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pH-sensitive immunoliposomes specific to the CD33 cell surface antigen of leukemic cells

Pierre Simard, Jean-Christophe Leroux*

Canada Research Chair in Drug Delivery, Faculty of Pharmacy, University of Montreal, P.C. 6128 Downtown Station, Montreal (Qc), Canada H3C 3J7

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ABSTRACT

A promising avenue in cancer therapy using liposomal formulations is the combination of site-specific delivery with triggered drug release. The use of trigger mechanisms in liposomes could be relevant for drugs susceptible to lysosomal hydrolytic/enzymatic degradation. Here, we propose a polymeric pH-sensitive liposome system that is designed to release its content inside the endosomes through a polymer structural change following receptor-mediated internalization. Specifically, pH-sensitive immunoliposomes (ILs) were obtained by including a terminally alkylated copolymer of *N*-isopropylacrylamide (NIPAM) in the liposome bilayer and by coupling the anti-CD33 monoclonal antibody to target leukemic cells. *In vitro* release of encapsulated fluorescent probes and cytosine arabinoside (ara-C) revealed that pH-sensitivity of the vector was retained in the presence of the antibody upon incubation in plasma. Flow cytometry and confocal microscopy analyses demonstrated that the pH-sensitive ILs were efficiently internalized by various CD33+ leukemic cell lines while limited interaction was found for liposomes decorated with an isotype-matched control antibody. Finally, the pH-sensitive ILs-CD33 formulation exhibited the highest cytotoxicity against HL60 cells, confirming the role of the NIPAM copolymer in promoting the escape of intact ara-C in the endosomes. These results suggest that this pH-sensitive liposomal formulation could be beneficial in the treatment of acute myeloid leukemia.

1. Introduction

The advent of liposomal delivery systems for various ailments, such as cancer, has brought about significant therapeutic benefits over standard chemotherapy. They are now considered as a mainstream drug delivery technology. The growing number of liposomal formulations already on the market or currently under clinical evaluation is a proof of the vast potential of these lipid-based carriers (Simard et al., 2007). The success of liposomes is mostly a consequence of their ability to reduce drug toxicity and prolong a drug's biological half-life. The long circulation times of these vectors are the combined effect of small particle size (<150 nm), adequate lipid composition, and PEGylation which limits recognition by the mononuclear phagocytes system (MPS) and enhances drug concentrations in tumors following intravenous (i.v.) administration (Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991; Working et al., 1994). Solid tumors are characterized by a leaky vasculature and impaired lymphatic drainage, leading to the enhanced permeation and retention effect, which can be exploited to improve passive drug accumulation. Liposomes also offer benefits in the treatment of leukemia by providing sustained drug levels in the bloodstream without being dependent on leaky blood vessels to access the neoplastic cells (Allen et al., 1992).

The newest generation of liposomes features direct molecular targeting of specific cells *via* ligand-mediated interactions. Blume et al. demonstrated that coupling plasminogen as a homing device to the end of the PEG chains combines long systemic vesicle circulation times with high target binding capability (Blume et al., 1993). The presence of targeting moieties such as antibodies can modify the biodistribution of the i.v. administered vesicles, increase the specificity of the interaction with target cells through receptors and bypass multidrug resistant transporters (Iden and Allen, 2001; Mastrobattista et al., 1999). While internalization of liposomes by receptor-mediated endocytosis increases the intracellular drug levels, the endocytosed material may eventually be delivered to the acidic lysosomal compartment, where it can be hydrolyzed by various enzymes, resulting in diminished biological activity. This is particularly critical for drugs that are sensitive to degradation, such as nucleic acids, peptidic drugs, as well as biologically unstable anticancer drugs such as cytosine β-D arabinofuranoside (ara-C) (Connor and Huang, 1986). For such fragile molecules, methods which can facilitate the release of the entrapped cargo in the cytosol are desirable.

Different triggered release mechanisms have been designed for liposomes in order to promote drug discharge in targeted tissues or cell compartments. These stimuli, including temperature

* Corresponding author. Tel.: +1 514 343-6455; fax: +1 514 343-6871.
E-mail address: Jean-Christophe.Leroux@umontreal.ca (J.-C. Leroux).

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(Weinstein et al., 1979), pH (Hope et al., 1983; Roux et al., 2002b), light (Zhang et al., 2002), enzymatic degradation (Davis and Szoka, 1998), and ultrasounds (Kiser et al., 2000), have been efficiently used to initiate a breakdown of the bilayers. In particular, pH-sensitive liposomes were introduced in the early 1980s as a means of increasing drug delivery in tumors having a pH lower than the normal physiological value (Yatvin et al., 1980).

The most studied class of pH-sensitive liposomes consists of dioleoylphosphatidylethanolamine (DOPE) vesicles that are stabilized in the bilayer phase by mildly acidic amphiphiles such as oleic acid or cholesteryl-hemisuccinate (CHEMS) (Connor et al., 1984; Düzgünes et al., 1985). Upon acidification, the amphiphile headgroups are protonated, resulting in charge neutralization and destabilization of the vesicles due to the conversion of the DOPE component to the inverted hexagonal phase (Litzinger and Huang, 1992). During this process, the liposomal structure is destroyed and the material encapsulated in the aqueous core is released. Although such liposomes have been shown to be efficient for cytoplasmic delivery in cultured cells, their moderate plasmatic stability and rapid clearance have hampered their use *in vivo* (Drummond et al., 2000). To increase the stability and prolong the circulation time of these DOPE-based formulations, different components have been added to the liposome membrane, such as PEG-derivatized lipids (Collins et al., 1990; Liu and Huang, 1989; Woodle and Lasic, 1992). As often reported for conventional liposomes, PEG-derivatives confer steric stability to the vesicles. However, they also hinder aggregation and dehydration of the membrane surface, thus reducing liposome fusion with cell membranes and the subsequent release of their contents (Holland et al., 1996; Kirpotin et al., 1996). To circumvent this drawback, Kirpotin et al. (1996) introduced approximately 10 years ago thiolytically cleavable PEG–lipid conjugates. This approach, which relies on the reduction of the disulfide linkage at the target site to form fusogenic DOPE/CHEMS vesicles, has shown little therapeutic benefit *in vivo*. Indeed, the system was found to be leaky in human plasma, and was rapidly eliminated from the circulation as the disulfide lipid derivative of PEG was cleaved in the bloodstream (Ishida et al., 2001). The groups of Thompson and Szoka investigated other strategies to prepare pH-sensitive liposomes (Gerasimov et al., 1999; Guo and Szoka, 2003). The latter are based on the acid-catalyzed hydrolysis of bilayer-stabilizing lipids into surfactants or conical lipids. The encapsulated content is released over several hours under mildly acidic conditions. This may be too slow to target the release into the endosomal compartment which has a transit half-life toward the lysosomes of less than 40 min. Many other pH-sensitive systems are based on pH-responsive peptides or proteins that can efficiently trigger membrane fusion/disruption at acidic pH. For example, the *N*-terminus of hemaglutinin (INF peptides from influenza) (Plank et al., 1994), GALA peptides (Simoes et al., 1998) and the listeriolysin O (Stier et al., 2005) have demonstrated reasonable enhancement of cytoplasmic delivery of biomacromolecules. Despite their interesting features, their clinical application may be limited due to potential immunogenicity (Huckriede et al., 2003).

Acid-triggered liposome destabilization/fusion can also be achieved by employing synthetic polyelectrolytes that undergo coil-to-globule phase transition upon protonation (Drummond et al., 2000; Roux et al., 2003; Yessine and Leroux, 2004). Most polymers investigated so far for the design of pH-sensitive liposomes are based on poly(alkyl acrylic acid)s (Chen et al., 1999; Jones et al., 2003; Kyriakides et al., 2002), succinylated PEG (Kono et al., 1994; Mizoue et al., 2002), biodegradable polyphosphazenes (Couffin-Hoarau and Leroux, 2004) and *N*-isopropylacrylamide (NIPAM) copolymers (Meyer et al., 1998; Roux et al., 2002a, 2003; Zignani et al., 2000). For example, poly(alkyl acrylate-*co*-methacrylic acid)s have been shown to express strong membrane destabilizing properties (Yessine et al., 2003) and have been used to construct pH-responsive lipoplexes (Yessine et al., 2006) and polyion complex micelles (Yessine et al., 2007) that were able to facilitate the transfer of oligonucleotides from the endosomes to the cytoplasm.

