysosomal enzymes due to a pH increase. In addition, PEI can promote the fusion of negatively-charged membranes (Oku et al., 1986) and act as a permeabilizing agent at high concentrations (Godbey et al., 1999). Depending on their state of ionization, polyanions such as poly(2-ethylacrylic acid) (PEAA), can also destabilize phospholipid membranes (Thomas and Tirrell, 1992; Chung et al., 1996). Upon protonation of the carboxylic groups, PEAA undergoes coil-to-globule transition, partitions into the membrane, and eventually causes membrane disruption (Thomas and Tirrell, 1992). Similarly, poly(propylacrylic acid) and random copolymers of acrylic acid and ethyl acrylate possess membrane-destabilizing properties within narrow pH ranges (Murthy et al., 1999; Stayton et al., 2000). These polymers have recently been used in the development of non-viral carriers for gene delivery (Cheung et al., 2001).

pH-sensitive liposomes represent another category of carriers that have been designed to enhance the cytoplasmic bioavailability of drugs (for a review, see Drummond et al., 2000). The most studied class of pH-sensitive liposomes consists of non-bilayer-forming lipids (e.g. dioleoylphosphatidylethanolamine (DOPE)) that are stabilized in bilayer structures at neutral pH by mildly acidic amphiphiles, such as oleic acid or cholesterylhemisuccinate. The protonation of the titratable amphiphile at acidic pH causes membrane destabilization and formation of fusion competent non-bilayer structures. The major concern about pH-sensitive DOPE formulations is their relative instability in plasma or serum (Connor et al., 1986), which results in premature drug leakage (Senior, 1987). Even though the stability can be increased by using double-chain amphiphiles, substantial loss in pH-sensitivity following incubation in plasma has been observed (Liu and Huang, 1989; Collins et al., 1990). Alternatively, intrinsically pH-sensitive liposomes can be prepared with pH-labile plasmalogens, which hydrolysis at acidic pH results in increased liposome permeability (Gerasimov et al., 1997). However, the addition of dihydrocholesterol to such formulations to increase the in vivo liposome stability leads to a significant decrease in pH-sensitivity (Gerasimov et al., 1997).

Polymers have also been investigated to confer pH-responsiveness to liposomes. One of the ad-

vantages of using stimuli-responsive polymers is the possibility to render almost any liposomal composition sensitive to pH. For example, egg phosphatidylcholine (EPC) liposomes can be destabilized at pH 6.5 by surface-bound PEAA (Maeda et al., 1988). Moreover, hydrophobically-modified PEAA has been shown to induce the fusion of EPC/cholesterol (Chol) liposomes at acidic pH (Chen et al., 1999). However, in vivo applications of PEAA-coated liposomes have not been investigated so far, and it may be expected that, upon intravenous injection, the abundance of the negative charges in the polymer would lead to a fast removal of liposomes by the mononuclear phagocyte system (Gabizon and Papahadjopoulos, 1992). Kono et al. (1994) have developed pH-responsive liposomes bearing succinylated poly(glycidol)s, a poly(ethylene glycol) (PEG) derivative with carboxylic groups. These liposomes undergo fusion and leakage below pH 5.5, and can efficiently deliver calcein to the cytoplasm of CV-1 kidney cells (Kono et al., 1994, 1997).

The use of *N*-isopropylacrylamide (NIPAM) copolymers to confer pH-sensitivity to liposomes was first reported by Meyer et al. (1998). NIPAM homopolymer (PNIPAM) collapses above its lower critical solution temperature (LCST), which is about 32 °C in aqueous solution (Heskins and Guillet, 1968). The addition of an ionizable moiety to PNIPAM can increase its LCST above the body temperature, and make the polymer responsive to pH (Hirotsu et al., 1987; Chen and Hoffman, 1995). Hydrophobically-modified NIPAM copolymers containing methacrylic acid (MAA) as the pH-sensitive moiety were shown to trigger the content release of EPC, EPC/Chol and EPC/Chol/1,2-distearoyl-*sn*-glycero-phosphatidylethanolamine-*N*-methoxy(PEG) liposomes under acidic conditions (Meyer et al., 1998; Zignani et al., 2000). While PEAA and succinylated poly(glycidol)s have been only used to confer pH-sensitivity to liposomes composed of fluid phase lipids, NIPAM copolymers were shown to destabilize liposomes made of high temperature phase transition lipids (distearoylphosphatidylcholine, $T_m = 55$ °C) (Roux et al., 2000).

The possible intracellular trafficking route of polymer-based pH-sensitive liposomes following receptor-mediated internalization is illustrated in Fig. 1. Following endocytosis, liposomes should be destabilized in response to polymer collapse and release rapidly their contents in the endosomes. By releasing liposomal content prior to reach the lysosomes, premature degradation by hydrolases and peptidases can be minimized (Huang et al., 1983). This may present an advantage for the delivery of labile macromolecules (e.g. peptides, antisense oligonucleotides) and certain anticancer drugs like cytosine arabinofuranoside (ara-C) that are degraded in lysosomes following their delivery by non pH-sensitive liposomes (Huang et al., 1983). Depending on the polymer and liposome properties, the endosomal membrane can also be destabilized, therefore facilitating the diffusion of the drug in the cytoplasm.