One of those polymers which have been shown to efficiently trigger the release of the content of liposomal formulations are NIPAM copolymers. The NIPAM homopolymer is characterized by a lower critical solution temperature (LCST), which is approximately 32 °C in aqueous solution (Heskins and Guillet, 1968). Below its LCST, the polymer is soluble, but precipitates when heated above this value. It is possible to increase the LCST above the physiological temperature and confer pH-sensitivity to the polymer by randomly copolymerizing a small proportion (<10 mol%) of a titrable comonomer, such as methacrylic acid (MAA) with NIPAM. Long alkyl chains can be introduced randomly or at one extremity of P(NIPAM-*co*-MAA) chains to allow anchoring of the polymer in the liposomal bilayer (Leroux et al., 2001). Upon acidification of the external medium, the polymer collapses, introducing a curvature in the bilayer plane, which induces membrane defects (Francis et al., 2001; Meyer et al., 1998; Pétriat et al., 2004; Zignani et al., 2000) and triggers the release of the entrapped cargo inside acidic organelles (Francis et al., 2001; Roux et al., 2002a). Alternatively, it was shown that the terminally alkylated NIPAM copolymer provided a steric barrier that enhanced, although marginally, the liposome circulation time *in vivo* (Roux et al., 2002b). The combination of both the terminally alkylated NIPAM copolymer and a PEGylated lipid in the vesicle structure was found to provide liposomes with both strong pH-responsive properties and a long half-life (Bertrand et al., 2009; Roux et al., 2004). This manuscript reports the *in vitro* evaluation and characterization of pH-sensitive liposomes based on NIPAM copolymers for the delivery of ara-C.

The encapsulation of ara-C into pH-responsive liposomes was demonstrated beneficial as this drug can be destroyed or inactivated easily by hydrolases or peptidases (Connor and Huang, 1986; Huang et al., 1983). Ara-C is a schedule-dependent antineoplastic drug used alone or in association with anthracycline agents in the treatment of acute myeloid leukemia (AML). When injected i.v. to animals or humans, the free drug is rapidly cleared, with most of it being deaminated to an inactive form in the first 20 min post-injection (Allen et al., 1992; Baguley and Falkenhaug, 1971). The incorporation of ara-C into long-circulating PEGylated liposomes has been found to substantially increase its therapeutic effect on L1210/C2 leukemia (Allen et al., 1992). However, some studies have shown that when incorporated inside conventional liposomes, ara-C was localized within lysosomal organelles where it degraded into its inactive form (Connor and Huang, 1986; Huang et al., 1983; Rustum et al., 1981).

The objective of the present work was to formulate pH-sensitive immunoliposomes (ILs) that would serve as effective chemotherapy agents against (AML). The ILs were designed to target CD33 (Gp67), a surface antigen expressed on over 80% of leukemia blasts from AML-suffering patients but not on healthy cells (Griffin et al., 1984). The murine anti-CD33 p67.6 monoclonal antibody (mAb) as targeting ligand binds the CD33 receptor with great avidity and is currently used in the clinic to treat AML. Indeed, the humanized p67.6 anti-CD33 conjugated with calicheamicin through an acid-labile linkage (gemtuzumab ozogamicin, Mylotarg®) has been approved in the United States in 2000 for the treatment of relapsed AML patients who are not candidates for conventional chemotherapy. Although this new treatment holds great promise, it is still associated with resistance to calicheamicin and serious side effects have been reported in a large proportion of treated patients (Giles et al., 2001). Thus, pH-sensitive ILs-CD33 with terminally alkylated P(NIPAM-*co*-MAA) loaded with ara-C could be an interesting avenue to target specifically myeloid leukemic cells and increase the intracellular bioavailability of the active drug (Fig. 1).

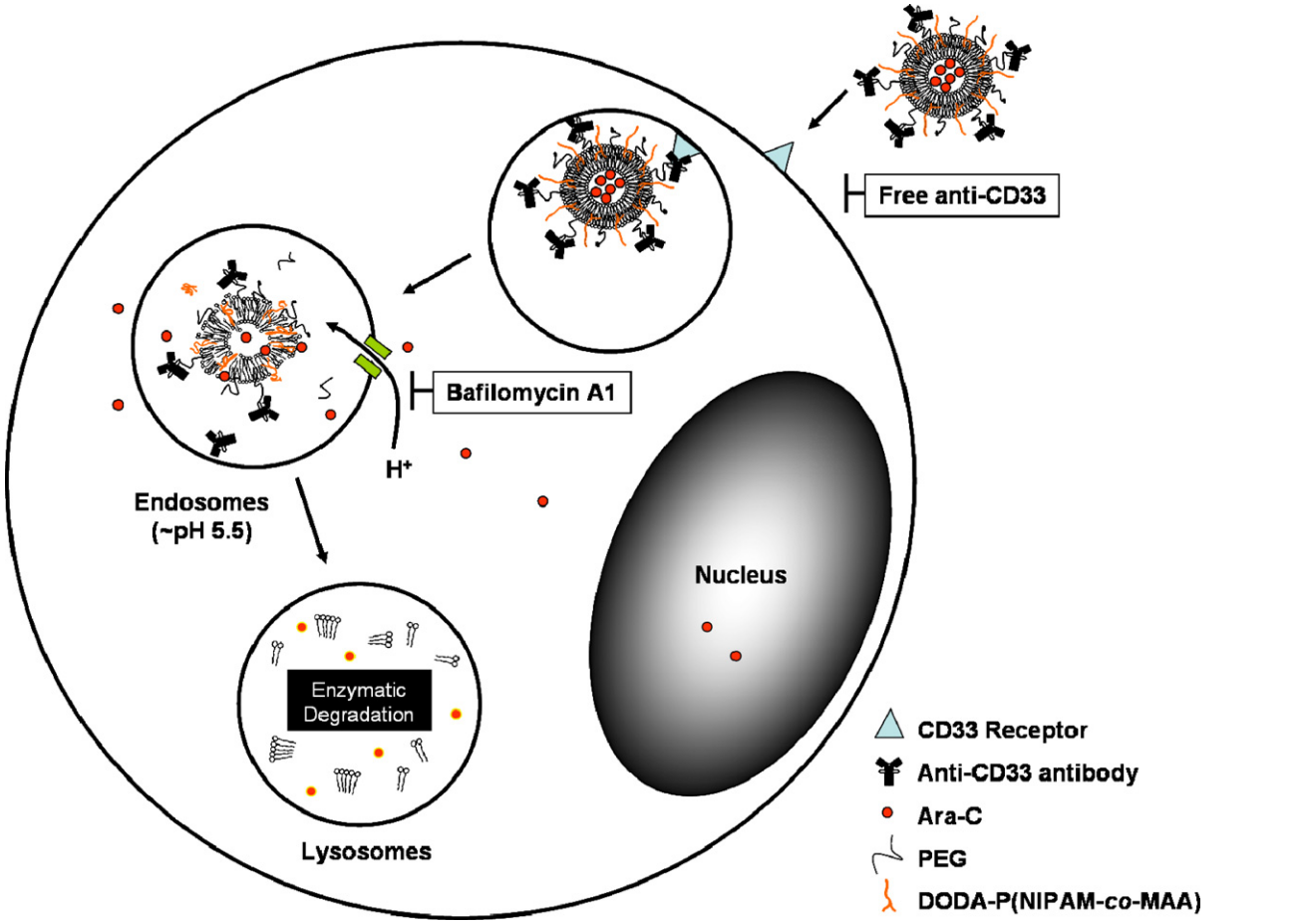

Fig. 1. Schematic representation of the binding and the internalization of the pH-sensitive ILs-CD33 through receptor-mediated endocytosis. Upon acidification of the endosomes, DODA-P(NIPAM-*co*-MAA) destabilizes the liposomal bilayer, thereby triggering the rapid release of encapsulated ara-C. In competitive assays free anti-CD33 binds the CD33 receptor and impedes the binding and the internalization of the ILs-CD33. Once internalized, the release of the pH-sensitive liposomal content can be slowed down by the addition of bafilomycin A1. The latter is a strong inhibitor of the vacuolar type H(+)-ATPase, which inhibits the acidification of the endosomes/lysosomes (Yoshimori et al., 1991).

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC, 760 g/mol), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[poly (ethylene glycol)] (DSPE-PEG) 2000 and cholesterol (Chol, 99.5% pure) were obtained from Northern Lipids Inc. (Vancouver, BC, Canada). DSPE-PEG 3400-maleimide was purchased from Laysan Bio Inc. (Arab, AL). NIPAM, MAA, Triton X-100, formaldehyde 37% (v/v), Sodium dodecyl sulfate (SDS), Sepharose® CL-4B, Sephadex® G-50, dithiotreitol (DTT), sodium *meta*-periodate, Ellman's reagent, ara-C, calcein, mouse isotype control IgG1b MOPC21 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Mowiol® was obtained from EMD Biosciences Inc. (Darmstadt, Germany). Purified anti-CD33 antibodies were obtained from AbD Serotec (Raleigh, NC). 3-(2-Pyridyldithio)propionyl hydrazide (PDPH) and the BCA protein kit were purchased from Pierce (Rockford, IL). [^{3}H]-Ara-C (15–30 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The HL60 (human promyelocytic leukemia cells), KG-1 (human myeloid cells), A549 (human lung carcinoma cells) were purchased from American Type Tissue Collection (ATCC, Rockville, MD). The THP-1 (human acute monocytic leukemia) cell line was donated by professor Ong (University of Montreal, Qc, Canada). Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoate (cholesteryl-BODIPY FL C12), trisodium 8-hydroxypyrene trisulfonate (HPTS), *p*-xylene-bis-pyrimidium (DPX), LysoTracker Red®, DAPI, RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin G (100 U/mL) and streptomycin (100 μg/mL) solution, and trypan blue were obtained from Invitrogen (Burlington, ON, Canada). All products were used without further purification. Water was deionized with a MilliQ purification system (Millipore, Bedford, MA) before use.

2.2. Preparation of copolymers

The terminally alkylated polymer was synthesized by free radical polymerization of NIPAM and MAA employing 4,4′-azobis(4-cyano-*N*,*N*-dioctadecyl)pentanamide (DODA-501) as the lipophilic initiator (NIPAM/MAA/DODA 93:5:2 mol%) (Leroux et al., 2001). A fluorescently labelled copolymer was also synthesized by adding methacryloxethyl thiocarbonyl rhodamine B during polymerization. The weight-average molecular weights (M_w) of the unlabelled and labelled copolymers were 11,000 (PI = 2.1) and 8600 (PI = 1.9), respectively. At 37 °C, these polymers undergo a coil-to-globule phase transition at pH 5.6, as ascertained by turbidimetry at 480 nm. The structure of the synthesized polymers was confirmed by H^1 NMR spectroscopy, and the purity of the labelled copolymers was verified by thin layer chromatography on silica using a mixture of methanol and chloroform (49:1) as eluent. The rhodamine

B content of the copolymer was assayed by spectrofluorimetry in PBS (pH 7.4) and was found to be 0.8 mol%.

2.3. Preparation of PEGylated pH-sensitive liposomes

Liposomes of EPC/Chol/DSPE-PEG2000/DSPE-PEG-maleimide (3:2:0.17:0.09 molar ratio) were prepared by the thin film hydration method. Briefly, lipids were dissolved in chloroform and mixed with 0.3 mol% of the fluorescent probe cholesteryl-BODIPY FL C12. This fluorescent marker is known to be a non-exchangeable lipidic probe (Dagar et al., 2001; Reaven et al., 1996). After solvent evaporation, the dried lipid film was placed *in vacuo* (~0.1 mBar) for at least 30 min to remove residual solvent. The film was then hydrated with a buffered solution of *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) 20 mM (pH 7.2) and NaCl (144 mM), or the appropriate solution of dye/drug. The liposomes were then extruded several times through polycarbonate membranes (400, 200 and 100 nm) using a LiposoFast extruder (Avestin, Ottawa, ON, Canada) to yield vesicles with diameters of ca. 160 nm (polydispersity index of 0.05). DODA-P(NIPAM-*co*-MAA) was added to the lipid mixture at a ratio of 0.1–0.3 (w/w) prior to the hydration step and excess polymer was removed by size exclusion chromatography (SEC). The liposome concentration was determined by the phosphorus assay (Bartlett, 1959).

2.4. Modification of the antibodies

The anti-CD33 (clone p67.6) and the isotype-matched control mouse IgG1 MOPC21 monoclonal antibodies (3–5 mg/mL) were oxidized at the carbohydrate sites with cold sodium *meta*-periodate (final concentration of 15 mM) at 4 °C during 40 min in sodium acetate buffer (0.1 M, pH 5.5) (Morehead et al., 1991). Excess of *meta*-periodate was quenched by adding a solution of glycerol (~15 mM) and removed by dialysis (cut-off 6–8000 g/mol) against acetate buffer (pH 5.5). After dialysis, the oxidized antibodies were reacted with PDPH (final concentration of 5 mM) (Ansell et al., 1996) during 5 h at room temperature under agitation. The PDPH-antibodies were dialyzed overnight against acetate buffer (0.1 M, pH 4.5). The following day, they were treated with DTT (25 mM) at room temperature for exactly 20 min. The reaction mixture was applied on Sephadex G-50 column and eluted with HBS (20 mM, pH 7.2) under a nitrogen flux. Twenty microliters of each of the collected fractions were treated with the Ellman's reagent (4 mg/mL in PBS) to verify the removal of excess DTT. The fractions containing the thiolated antibodies were pooled together under nitrogen atmosphere and the final protein concentration assayed with a BCA kit. A gel electrophoresis (SDS-PAGE) under non-reducing conditions using 10% acrylamide was conducted to verify the integrity of the antibodies following the modification process.

Immediately after the antibody modification, the functionalized liposomes containing DSPE-PEG-maleimide were coupled to the thiolated antibodies at a ratio of 100 μg proteins/μmol of lipids under nitrogen atmosphere. The mixture was incubated for 30 min at room temperature, followed by an overnight incubation at 4 °C on a rotating plate set at low speed. After the coupling period, all the formulations were incubated with β-mercaptoethanol for 20 min at room temperature to quench free maleimide groups. The vesicles were then chromatographed over a Sepharose® CL-4B column equilibrated with isotonic HBS (pH 7.2), to separate the liposomes from the excess β-mercaptoethanol and free antibodies. The amount of antibody conjugated was determined according to the modified-BCA protein assay method which included the use of 2% (w/v) SDS. With this coupling technique, it was estimated that 30–40 monoclonal antibody molecules were attached per individual liposome.

2.5. Particle size

Particle size was determined at 25 °C by dynamic light scattering (DLS) at a 173° angle on a Zetasizer Nanoseries (Malvern Instruments, Worcestershire, United Kingdom) using the Contin algorithm. The measurements of the mean hydrodynamic diameters (*z*-average) were performed in triplicate.