This manuscript summarizes our recent progress on pH-sensitive vesicles (i.e. liposomes and niosomes) obtained through complexation with hydrophobically-modified NIPAM copolymers.

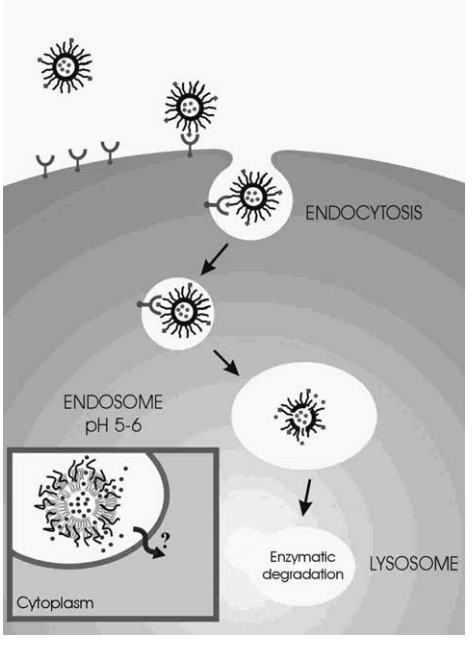

Fig. 1. Potential intracellular trafficking route of polymer-based pH-sensitive vesicles following receptor-mediated internalization.

Table 1
Characteristics of the different pH-sensitive polymers

Entry	Formula[a]	Molecular weight	pH of phase transition at 37 °C	References
A	P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$)	$M_w = 52\,500$	3.8	Spafford et al., 1998
B	P(NIPAM$_{93}$-*co*-Gly$_5$-*co*-ODAm$_2$)	$M_v = 260\,000$	4.4	Francis et al., 2001
C	P(NIPAM$_{90}$-*co*-VP$_4$-*co*-MAA$_2$-*co*-ODA$_4$)	$M_w = 21\,000$	6.3	Leroux et al., 2001
D	DODAm-P(NIPAM$_{90}$-*co*-MAA$_4$)	$M_w = 8200$	5.6	Roux et al., 2002

[a] Subscripts indicate mol% of each monomer.

The pH-sensitive vesicles are characterized with respect to their interaction with the copolymers, stability in plasma, pharmacokinetics and ability to release their contents under mildly acidic conditions. Most of the data presented in this manuscript is taken from previously published work.

2. Materials and methods

2.1. Polymer synthesis

The randomly- and terminally-alkylated copolymers of NIPAM were prepared by free radical polymerization as described previously using either MAA (Leroux et al., 2001) or glycine acrylamide (Gly) as pH-sensitive moiety (Spafford et al., 1998). The selected hydrophobic anchors were octadecylacrylate (ODA) or octadecylacrylamide (ODAm) (randomly-alkylated polymers), and dioctadecylamide (DODAm) (terminally-alkylated polymer). *N*-vinylpyrrolidone (VP) was sometimes added to increase the copolymer hydrophilicity. The fluorescently-labeled polymer was prepared by randomly introducing *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide (Py) in the polymeric chain (Spafford et al., 1998). The characteristics of the different polymers are reported in Table 1.

2.2. Liposome and niosome preparation

Large unilamellar EPC or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) liposomes were prepared by mechanical dispersion of lipids (including Chol) in the appropriate buffer or marker solution, followed by extrusion through 50–200 nm pore-size filters as described elsewhere (Szoka et al., 1980). Niosomes were prepared using either of *n*-octadecyldiethylene oxide ($EO_2C_{18}H_{37}$) or *n*-octadecyltriethylene oxide ($EO_4C_{18}H_{37}$) and Chol, by the reverse-phase evaporation procedure (Szoka and Papahadjopoulos, 1978) or by mechanical dispersion, followed by extrusion. The polymer was added during the vesicle preparation or incubated with preformed vesicles at different polymer to lipid ratios. For more details on the preparation of the vesicles please refer to the studies of Polozova and Winnik (1999), Francis et al. (2001), Zignani et al. (2000). Cationic and anionic vesicles were prepared by adding, respectively, dimethyldioctadecylammonium bromide (DDAB) or dioctadecyl phosphate (DP) to the lipid/surfactant mixture. For the in vitro release studies, untrapped marker/drug and free polymer were removed by gel permeation chromatography in HEPES/dextrose buffer, pH 7.2 (20 mM HEPES, 5% w/v dextrose) (Zignani et al., 2000). Vesicle size was determined by dynamic light scattering (N4 Plus, Coulter Electronics, Miami, FL).

2.3. Fluorescence measurements

Emission spectra were recorded with a SPEX Fluorolog 212 spectrometer (Edison, NJ) with an excitation wavelength of 330 nm for pyrene. The ratio I_E/I_M of the excimer emission intensity to the monomer emission intensity was calculated as the ratio of the intensity of the emission of the excimer at 480 nm to the half-sum of the emission intensities of the monomer at 377 and 397 nm (Polozova and Winnik, 1999).