2.6. In vitro release of HPTS

ILs were prepared as mentioned above except that the lipid film was hydrated with a buffered solution of HEPES (20 mM, pH 7.4) containing the water-soluble fluorophore HPTS (35 mM) and the collisional quencher DPX (50 mM) (Daleke et al., 1990). After liposome formation, untrapped dye was removed by gel filtration over a Sephadex® G-50 column. The release of liposomal content was monitored during 30 min at 37 °C in 2-*N*-(morpholino)ethanesulfonic acid (MES)-saline buffer (100 mM MES, 144 mM NaCl, pH 5.0 or 5.5) or in PBS (35 mM, pH 7.4) on a Series 2 Aminco Bowman fluorimeter (Spectronics Instruments Inc., Rochester, NY). Some formulations were incubated in 50% (v/v) pre-heated human plasma for 1 h at 37 °C and then chromatographed onto CL-4B sepharose gel to remove the excess of plasma components before monitoring the release of HPTS. The extent of content release was calculated from HPTS fluorescence intensity (λ_{ex} = 413, λ_{em} = 512 nm) relative to measurement after vesicle disruption with Triton X-100 (10%, w/v), which triggered complete release of encapsulated HPTS and DPX.

2.7. Encapsulation and in vitro release of ara-C

The ara-C-loaded liposomes were prepared by hydrating the lipid film with a solution of ara-C (230 mM, spiked with [^{3}H]-ara-C, 5 μCi/mL) in HBS (5 mM, pH 7.4, 350 mOsm) for 5 h at 4 °C. After the extrusion process, the non-encapsulated drug was removed by gel filtration (1.5 cm × 20 cm) on a Sepharose® CL-4B column. The ara-C loading was determined by measuring the radioactivity in the eluted fractions using a scintillation counter (Liquid Scintillation Analyser Tri-Carb 2100TR, Packard, Meriden, CT). The encapsulation efficiency (EE) was calculated using Eq. (1):

$$\mathrm{EE}\,(\%) = \frac{\mathrm{AUC_E}}{\mathrm{AUC_T}} \times 100 \quad (1)$$

where $\mathrm{AUC_E}$ and $\mathrm{AUC_T}$ stand for area under the elution profile curve of the encapsulated and total drug in the feed, respectively.

The effect of plasma on pH-triggered release was assessed after incubation of the liposomes in 50% (v/v) human plasma at 37 °C for 1 h. The excess of plasma components was removed by gel filtration, and the vesicles were incubated for 30 min at 37 °C in PBS (pH 7.4) or isotonic MES buffer (pH 5.0). After incubation, the ara-C released was separated from the liposomes by ultrafiltration (5000 g × 15 min) using a Centricon® tubes (Millipore, cut-off 50 kDa). In a parallel experiment, the amount of drug directly released in 50% plasma was also measured. The extent of content released was calculated by radioactivity counting relative to measurement conducted after vesicle disruption with Triton X-100.

2.8. Cell culture

The human monocyte cell lines HL60, KG-1 and THP-1 were grown as suspension cultures in RPMI 1640, supplemented with 10% (v/v, KG-1 and THP-1) or 20% (v/v, HL60) heat-inactivated FBS, 2 mM glutamine, and 1% (v/v) penicillin G (100 units/mL) and streptomycin (100 μg/mL). The A549 cells were grown in monolayer in 75 cm^2-flasks containing 15 mL of DMEM supplemented with 10% (v/v) heat-inactivated FBS, penicillin G, and streptomycin. Cells

were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were resuspended or scrapped, and counted using Trypan blue exclusion assay with a hemacytometer. All experiments were performed on mycoplasma-free cell lines. Only cells in the exponential phase of growth were used.

2.9. Binding and internalization assays

Leukemic HL60, KG-1, THP-1 (CD33+ expression) and lung carcinoma A549 (CD33−) cells (5×10^5/tube) were incubated at 37 °C for 2 h with 0.2 μmol of ILs labelled with the hydrophobic probe BODIPY FL C12. Competitive binding assays were performed in the presence of 20-fold excess free antibody. Unbound liposomes were removed by washing three times with cold PBS and the cells were fixed with 1% (v/v) formalin/PBS during 10 min at 4 °C. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were acquired and analyzed with CellQuest software (Becton Dickinson). The mean fluorescence intensity (MFI) of single cells was recorded. Cell profiles were constructed according to parameters of side scatter (SSC) and forward scatter (FSC). This region was gated in order to exclude dead cells and cell debris. The excitation of cholesteryl-BODIPY FL C12 was obtained with an argon ion laser (488 nm) and the green fluorescence emission was recorded in the FL1 channel (530/30 nm). A total of 10,000 events were analyzed for each sample. The upper limit of background fluorescence was set such that no more than 1% of the events with the autofluorescence controls (attributable to native cells) occurred in the positive region. The internalization of the ILs-CD33 formulations containing rhodamine-labelled DODA-P(NIPAM-*co*-MAA) was confirmed by confocal microscopy (λ_{ex} = 543, λ_{em} = 560 nm), following the same experimental conditions as mentioned above.

2.10. Evaluation of the binding affinity of ILs

To evaluate the affinity of the anti-CD33 ILs, a fixed number of HL60 cells (3×10^3) were incubated at 4 °C with serial dilutions of the ILs during 3 h. Bound antibody was then detected with a goat anti-mouse FITC-conjugated antibody by flow cytometry. The apparent dissociation constant (K_{Dapp}) was calculated from Eq. (2):

$$\frac{1}{F} = \frac{1}{F_{max}} + \left(\frac{K_{Dapp}}{F_{max}}\right)\left[\frac{1}{C_{Ab}}\right] \quad (2)$$

where C_{Ab} is the molar concentration of the antibody, F is the measured fluorescence value after subtracting the background fluorescence, and F_{max} is the fluorescence value obtained when the cells are incubated with saturating concentrations of antibody. When plotting the F_{max}/F ratio as a function of the inverse of C_{Ab}, a regression line, whose slope represents the K_{Dapp}, can be drawn (Benedict et al., 1997; Occhino et al., 2004).

2.11. Intracellular release of calcein

In order to determine the intracellular release of a fluorescent probe in acidic cell compartments, calcein was encapsulated in ILs at a self-quenched concentration (120 mM) (Francis et al., 2001). Leakage of calcein from the liposomes in the cells results in an increase in fluorescence. Ten microliters of ILs or pH-sensitive ILs (2 μmol/mL lipids) were incubated 30 min at 37 °C with 5×10^5 HL60 cells in RPMI 1640 (10%, v/v FBS, without red phenol). In some samples, 500 nM bafilomycin A1 was added to the cells 30 min before the liposome formulations to block the acidification of the endosomes (Yoshimori et al., 1991). In the case where LysoTracker Red® was used for the staining of acidic cell organelles (*i.e.* endosomes and lysosomes), the probe was incubated 30 min at a final concentration of 80 nM. After incubation, cells were washed three times with cold PBS (pH 7.4), and fixed with 4% (w/v) paraformaldehyde during 25 min. The cells were washed again with PBS, and the DAPI probe was added in each tube (300 μL, 0.1 μM, 20 min at room temperature) to counterstain the nucleus in blue. They were subsequently washed three times with PBS to remove excess dye. For fluorescence imaging, the cover slips were mounted on glass slides containing a mixture of concentrated cells and Mowiol®. Cells were then analyzed on a Zeiss LSM 410 inverted confocal microscope (Zeiss, Jena, Germany) equipped with a high pressure mercury lamp (HBO 100) for excitation and a triple bandpass filter set. The cells were excited at 405, 488 and 543 nm, and fluorescence was collected by using emission windows set at 420, 505–530 and 560 nm, respectively.

2.12. Antiproliferative assay

Inhibition of cell proliferation was measured by MTT assay (Mosmann, 1983). Briefly, HL60 in logarithmic phase of growth were synchronized in S-phase following exposure to 2 mM thymidine for 24 h. The synchronized cells were rinsed and resuspended in RPMI 1640 containing 10% (v/v) heat-inactivated FBS (thymidine-free) and were seeded in 96-well round bottom tissue culture plates (100 μL RPMI–FBS containing 3×10^4 viable cells) at 37 °C in a humid atmosphere containing 5% CO_2. The liposomal formulations encapsulating 300 mM of ara-C (spiked with [^{3}H]-ara-C (16.7 μCi/mL)) were sterilized by filtration (0.45-μm pore size) and adjusted to the same ara-C concentration prior to their incubation with the cells. The encapsulated or free drug (20–40 μg/mL) were added to the cells 2 h after removing the thymidine, and incubated at 37 °C for 2 h. Controls (100% cell survival) were prepared by adding sterile HBS (10 mM, pH 7.2) to the cells. After incubation, the cells were washed 3 times with non-complete RPMI by centrifugation (800 *g* × 2 min), and resuspended in 100 μL complete medium. They were incubated for another 48 h, and MTT dissolved in PBS (10 μL of a 5 mg/mL solution) was added to each well. After 3 h, SDS (100 μL of a 10% (w/v) solution containing 0.01 N HCl) was added to each well to dissolve the reduced MTT. Absorbance was measured 24 h later at 570 nm using a Safire plate reader (Tecan, Medford, MA). Each experiment was undertaken at least in triplicate. To ascertain the significance of the differences observed between the liposomal formulations, statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Characterization of the pH-sensitive liposomes

In the past decade, we have extensively published on the synthesis, interaction with lipid bilayers and capacity of various pH-sensitive NIPAM copolymers to destabilize liposome membranes by introducing a curvature in the bilayer plane at acidic pH (Francis et al., 2001; Leroux et al., 2001; Meyer et al., 1998; Roux et al., 2002a, 2003, 2004; Zignani et al., 2000). We demonstrated that the interaction area between the phospholipids and the polymer increased at acidic pH while the copolymer was found to be partially dehydrated at neutral pH and physiological temperature. In this work, a terminally alkylated copolymer was used in this study instead of a randomly alkylated one, because it was shown to confer steric stability to the liposomes, while preserving its ability to destabilize liposomes at endosomal pH (Roux et al., 2004). When incorporated into PEGylated liposomes, the average particle size was ca. 160 nm, regardless of the initial ratio of polymer used (0.12–0.3 w/w). Experiments carried out with rhodamine-labelled

DODA-P(NIPAM-*co*-MAA) revealed that 34 ± 3% of the polymer was incorporated into the liposomal formulation. This polymer incorporation efficiency was about half that previously reported with randomly alkylated NIPAM copolymers incorporated by the same method (Zignani et al., 2000). Such a difference in the binding efficacy can be rationalized in terms of reduced anchoring points in the case of the terminally alkylated polymer. The formulation was physically stable as no change in terms of size distribution was noted after storage of this unloaded pH-sensitive PEGylated formulation at 4 °C over 1 year.

3.2. Modification of the antibodies

Different conjugation procedures have been reported for the coupling of targeting ligands to liposomes (Harasym et al., 1998; Nobs et al., 2004). The method chosen is important as it may modify the binding affinity of the antibody to its target. It may also affect its orientation at the vesicle surface and therefore its propensity to activate the complement system and be recognized by the MPS (Aragnol and Leserman, 1986). The antibody modification method applied here relies on the use of carbohydrate sites located in the Fc portion of the antibody. The latter is therefore expected to adopt a spacial conformation whereby the Fc region is oriented toward the liposome surface and less accessible against undesirable recognition by the MPS, while the Fab' domains are oriented outward for optimal antibody–antigen interaction (Harasym et al., 1998). As mentioned in Section 2, the sugar site on the antibody was oxidized to create aldehyde functions which were then reacted with the hydrazide group of the spacer arm PDPH. The 2-pyridyldisulfide moiety of PDPH was reduced with DTT in order to introduce sulfhydryl groups in the protein without altering the native disulfide bonds (Ansell et al., 1996). Gel electrophoresis (SDS-PAGE) experiments and incubation of the antibodies at the different stages of modification with HL60 cell line (CD33+) confirmed that the integrity and the antigen specificity were not affected during the process (data not shown). The PDPH-thiolated antibodies were coupled to the PEG termini of liposomes using a conventional DSPE-PEG-maleimide coupling method (Iden and Allen, 2001; Kirpotin et al., 1997; Mastrobattista et al., 1999). A 10–15 nm increase in the vesicle diameter (*z*-average) was observed following grafting of the antibodies and a coupling efficiency of approximately 15–20% was obtained. Despite a large number of maleimide reactive groups available on the surface of liposomes, this value lies within the normal range for this coupling technique (Huwyler et al., 1996). Better coupling efficiencies can be obtained with other conjugation methods (Nobs et al., 2004), but the final orientation of the antibody at the vesicle surface is not controlled. Furthermore, a ratio of ~20 μg antibody/μmol of lipids is generally sufficient to ensure efficient targeting (Ansell et al., 1996). The addition of DODA-P(NIPAM-*co*-MAA) to the liposome bilayer reduced the binding level of the CD33 antibody from 18 to 14 μg/μmol lipids, possibly through a steric hindrance effect. Indeed, the molecular weight

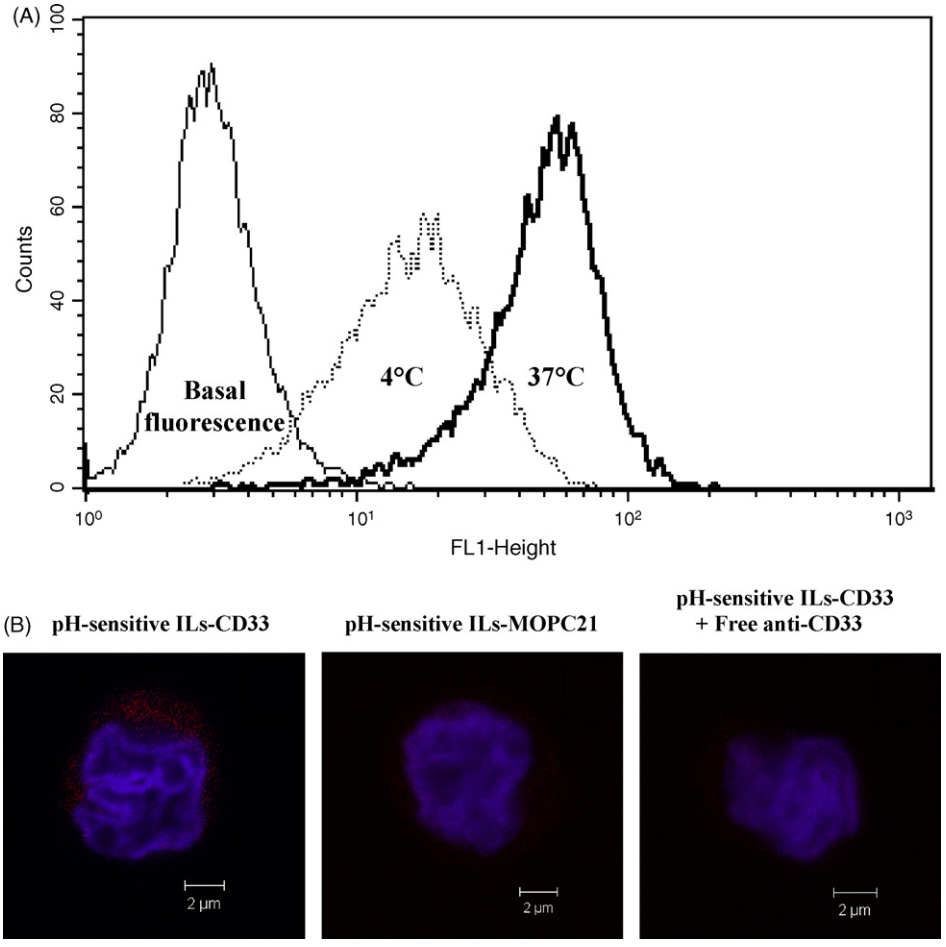

Fig. 2. (A) Fluorescent labelling of HL60 (CD33+) cells after 2 h incubation of pH-sensitive ILs-CD33 labelled with BODIPY FL C12 at 4 °C (dotted light line) and 37 °C (plain dark line) determined by flow cytometry. The *x*-axis represents the logarithm of green florescence signal, and the *y*-axis represents cell count. The first plain line represents basal cellular fluorescence (without any probe). (B) Confocal microscopy micrographs of an HL60 cell treated with ILs-CD33 (left panel), ILs-MOPC21 (middle panel), and pre-incubated with free anti-CD33 during 30 min before the addition of the ILs-CD33 (right panel). All the formulations contain rhodamine-labelled DODA-P(NIPAM-*co*-MAA) (red). Nuclei were stained with DAPI (blue).

of DODA-P(NIPAM-*co*-MAA) being greater than that of the PEG, the maleimide groups may be less accessible in the presence of the pH-sensitive polymer.