2.4. In vitro release kinetics

pH-sensitive vesicles (20 mM lipids/surfactants) containing 35 mM trisodium 8-hydroxypyrene trisulfonate (HPTS), 50 mM p-xylene-bis-pyrimidium as the collisional quencher (Molecular Probes, Eugene, OR), and 20 mM HEPES were added to 2-*N*-(morpholino)ethanesulfonic acid (MES)-saline buffer (100 mM MES, 144 mM NaCl, pH 3.0–7.2) or to HEPES-saline buffer (20 mM HEPES, 144 mM NaCl, pH 7.2) preheated at 37 °C. The content release was calculated from the fluorescence intensity of HPTS ($\lambda_{ex} = 413$ nm, $\lambda_{em} = 512$ nm) over that obtained following sample lysis in 0.5% (w/w) Triton X-100. The in vitro release following incubation in serum was done by incubating first the vesicles in 75% (v/v) preheated human serum for 1 h at 37 °C. The excess of serum components was then removed by gel permeation chromatography and the release of HPTS from the vesicles was determined as described above (Daleke et al., 1990).

2.5. In vitro uptake of vesicles by macrophages and cytotoxicity assays

J774 macrophage-like cells (kind gift from professor Michel Desjardins, University of Montreal, Quebec, Canada) were grown in Dulbecco's modified eagle medium (DMEM, Invitrogen Corp., Ont., Canada) supplemented with ten percent heat-inactivated fetal bovine serum (FBS) and containing 1 U/ml penicillin G and 1 μg/ml streptomycin (Invitrogen). J774 cells were plated in six-well tissue culture plates (1 ml DMEM-FBS containing 5×10^5 viable cells) and allowed to adhere and proliferate for 24 h. Empty liposomes labeled with [^{3}H]cholesteryl hexadecyl ether (130–160 nm, unimodal distribution) were added (200 μl) to each well, and incubated at 37 °C with the cells for 3 h in a humidified atmosphere of 5% CO_2. Cells were then rinsed three times with 2 ml cold phosphate buffered saline (PBS). Cells were lysed by adding 1 ml of triton X-100 (1% w/w in water). Radioactivity of 800-μl aliquots was counted to assess the liposome uptake.

The ability of pH-sensitive and non pH-sensitive liposomes to inhibit J774 cell proliferation in vitro was determined using cell proliferation assay. EPC/Chol liposomes (4:1 mol/mol, 40 mM lipids, 150–170 nm, unimodal distribution) containing ara-C (Sigma, St. Louis, MO) were prepared by hydrating the lipid film with a 200 mM ara-C solution spiked with [^{3}H]ara-C (1 nCi/ml) (Amersham Pharmacia Biotech, Piscataway, NJ). Liposomes were extruded as described above. After removal of free ara-C by passage over Sephadex G-100 (column i.d. 1 cm, length 10 cm), liposomes were incubated for 20 h at 4 °C with polymer C.

J774 cells were plated in 24-well tissue culture plates (500 μl DMEM-FBS containing 15×10^3 viable cells) and allowed to adhere and proliferate for 24 h. Liposomes (100 μl) were added to each well, after which the cells were incubated for 4 h. Cells were then rinsed twice with 1.5 ml DMEM and then 1 ml of DMEM-FBS was added. Cell proliferation was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) as described by Tada et al. (1986). Briefly, MTT (100 μl, 5 mg/ml in PBS) was added to the cell cultures after 48 h, and incubation was carried-out for a further 3 h at 37 °C. One ml of 10% (w/v) sodium dodecyl sulfate in 0.01 M HCl was added to each well to dissolve reduced MTT, and the absorbance was measured at 570 nm after overnight incubation at 37 °C.

3. Results and discussion

The introduction of alkyl chains in PNIPAM allows its anchoring into lipid membranes, and this has been exploited for the acid- (Meyer et al., 1998) and temperature-triggered (Kim et al., 1997) destabilization of liposomes. An important consideration in the development of stimuli-responsive liposomal formulations is their stability in biological fluid, in the absence of stimulus. The physical state of the bilayer plays a critical role in the retention of liposomal content as well as in the circulation time in vivo (Senior and Gregoriadis, 1982). Fluid-phase liposomes are known to be leaky in serum, but the addition of Chol in the membrane increases their stability (Kirby et al.,

1980). In a previous study (Zignani et al., 2000), we showed that EPC/Chol liposomes bearing a randomly-alkylated NIPAM copolymer containing 5 mol% MAA were stable at pH 7.2 but could release up to 80% of their contents at pH 4.9. We also reported that NIPAM copolymers conferred pH-sensitivity to DSPC/Chol liposomes, which are intrinsically more stable because of the presence of a high temperature phase transition lipid. However, experiments undertaken in biological fluids suggested a poor anchoring of the polymer in such liposomal membranes (Roux et al., 2000). In order to increase the retention of the polymer at the liposomal surface, two different approaches were considered: (1) changing the vesicle components and (2) modifying the polymer composition to increase its affinity to the lipid bilayer.

3.1. Approach 1—evaluation of a non-phospholipid pH-sensitive vesicle formulation

Non-phospholipid liposomes, also known as niosomes, are physically similar to phopholipid vesicles in that an aqueous core is entrapped in one or several membrane bilayers (Baillie et al., 1985). Depending on the main amphiphile which is used for their preparation (e.g. polyoxyethylene alkyl ether), they can form hydrogen bonds via ether and hydroxyl groups at their surface. The addition of Chol is usually required to avoid micelle formation and increase stability since the small hydrophilic head group of Chol compensates for the large hydrophillic group of the surfactant (Bouwstra and Junginger, 1995). The charge of the niosomes can be modulated by adding cationic or anionic amphiphiles such as DDAB or DP, respectively. As NIPAM copolymers can form hydrogen bonds through their amide and carboxylic groups, this property was examined as a possible means of increasing the affinity of the pH-sensitive polymer for the vesicles (Polozova and Winnik, 1999).

The interactions between P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$) and different vesicles have been studied by fluorescence spectroscopy, using covalently-attached pyrene as a probe (Polozova and Winnik, 1999). When pyrene molecules are located in close proximity to each other as in micellar assemblies, the fluorescence spectrum presents a broad signal, associated with chromophore excimers, at 480 nm, as well as more resolved signals at 377 and 397 nm, due to the emission of monomers that are locally set apart (Fig. 2). However, the association of the polymer with vesicles causes an increase in the intensity of the monomer emission, as the incorporation of the hydrophilic groups in the bilayers spaces pyrene molecules out from each other (Fig. 2).

One possible mechanism involved in the complexation of P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$) with vesicle membranes is the insertion of the alkyl chains in the bilayers via hydrophobic interactions. However, it has been shown that the addition of neutral POPC liposomes to P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$) induces only a slight change in the emission spectrum of the polymer, suggesting that relatively poor interactions take place between the polymer and liposomes (Polozova and Winnik, 1999). Fig. 3 shows that the addition of increasing amount of cationic surfactant (DDAB) into POPC liposomes triggers an important decrease in the ratio of excimer to monomer intensity of P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$). As this effect increases with the charge density of the cationic liposomes, attractive electrostatic forces seem, in this case, to be the main driving force for the formation of the complexes. This change in the emission spectrum of

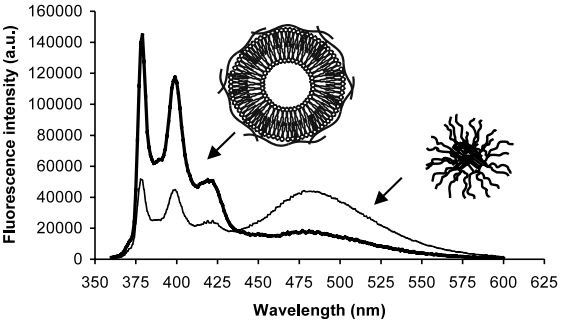

Fig. 2. Variation in the fluorescence spectrum of P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$) (polymer A) upon their complexation with cationic niosomes. $\lambda_{exc} = 330$ nm; polymer concentration, 0.002 g/l; total lipid concentration, 0.1 g/l; niosome composition (w/w), 75% $EO_2C_{18}H_{37}$, 20% Chol, 5% DDAB. Adapted from Polozova and Winnik (1999) with permission from the American Chemical Society.

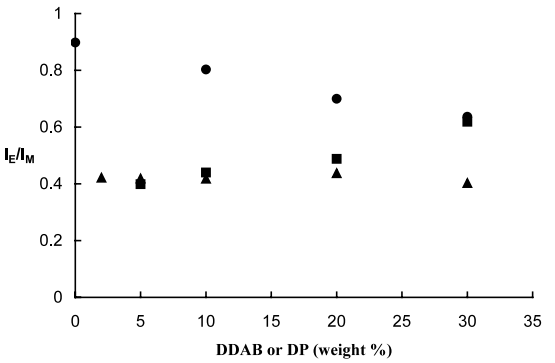

Fig. 3. Change in the ratio I_E/I_M as a function of the amount of charged surfactant in mixtures of P(NIPAM$_{83}$-*co*-Gly$_{16}$-*co*-Py$_1$) (polymer A) with vesicles of different types: $EO_2C_{18}H_{37}$/Chol/DDAB niosomes (triangles), $EO_2C_{18}H_{37}$/Chol/DP niosomes (squares) and POPC/DDAB liposomes (circles). Polymer concentration, 0.002 g/l; total lipid concentration, 0.01 g/l; Tris buffer (5 mM) containing 0.15 M NaCl. Reproduced from Polozova and Winnik (1999) with permission from the American Chemical Society.

the copolymer is even more significant in the presence of cationic niosomes ($EO_2C_{18}H_{37}$/Chol/DDAB). Moreover, the I_E/I_M ratio reaches its lowest value with a small amount of cationic component (2 wt.%) and remains unchanged with further increase of DDAB. Fig. 3 also shows that, upon addition of low charge density anionic niosomes ($EO_2C_{18}H_{37}$/Chol/DP), the I_E/I_M ratio reaches values close to those obtained with cationic niosomes. As the negative charge density further increases, the electrostatic repulsion forces overcome these interactions. This unusual behavior is indicative of a peculiar binding mechanism between niosomes and NIPAM copolymers.

Indeed, these results and those reported by Polozova and Winnik (1999) suggest that the binding mechanism of PNIPAM to niosomes is different than liposomes. Hydrophobic interactions via the non-polar side group of the copolymer with neutral liposomes are relatively weak and the addition of a cationic amphiphile promotes the formation of electrostatic interactions, causing a stronger complexation. In contrast, the entire surface of niosomes seems to participate in the formation of hydrogen bonds between the amide groups of NIPAM and the hydroxyl groups of the ethoxylated surfactant, or between the amide proton of NIPAM and the ether oxygen of the surfactant (Polozova and Winnik, 1999). Moreover, this strong interaction prevails in the control of polymer adsorption to niosomes, as it can overcome repulsive electrostatic interactions between the polymer and anionic niosomes. As stability of the formulation is a prerequisite for efficient vesicle-mediated delivery of therapeutic agents, this unique feature in the mechanism of interaction renders niosomes attractive vesicles for the preparation of pH-sensitive carriers.