3.3. *In vitro* cellular association of ILs

In vitro cell association studies were performed to characterize the targeting efficiency of ILs to CD33+ leukemia cell lines. Fig. 2A shows the binding of the pH-sensitive ILs labelled with the hydrophobic probe cholesteryl-BODIPY FL C12 at 4 °C (no endocytosis) and at 37 °C (internalization). The ILs were recognized and internalized by the tumoral cells, corroborating previous data obtained with radiolabelled anti-CD33 antibodies (Caron et al., 1994; Press et al., 1996; Scheinberg et al., 1991). To determine the specificity of ILs-CD33 toward CD33+ cells, negative control binding experiments were performed using isotype-matched control ILs (ILs-MOPC21) or undecorated PEGylated liposomes (Fig. 3). These negative controls resulted in significantly lower internalization values after 2 h incubation with HL60 cells compared to ILs-CD33. Confocal micrographs of the ILs-CD33 labelled with cholesteryl-BODIPY FL C12 (data not shown) or rhodamine DODA-P(NIPAM-*co*-MAA) (Fig. 2B, first two panels) confirmed the cytofluorometry analysis. The ILs-CD33 were taken up by the cells by a specific receptor-endocytosis process. The copolymer was distributed equally within the cells, and the intensity of the fluorescence was lower for the control ILs-MOPC21 formulation.

A slight reduction of cell-associated fluorescence was observed when comparing pH-sensitive ILs-CD33 to non-pH-sensitive ILs-CD33 (Fig. 3A). A competitive binding assay was also conducted on HL60 cells by adding free anti-CD33 antibodies (20-fold) to the medium 30 min before adding the pH-sensitive ILs-CD33 formulation. The presence of free excess antibody produced a significant decrease in vesicle uptake, confirming the specificity of the interaction (Fig. 2B last panel and Fig. 3A). Finally, uptake experiments conducted with different CD33+ leukemia cells (HL60, KG1, THP-1) demonstrated that pH-sensitive ILs-CD33 were internalized by all cell lines expressing the receptor while limited interaction was found with the A549 (CD33−) cell line (Fig. 3B).

It was reported that the presence of a PEG corona may reduce the lateral mobility of the liposome-conjugated ligand and therefore limit the number of antibody molecules fully exposed at the surface of the liposome capable of interacting with the cell membrane receptor (Harasym et al., 1998; Mercadal et al., 1999). The repulsion between the cell surface and PEGylated liposomes may

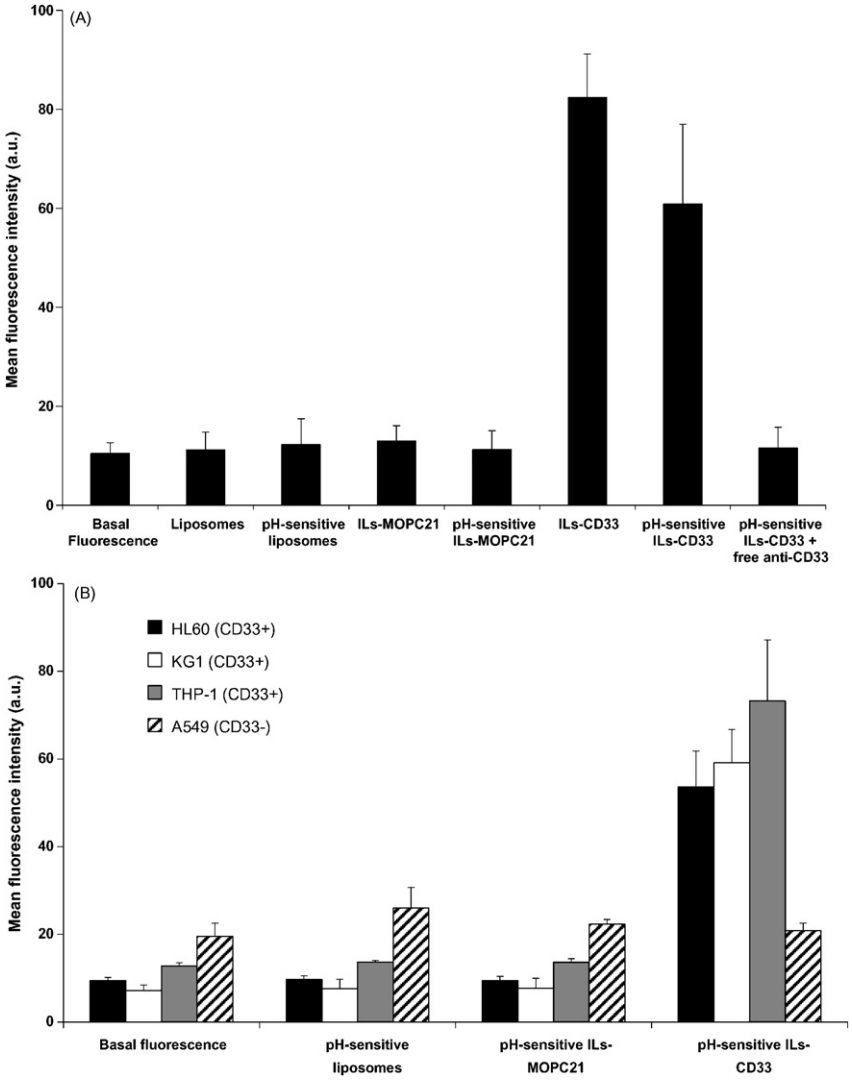

Fig. 3. (A) Uptake of different liposome formulations by HL60 cells. The last bar represents competitive binding assays of pH-sensitive IL-CD33 performed in the presence of a 20-fold excess free anti-CD33 antibody. Mean ± SD, $n = 4$. (B) Uptake of different pH-sensitive formulations by HL60 (black bars), KG1 (white bars), THP-1 (grey bars) and A549 (stripped bars) cells. Mean ± SD, $n = 3$.

decrease the free energy gain of the ligand binding to its receptor, therefore reducing the apparent affinity constant. Kirpotin et al. (1997) demonstrated that by increasing the DSPE-PEG content (0–5.7 mol%) in anti-HER2 liposomes prepared with a short spacer, 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (MMC), the liposome-cell binding affinity was decreased by 60–100-fold. However, when Fab' fragments were directly coupled to the extremity of the PEG chain (without a spacer), the surface grafted PEG did not affect the binding affinity. As lower internalization of the ILs-CD33 was obtained with the ILs decorated with DODA-P(NIPAM-*co*-MAA) compared to the control ILs-CD33 (Fig. 3A), it was first hypothesized that the binding site on the antibodies may be hidden by the flexible chain of the pH-sensitive polymer, subsequently reducing the binding affinity for the receptor. Therefore the K_{Dapp} values were calculated for both formulations. The pH-sensitive and pH-insensitive ILs-CD33 were found to exhibit a comparable affinity for HL60 cells with K_{Dapp} of $7.7 \pm 1.1 \times 10^{-10}$ M and $7.9 \pm 1.7 \times 10^{-10}$ M, respectively. These data suggest that DODA-P(NIPAM-*co*-MAA) did not alter the affinity of ILs for the CD33 receptor and that the lower amount of antibody grafted on the pH-sensitive vesicles (14 μg antibody/μmol lipid *vs.* 18 μg antibody/μmol lipid) is probably accountable for the reduced internalization in HL60 cells.