As shown in Fig. 4, the hydrophobically-modified NIPAM/Gly copolymer (Table 1, polymer B) is able to trigger the release of an encapsulated fluorescent dye (HPTS) from both niosomes and liposomes at acidic pH, whereas the vesicles remain stable at neutral pH (data not shown). However, the range of pH over which the copolymer destabilizes the vesicles is different in both cases, starting at about pH 4.5 and 5.2 for liposomes and niosomes, respectively. The difference between both formulations might be explained by the interactions that take place between the copolymer and the two types of vesicles. It has been previously demonstrated that binding of hydrophobically-modified NIPAM/MAA copolymer to liposomes does not signifi-

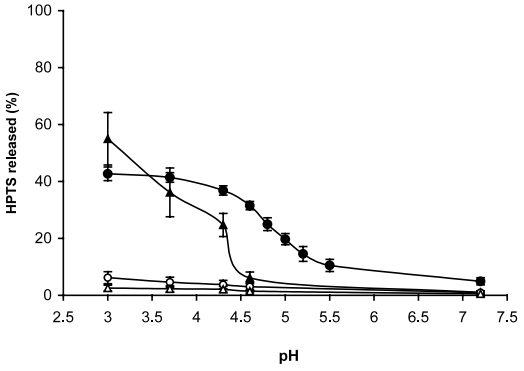

Fig. 4. Percent HPTS release after 3 min at 37 °C from $EO_4C_{18}H_{37}$/Chol niosomes (3:2 mol/mol) (circles) and POPC/Chol liposomes (3:2 mol/mol) (triangles) incubated with 0.3 (*w*/*w*) of P(NIPAM$_{93}$-*co*-Gly$_5$-*co*-ODAM$_2$) (polymer B) as a function of pH (3.0–7.0), before (closed symbols) and after (open symbols) 1-h incubation in 75% human serum at 37 °C. Adapted from Francis et al. (2001) with permission from the American Chemical Society.

cantly modify its phase transition pH (Meyer et al., 1998). The acid-triggered destabilization of the liposomes occurred at the phase transition pH of the polymer, which is about 4.4 at 37 °C (Francis et al., 2001; Table 1). However, for niosomes, destabilization took place at a higher pH than that of polymer collapse. In this case, the complex formation is predominantly controlled by hydrogen bonding, and this may affect the hydration of the isopropylacrylamide group and thus, the pH at which the polymer collapses.

We previously found that incubation of DSPC/Chol liposomes complexed with NIPAM copolymer in human serum leads to a substantial shift in the pH of destabilization, suggesting partial extraction of the copolymer from the bilayers (Roux et al., 2000). In the case of niosomal formulations, the cooperativity of hydrogen bonding in the complexation of PNIPAM to vesicles was thought to favor a more stable anchoring of the polymer. However, Fig. 4 shows that, following incubation in serum, the niosomes completely lost their pH-sensitivity. This was partially attributed to premature collapse of the polymer in serum (Francis et al., 2001). It is known that some ions influence the phase transition of PNIPAM (Park and Hoffman, 1993). Indeed, niosomes complexed with hydrophobically-modified NIPAM/Gly polymer (polymer B) released almost 20% of their contents at pH 7.2 in the presence of 4 mM calcium ions (Francis et al., 2001). A similar loss of pH-sensitivity after incubation in serum was also observed for the control liposome formulation (Fig. 4), although in this case, the liposomes were shown to retain their contents in the presence of calcium, as well as in serum (Francis et al., 2001). Still, for both formulations, possible extraction of the polymer by serum components or adsorption of serum proteins on the vesicle can decrease pH-responsiveness.

The ability of these pH-sensitive vesicles to facilitate the transfer of a fluorescent marker (i.e. calcein) in the cytoplasm of J774 macrophage-like cells has been qualitatively assayed by fluorescence microscopy (Francis et al., 2001). Upon liposome phagocytosis, the cells displayed a weak and vesicular fluorescence reflecting the presence of the probe in the endosomal/lysosomal compartments. Unfortunately, niosome/polymer complexes were unable to efficiently deliver calcein to macrophages cytoplasm, as demonstrated by the punctuated fluorescence pattern observed (Francis et al., 2001). This was attributed to a premature collapse of the copolymer in the incubation medium causing a release of the dye before internalization of the vesicles. In contrast, cells treated with liposome/polymer complexes displayed a more diffuse fluorescence, indicating rapid diffusion of the dye in the cell cytoplasm (Francis et al., 2001). However, these experiments were carried in the absence of serum, thus minimizing extraction of the polymer from vesicles by the incubation medium. Accordingly, even though PNIPAM can bind with strong avidity to niosomes, the overall stability of the resulting formulation remains inadequate in physiological fluids and would require further optimization.

3.2. Approach 2—adjustment of the polymer composition

As extraction of the copolymer from the vesicle bilayer is thought to be the major limitation in the stability of liposome/copolymer complexes, another approach was to modify the polymer composition to increase its affinity to the lipid bilayer. In comparison with the first generation of P(NIPAM-*co*-MAA-*co*-ODA) typically containing 2 mol% ODA (anchor) (Meyer et al., 1998; Zignani et al., 2000; Leroux et al., 2001), the ODA content was increased to 4 mol%. The MAA content was also decreased to 2 mol%, in order to bring the phase transition pH (Table 1, polymer C) to a value close to the endosomal pH (5.5–6.5). Finally, VP was added to provide the polymer enough water-solubility. Fig. 5 shows that this copolymer triggered, within 8 min, the release of 24 and 59% liposomal contents at pH 4.9, depending on whether the copolymer was simply incubated with preformed liposomes or incorporated during the liposome preparation procedure. These liposomes were stable at neutral pH. The lower content release obtained with the complexes prepared by the incubation method could be explained by a lower binding to the lipid bilayer due to the presence strong intra/interchain hydropho-

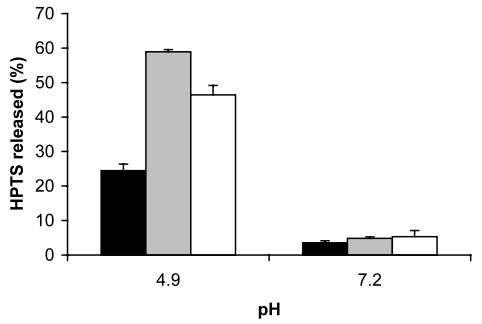

Fig. 5. Percent HPTS release after 8 min at 37 °C from EPC/Chol liposomes (3:2 mol/mol) at pH 4.9 and 5.8, before (closed bars) and after (open bars) 1-h incubation in 75% human serum. P(NIPAM$_{90}$-*co*-VP$_4$-*co*-MAA$_2$-*co*-ODA$_4$) was either incorporated during liposome preparation (open and shaded bars) or incubated overnight at 4 °C with liposomes (black bars). Mean ± S.D. ($n = 3$).

bic domains. Our results also demonstrate that the pH-sensitivity of the formulation prepared by incorporating the polymer during the liposome preparation procedure was essentially preserved following 5-h incubation in human serum, with 46% of HPTS released at pH 4.9 (Fig. 5). Another method to increase the affinity of the copolymer to the lipid bilayer is to prepare a terminally-alkylated copolymer bearing two octadecyl chains at one extremity and a shorter PNIPAM segment (Table 1, polymer D). Such pH-sensitive liposomes were recently shown to maintain their pH-sensitivity in serum and exhibited, in vivo, a circulation time which was even higher than the control formulation (60% increase in AUC) (Roux et al., 2002). This suggests that the terminally-alkylated NIPAM copolymer can to some extent sterically-stabilize liposomes at neutral pH, although this effect remains marginal in comparison to PEG.

As the ultimate goal is to produce a pH-sensitive liposomal formulation that will facilitate the cytoplasmic delivery of drugs, the in vitro efficiency of the formulation was assessed on J774 macrophage-like cells using ara-C as model anticancer drug. Ara-C was chosen because of its susceptibility to degradation by lysosomal enzymes (Huang et al., 1983). First, we verified whether the randomly-alkylated polymer/liposome complexes and the control liposomes were taken up to the same extent by J774 cells. Indeed, below the LCST, it has been demonstrated that PNIPAM copolymers could reduce liposome-cell interactions (Yamazaki et al., 1999). No significant difference was observed between the uptake of the copolymer-coated liposomes versus the non-coated one (data not shown). Compared with the control formulation, a small but significant increase in the ara-C efficiency was observed when the drug was loaded into the pH-sensitive liposomes (Fig. 6). The polymer alone was not toxic at the concentrations tested (data not shown). Thus, rapid release in the endosome probably ensures more efficient delivery of ara-C to the cytoplasm. Increased drug efficacy with liposomal ara-C has been observed with other pH-sensitive formulations. Compared with pH-insensitive formulations, ara-C loaded in pH-sensitive targeted liposomes was found to be more cytotoxic to L-929 cells (Connor and Huang, 1986), CV-1 cells (Brown and Sylvius, 1990) and KB cells (Rui et al., 1998).

We have to point out that ara-C is not an optimal model for evaluating the performance of this specific pH-sensitive formulation. Indeed, it turned out that in the presence of the copolymer, ara-C precipitated. Accordingly, the polymer was incorporated in the bilayer by incubation with preformed liposomes, a procedure which does not lead to maximal drug release under acidic conditions (Fig. 5).

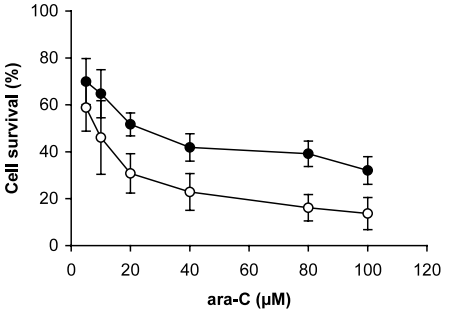

Fig. 6. Toxicity of ara-C encapsulated in control (closed circles) and pH-sensitive (open circles) EPC/Chol liposomes (4:1 mol/mol) toward J774 cells. Mean ± S.D. ($n = 5$).