3.4. In vitro release of HPTS and ara-C

A main concern for DOPE-based pH-sensitive liposomes, or any other polymer configuration such as randomly alkylated NIPAM copolymer (Roux et al., 2003; Zignani et al., 2000) is the detrimental effect of PEGylation on the pH-sensitivity. Another issue regarding polymer-coated liposomes is their partial loss of pH-responsiveness (15–25%) following incubation in biological fluids (Roux et al., 2002a,b, 2003). Protein adsorption onto pH-sensitive vehicles may stabilize the system and/or partially extract the copolymer from the lipid bilayers (Roux et al., 2002a). In a former study, it was demonstrated that pH-sensitivity of PEGylated liposomes could be largely preserved after incubation in human serum when DODA-P(NIPAM-*co*-MAA) was employed as the triggered release polymer (Roux et al., 2004). Therefore, using the fluorescent probe HPTS co-encapsulated with the collisional quencher DPX, we verified whether this polymer would also maintain its activity when complexed to PEGylated liposomes decorated with an antibody. Release kinetics performed in the absence of plasma proteins showed that the polymer efficiently triggered, within the first 10 min, the release of 80 ± 1% of liposomal content at pH 5.0 (data not shown). Fig. 4 illustrates the percent of dye released from ILs bearing DODA-P(NIPAM-*co*-MAA) following 1 h incubation in 50% (v/v) human plasma. As expected, minimal dye leakage was observed at pH 7.4 (~5%, after 30 min). On the contrary, lowering the pH to 5.8 and 5.0 brought about a major increase in the release rate with 48 and 78% of HPTS released after 30 min, respectively. These data confirm that the pH-sensitive polymer fully preserved its ability to destabilize the liposomes at acidic pH after having been in contact with blood proteins, independently of the presence of the antibody at the liposome surface.

We then verified whether the anticancer drug ara-C could be released in a pH-dependent fashion from the ILs. For the pH-insensitive and pH-sensitive liposomes an entrapment efficiency of 10.7 ± 1.0 and 9.9 ± 2.3% was achieved, respectively. This indicated that DODA-P(NIPAM-*co*-MAA) did not affect the encapsulation of the drug. This entrapment efficiency is comparable to previously reported values which ranged from 1 to 20% (Hong and Mayhew, 1989). Incubation of ara-C-loaded pH-insensitive or pH-sensitive ILs in 50% (v/v) human plasma during 1 h was accompanied by a small drug loss of 4.3 ± 0.8 % and 5.6 ± 0.9% respectively, demonstrating that the stability of ILs in plasma was maintained in the

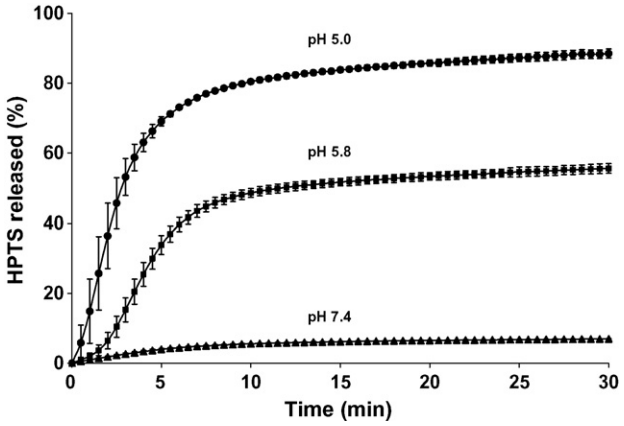

Fig. 4. *In vitro* release of encapsulated HPTS at 37 °C for pH-sensitive ILs at pH 5.0 (circle), 5.8 (square), and 7.4 (triangle) after 1 h incubation in 50% (v/v) human plasma. DODA-P(NIPAM-MAA) was incorporated during liposome preparation. The extent of content release was calculated from HPTS fluorescence intensity (λ_{ex} = 413 nm, λ_{em} = 512 nm) relative to the intensity obtained upon vesicle disruption with 10% (v/v) Triton X-100. Mean ± SD, n = 3.

presence of the pH-sensitive polymer. After removing the excess blood proteins, the ability of the pH-sensitive liposomes to release the encapsulated ara-C was also tested at pHs 5.0 and 7.4. As shown in Fig. 5, after 30 min incubation at pH 5.0, the pH-sensitive formulations released ~90% of their content, whereas less than 6% leaked from the pH-insensitive liposomal formulations. At neutral pH, the DODA-P(NIPAM-*co*-MAA)-coated liposomes appeared slightly more permeable to ara-C than HPTS. The antibody had apparently no impact on the pH-triggered drug release. The data presented herein indicate that pH-sensitive ILs formulated with DODA-P(NIPAM-*co*-MAA) would be stable in the blood but would rapidly release ara-C under the mildly acidic conditions found in the endosomal compartment.

3.5. Intracellular release of quenched calcein

In order to verify that cargo release could be achieved in the endosomes, ILs containing calcein encapsulated at a self-quenched concentration were prepared and incubated 30 min with HL60 cells. Upon release of the dye from the liposomes into the endosomal/lysosomal lumen, the cellular fluorescence is expected to increase significantly. Fig. 6 shows confocal microscopy photographs of HL60 cells exposed to pH-insensitive ILs-CD33 (panel A) and pH-sensitive ILs-CD33 (panel B). It can be observed that after 30 min exposure, the calcein fluorescence remained largely quenched in the case of the pH-insensitive formulation, indicating minimal dye release. On the opposite, the pH-sensitive ILs rapidly released their content as revealed by the more intense and diffuse fluorescence signal. Similar findings were obtained using phagocytic RAW264.7 (unpublished data) or J774 cells (Francis et al., 2001) in the absence of an antibody. In order to confirm that the intracellular release of calcein was a consequence of the acidification of intracellular organelles, pH-sensitive ILs-CD33 were then incubated with HL60 previously exposed to bafilomycin A1, an inhibitor of endosome/lysosmes acidification (Yoshimori et al., 1991). As shown in Fig. 6C, bafilomycin A1 suppressed the release of calcein, thereby confirming the pH-dependent release mechanisms of DODA-P(NIPAM-*co*-MAA)-coated ILs-CD33.

3.6. Antiproliferative assay

The anticancer agent ara-C was encapsulated into different liposomal formulations and its cytotoxic activity monitored on HL60

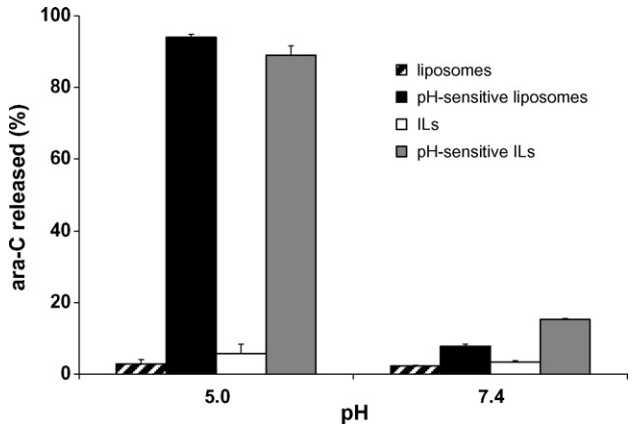

Fig. 5. Percent of ara-C released from pH-insensitive liposomes (stripped bars), pH-sensitive liposomes (black bars), ILs (white bars), and pH-sensitive ILs (grey bars) after 30 min incubation at 37 °C. The formulations were previously incubated in 50% (v/v) human plasma during 1 h at 37 °C. Mean ± SD, $n = 3$.

cells. Ara-C is generally thought to act specifically on the process of DNA synthesis (S-phase) and, therefore, is regarded as cell-cycle-specific (Hamada et al., 2002). This drug was chosen because of its susceptibility to degradation of the *N*-glycosidic linkage upon exposure to lysosomal hydrolases. Indeed, with ara-C, the benefits associated to increased transport through internalizing epitopes are partly offset by drug inactivation within the lysosomal organelles (Huang et al., 1983). In theory, this problem can be circumvented by using vesicles that release ara-C upon pH-triggered destabilization in the endosomes (Brown and Silvius, 1990; Connor and Huang, 1986; Rui et al., 1998). The free drug is then translocated in the cytoplasm by nucleoside transporters located in the endosomal/lysosomal membranes (Brown and Silvius, 1990; Pisoni and Thoene, 1989).