4. Conclusion

The concept of pH-responsive vesicles was originally proposed more than two decades ago. Despite initial promising results, this approach is still granted with limited success. In order to be efficient, a pH-sensitive colloidal formulation should bear a number of essential properties, which include biocompatibility of the carrier, stability in serum, long circulation time in vivo, interaction with targeted cells and internalization, rapid release following the application of the stimulus, and finally escape of the drug from the endosomal/lysosomal compartments. To date, all these characteristics have not been combined into one formulation with great success. Still a lot of work remains to be carried out to produce a clinically viable formulation. Because of their great versatility polymer/vesicle complexes may provide a means of achieving such a goal. PNIPAM offers interesting characteristics that have not yet been fully exploited in the design of pH-sensitive carriers. Stability in serum and rapid release under acidic conditions can be achieved with PNIPAM formulations. However, it is still unknown whether the attachment of a targeting ligand will compromise pH-responsiveness. Moreover, despite the recent progress, PNIPAM liposomal formulations need to be further optimized with respect to their circulation time in vivo and ability to efficiently deliver their contents to the cytoplasm.

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References

Baillie, A.J., Florence, A.T., Hume, L.R., Muirhead, G.T., Rogerson, A., 1985. The preparation and properties of niosomes-non-ionic surfactant vesicles. J. Pharm. Pharmacol. 37, 863–868.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. USA 92, 7297–7301.

Bouwstra, J.A., Junginger, H.E., 1995. Non-ionic surfactant vesicles (niosomes) for oral and transdermal administration of drugs. In: Puiseux, F., Couvreur, P., Delattre, J., Devissaguet, J.-P. (Eds.), Liposomes, New Systems and New Trends in Their Applications. Editions de Santé, Paris, pp. 99–121.

Brown, P.M., Sylvius, J.R., 1990. Mechanisms of delivery of liposome-encapsulated cytosine arabinoside to CV-1 cells in vitro. Fluorescence-microscopic and cytotoxicity studies. Biochim. Biophys. Acta 1023, 335–341.

Carrasco, L., 1994. Entry of animal viruses and macromolecules into cells. FEBS Lett. 350, 151–154.

Chen, G., Hoffman, A.S., 1995. Graft polymers that exibit temperature-induced phase transitions over a wide range of pH. Nature 373, 49–52.

Chen, T., Choi, L.S., Einstein, S., Klippenstein, M.A., Scherrer, P., Cullis, P.R., 1999. Proton-induced permeability and fusion of large unilamellar vesicles by covalently conjugated poly(2-ethylacrylic acid). J. Liposome Res. 9, 387–405.

Chung, J.C., Gross, D.J., Thomas, J.L., Tirrell, D.A., Opsahl-Ong, L., 1996. pH-sensitive, cation-selective channels formed by simple synthetic polyelectrolyte in artificial bilayer membranes. Macromolecules 29, 4636–4641.

Cheung, C.Y., Murthy, N., Stayton, P.S., Hoffman, A.S., 2001. A pH-sensitive polymer that enhances cationic lipid-mediated gene transfer. Bioconjugate Chem. 12, 906–910.

Collins, D., Litzinger, D.C., Huang, L., 1990. Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols. Biochim. Biophys. Acta 1025, 234–242.

Connor, J., Huang, L., 1986. pH-sensitive immunoliposomes as an efficient and target-specific carrier for antitumor drugs. Cancer Res. 46, 3431–3435.

Connor, J., Norley, N., Huang, L., 1986. Biodistribution of pH-sensitive immunoliposomes. Biochim. Biophys. Acta 884, 474–481.

da Costa, M.H., Chaimovich, H., 1997. Structure-activity relationships in the fusion of small unilamellar phosphatidylcholine vesicles induced by a model peptide. Biochimie 79, 509–516.

Daleke, D.L., Hong, K., Papahadjopoulos, D., 1990. Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay. Biochim. Biophys. Acta 1024, 352–366.

Drummond, D.C., Zignani, M., Leroux, J.-C., 2000. Current status of pH-sensitive liposomes in drug delivery. Prog. Lipid Res. 39, 409–460.

Francis, M.F., Dhara, G., Winnik, F.M., Leroux, J.-C., 2001. In vitro evaluation of pH-sensitive polymer/niosome complexes. Biomacromolecules 2, 741–749.

Gabizon, A., Papahadjopoulos, D., 1992. The role of surface charge and hydrophilic groups on liposome clearance in vivo. Biochim. Biophys. Acta 1103, 94–100.

Gerasimov, O.V., Schwan, A., Thompson, D.H., 1997. Acid-catalized plasmenylcholine hydrolysis and its effect on bilayer permeability: a quantitative study. Biochim. Biophys. Acta 1324, 202–214.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999. Poly(ethylenimine) and its role in gene delivery. J. Control. Release 60, 149–160.

Hahn, K.H., Kim, H., 1991. Fusion and fragmentation of phospholipid vesicles by apohemoglobin at low pH. J. Biochem. 110, 635–640.

Heskins, M., Guillet, J.E., 1968. Solution properties of poly(*N*-isopropylacrylamide). J. Macromol. Sci. Chem. A2, 1441–1455.

Hirotsu, S., Hirokawa, Y., Tanaka, T., 1987. Volume-phase transitions of ionized *N*-isopropylacrilamide gels. J. Chem. Phys. 87, 1392–1395.

Huang, A., Kennel, S.J., Huang, L., 1983. Interactions of immunoliposomes with target cells. J. Biol. Chem. 258, 14034–14040.

Kim, J.C., Bae, S.K., Kim, J.D., 1997. Temperature-sensitivity of liposomal lipid bilayers mixed with poly(*N*-isopropylacrylamide). J. Biochem. 121, 15–19.

Kirby, C., Clarke, J., Gregoriadis, G., 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem. J. 186, 591–598.

Kono, K., Igawa, T., Takagishi, T., 1997. Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative. Biochim. Biophys. Acta 1325, 143–154.

Kono, K., Zenitani, K.I., Takagishi, T., 1994. Novel pH-sensitive liposomes: liposomes bearing a poly(ethylene glycol) derivative with carboxyl groups. Biochim. Biophys. Acta 1193, 1–9.

Leroux, J.-C., Roux, E., Le Garrec, D., Hong, K., Drummond, D.C., 2001. *N*-isopropylacrylamide copolymers for the preparation of pH-sensitive liposomes and polymeric micelles. J. Control. Release 72, 71–84.

Liu, D., Huang, L., 1989. Small, but not large, unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid can be stabilized by human plasma. Biochemistry 28, 7700–7707.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284.

Maeda, M., Kumano, A., Tirrell, D.A., 1988. H + -induced release of contents of phosphatidylcholine vesicles bearing surface-bound polyelectrolyte chains. J. Am. Chem. Soc. 110, 7455–7459.

Meyer, O., Papahadjopoulos, D., Leroux, J.C., 1998. Copolymers of *N*-isopropylacrylamide can trigger pH sensitivity to stable liposomes. FEBS Lett. 42, 61–64.

Murthy, N., Robichaud, J.R., Tirrell, D.A., Stayton, P.S., Hoffman, A.S., 1999. The design and synthesis of polymers for eukaryotic membrane disruption. J. Control. Release 61, 137–143.

Oku, N., Yamaguchi, N., Yamaguchi, N., Shibamoto, S., Ito, F., Nango, M., 1986. The fusogenic effect of synthetic polycations on negatively charged lipid bilayers. J. Biochem. 100, 935–944.

Park, T.G., Hoffman, A.S., 1993. Sodium chloride-induced phase transition in nionic poly(*N*-isopropylacrylamide) gel. Macromolecules 26, 5045–5048.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E., 1994. The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J. Biol. Chem. 269, 12918–12924.

Polozova, A., Winnik, F.M., 1999. Contribution of hydrogen bonding to the association of liposomes and an anionic hydrophobically modified poly(*N*-isopropylacrylamide). Langmuir 15, 4222–4229.

Roux, E., Stomp, R., Giasson, S., Pézolet, M., Moreau, P., Leroux, J.-C., 2002. Steric stabilization of liposomes by pH-responsive *N*-isopropylacrylamide copolymer, J. Pharm. Sci., in press.

Roux, E., Zignani, M., Daigle, C., Moreau, P., Drummond, D.C., Hong, K., Leroux, J.-C., 2000. pH-sensitive gel phase liposomes. Proc. Int. Symp. Control. Release Bioact. Mater. 27, 215.

Rui, Y., Wang, S., Low, P.S., Thompson, D.H., 1998. Dismenylcholine-folate liposomes: an efficient vehicle for intracellular drug delivery. J. Am. Chem. Soc. 120, 11213–11218.

Schatzlein, A.G., 2001. Non-viral vectors in cancer gene therapy: principles and progress. Anti-Cancer Drugs 12, 275–304.

Senior, J., Gregoriadis, G., 1982. Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. Life Sci. 30, 2123–2136.

Senior, J.H., 1987. Fate and behavior of liposomes in vivo: a review of controlling factors. Crit. Rev. Ther. Drug Carrier Syst. 3, 123–193.

Spafford, M., Polozova, A., Winnik, F.M., 1998. Synthesis and characterization of a hydrophobically modified copolymer of *N*-isopropylacrylamide and glycinyl acrylamide. Macromolecules 31, 7099–7102.

Stayton, P.S., Hoffman, A.S., Murthy, N., Lackey, C., Cheung, C., Tan, P., Klumb, L.A., Chilkoti, A., Wilbur, F.S., Press, O.W., 2000. Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics. J. Control. Release 65, 203–220.

Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., Papahadjopoulos, D., 1980. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim. Biophys. Acta 601, 559–571.

Szoka, F.J., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA 75, 4194–4198.

Tada, H., Shiho, O., Kuroshima, K., Koyama, M., Tsukamoto, K., 1986. An improved colorimetric assay for interleukin 2. J. Immunol. Methods 93, 157–165.

Thomas, J.L., Tirrell, D.A., 1992. Polyelectrolyte-sensitized phospholipid vesicles. Acc. Chem. Res. 25, 336–342.

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., Birnstiel, M.L., 1992. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc. Natl. Acad. Sci. USA 89, 7934–7938.

Yamazaki, A., Winnik, F.M., Cornelius, R.M., Brash, J.L., 1999. Modification of liposomes with *N*-sustituted polyacrylamides: identification of proteins adsorbed from plasma. Biochim. Biophys. Acta 1421, 103–115.

Zignani, M., Drummond, D.C., Meyer, O., Hong, K., Leroux, J.-C., 2000. In vitro characterization of a novel polymeric-based pH-sensitive liposome system. Biochim. Biophys. Acta 1463, 383–394.