To avoid prolonged incubation time, and reduce potential indirect cellular toxicity caused by the leakage of ara-C in the incubation medium, the cells were first exposed to thymidine. This procedure allowed to synchronize a maximum of HL60 cells in the S-phase, and subsequently increased the antiproliferative outcome of the drug. The cytotoxicity experiments were conducted 2 h after removing the thymidine, because free ara-C alone showed the highest level of toxicity at that time after synchronization (unpublished data). Fig. 7 shows the percentage of surviving cells incubated with free ara-C or the following formulations: liposomes, ILs-MOPC21 and ILs-CD33 coated or not with DODA-P(NIPAM-*co*-MAA), at ara-C concentrations of 20 and 40 μg/mL. These concentrations were slightly above the IC_{50} of free ara-C (5 μg/mL). Free ara-C was more toxic than the control non-targeted pH-insensitive formulations (liposomes and ILs-MOPC21). This phenomenon was also observed with other pH-sensitive systems, such as non-targeted liposomes prepared with DOPE and oleic acid (8:2 molar ratio) where the liposomal formulations were found less cytotoxic than the free ara-C when incubated 3 h with the adherent L-929 cells (Connor and Huang, 1986).

The decoration of the liposomes with the CD33 antibody produced a significant increase ($p < 0.05$) in the cytotoxic activity of ara-C, reflecting the importance of receptor-mediated internalization for enhanced uptake of liposomal ara-C. Likewise, the addition of DODA-P(NIPAM-*co*-MAA) to the non-targeted liposomes improved significantly ($p < 0.05$) their efficiency, possibly through facilitated release of ara-C from the endocytosed liposomes. Indeed, the higher cytotoxicity of the pH-insensitive *vs.* pH-sensitive liposomes cannot be attributed to differences in the concentration of unencapsulated ara-C in the cell culture medium as it was verified in a control experiment that the free ara-C levels in the culture medium were low and independent of the formulation (data not shown). It is important to mention that the polymer alone did not exert any cytotoxic activity even at concentrations largely exceeding those used in this experiment (data not shown).

The combination of both the CD33 antigen and pH-sensitive polymer resulted in the highest cytotoxic activity, although statistical significance *vs.* the ILs-CD33 formulation could be achieved only at 20 μg/mL. Similarly, the combination of site-specific and other types of pH-sensitive liposome formulations was reported as a promising system to increase the cytotoxicity of loaded ara-C against L-929 cells (Connor and Huang, 1986), CV-1 cells (Brown and Silvius, 1990) and KB cells (Rui et al., 1998; Sudimack et al., 2002) when compared to pH-insensitive formulations. For example, a DOPE-based pH-sensitive formulation targeting folate receptors showed a 16-fold decrease in ara-C IC_{50} on adherent KB cells when compared to targeted pH-insensitive liposomes (Sudimack et al., 2002). It was hypothesized that this enhanced cytotoxicity of the DOPE-based formulation was conferred by the destabilization and/or fusion of the liposomes with the endosomal bilayer. Since NIPAM copolymers are devoid of membrane fusion activity (Zignani et al., 2000), it is possible that they are less efficient than DOPE-based liposomes in augmenting the intracellular bioavailability of ara-C. However, from a biological viewpoint, it may be difficult to compare different IL formulations, as the expression of target epitopes on cancer cells varies greatly, the turnover process is also divergent. Finally, these receptors may also be up- or down-regulated depending on the cell cycle or the differentiation state of the cancer cells (Mastrobattista et al., 1999). Compared to DOPE-based liposomes, pH-sensitive liposomes prepared with NIPAM copolymers present clear advantages such as better plasma stability and long-circulating properties (Roux et al., 2004), which may eventually translate into better *in vivo* efficacy.

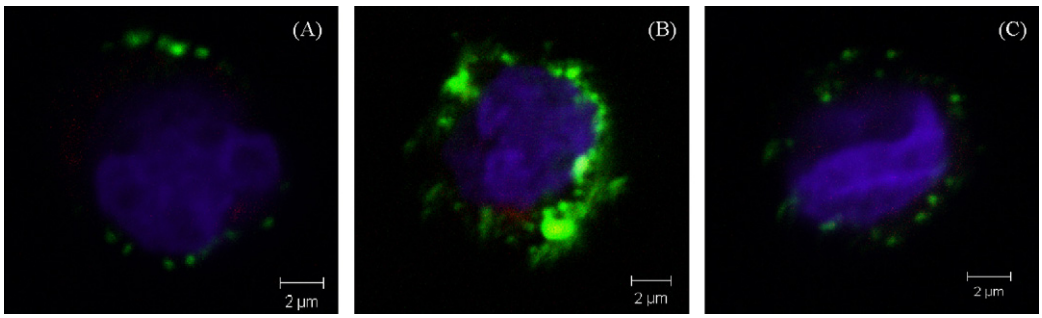

Fig. 6. Confocal microscopy micrographs of HL60 cells treated with pH-insensitive ILs-CD33 (A) or pH-sensitive ILs-CD33 (B) containing self-quenched calcein (green). Panel (C) shows HL60 cells treated with bafilomycin A1 30 min before addition of pH-sensitive ILs-CD33. The Nuclei were stained with DAPI (blue), and the acidic compartments were stained with Lysotracker Red® (red).

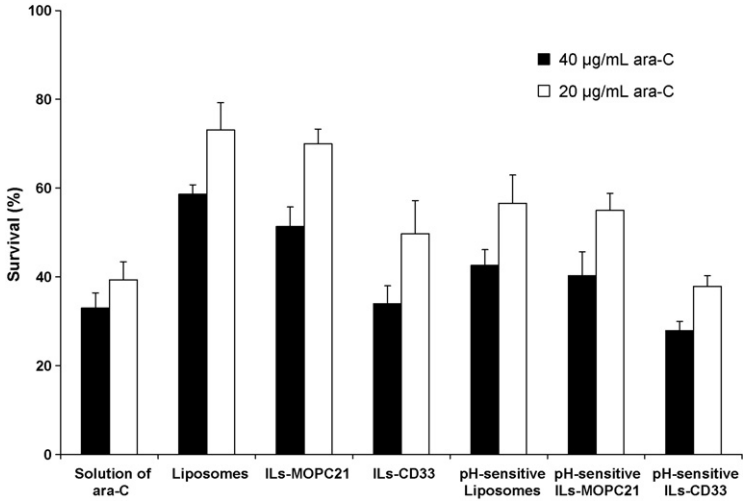

Fig. 7. Toxicity of encapsulated and free ara-C at a final concentration of 20 (open bars) and 40 μg/mL (closed bars) on HL60 cells after an incubation time of 2 h. Mean ± SD, $n = 3$. At 20 μg/mL of ara-C, the cytotoxicity induced by pH-sensitive ILs-CD33 is statistically superior ($p < 0.05$) to all the other liposomal formulations.

4. Conclusion

This study describes the preparation and *in vitro* evaluation of a targeted liposomal formulation containing pH-responsive properties that is selective toward leukemic cells expressing the CD33 antigen. The pH-sensitive copolymer DODA-P(NIPAM-*co*-MAA) was shown to destabilize the liposomal membrane of ILs under mildly acidic conditions and may play a beneficial role in the intracellular trafficking of encapsulated agents by facilitating the release of the encapsulated agent in the endosomes prior to its degradation in the lysosomes. However, at this stage the precise locus and extent of delivery of ara-C to the cytoplasm is still unknown. Future work will aim at evaluating the biodistribution and pharmacokinetic parameters of the pH-sensitive ILs-CD33 formulation in animal models and track the fate of the polymer under *in vivo* conditions. Considering these recent results and those previously published on pH-sensitive liposomes, the combination of site-specific and triggered drug release mechanisms may constitute a promising avenue in the treatment of AML.

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