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In Vivo Evaluation of pH-Sensitive Polymer-Based Immunoliposomes Targeting the CD33 Antigen

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Abstract: The purpose of this study was to evaluate *in vivo* a targeted pH-sensitive liposomal formulation tailored to promote the efficient intracellular delivery of 1-β-D-arabinofuranosylcytosine (ara-C) to human myeloid leukemia cells. Specifically, pH-sensitive immunoliposomes were obtained by anchoring a copolymer of dioctadecyl, *N*-isopropylacrylamide and methacrylic acid in bilayers of PEGylated liposomes (LP) and by coupling the whole anti-CD33 monoclonal antibody (mAb) or its Fab′ fragments. Their pharmacokinetic and biodistribution profiles were assessed in Balb/c and leukemic HL60-bearing immunodepressed (SCID) mice. In naive mice, nontargeted and pH-sensitive Fab′-LP had longer circulation times than LP with whole mAb. In SCID/HL60 ($CD33^+$) mice, the pharmacokinetic and biodistribution profiles of LP and encapsulated ara-C were comparable between nontargeted and pH-sensitive Fab′-LP. In leukemic mice, only pH-insensitive, ara-C-loaded Fab′ induced prolonged survival times. The apparent absence of pH-sensitive Fab′-LP effect could be related to lower exposure to ara-C in SCID mice.

Keywords: Immunoliposomes; pH-sensitive copolymers; *N*-isopropylacrylamide; acute myeloid leukemia; ara-C; pharmacokinetics

1. Introduction

Acute myeloid leukemia (AML) is a disorder of hematopoietic stem cells where a disruption of the differentiation process allows uncontrolled growth of blast clones in bone marrow. The accumulation of these abnormal cells eventually causes loss of normal hematopoietic function, eliciting neutropenia, anemia and thrombocytopenia.[1] Unless treated, AML has been associated with a high mortality rate due to increased susceptibility to infections or bleeding, both of which can occur within weeks after onset of the disease.

1-β-D-Arabinofuranosylcytosine (ara-C) remains the gold standard for first-line treatment of AML. It is part of current induction therapy, often in association with an anthracycline to improve the antitumoral response.[2] Ara-C is a pyrimidine analogue pro-drug which penetrates cells by a carrier-mediated transporter used by other nucleosides.[3] Once internalized, it needs to be metabolized intracellularly into its active triphosphate form (cytosine arabinoside triphosphate, ara-CTP) to exert its toxicity. Ara-CTP acts by inducing DNA breakdown through the inhibition of DNA synthesis and repair because of its effect on α- and β-DNA polymerases and its incorporation into DNA.[4,5] *In vitro*, the efficiency of ara-C is dependent on its intracellular bioavail-

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ability and exposure time.[6] In humans, the compound is rapidly converted to an inert metabolite, 1-β-D-arabinofuranosyluracil, by the ubiquitous enzyme cytidine deaminase, and eliminated by renal clearance within 7−20 min after intravenous (iv) administration.[7] As a consequence, repetitive dosing schedules or continuous iv infusion over 5−7 days and a high-dose regimen are required for optimal therapy.[8] This intensive treatment is not always well tolerated, especially in the elderly. Furthermore, for about 10−50% of newly diagnosed AML patients, treatment may not lead to remission, and the risk of developing resistance increases with each relapse. The chemoresistance of AML patients appears to be related to many different mechanisms, such as ara-C efflux by multidrug resistance *P*-glycoprotein from malignant cells, the expression of cytoplasmic 5′-nucleotidase, an enzyme preventing ara-C phosphorylation of ara-C, and human equilibrative nucleoside transporter 1 deficiency involved in ara-C internalization.[9,10] Therefore, efforts to facilitate ara-C delivery are expected to enhance the intracellular bioavailability of ara-CTP and improve the outcome of AML.

Previous studies have demonstrated the ability of liposomes (LP) to encapsulate ara-C, protect it from extracellular deamination and prolong its half-life.[11,12] Moreover, opportunities for new promising liposomal treatments have come from the identification of novel targets. Different targeting moieties have been investigated to facilitate the cellular uptake of ara-C-loaded LP. They include transferrin,[13] anti-D4.2 monoclonal antibody (mAb),[14] anti-H-2K^k mAb,[15] and anti-CD33 mAb.[16] The latter binds the CD33 receptor, a 67 kDa glycoprotein expressed on the surface of leukemia cells, from more than 80−90% of patients with AML.[17] This receptor is expressed neither on normal hematopoietic stem cells nor on nonmyeloid tissue.[18] Because CD33 is rapidly internalized after antibody binding, an antibody-cytotoxic agent conjugate or a targeted nanocarrier can effectively be taken up specifically by leukemia cells. The anticancer agent, calicheamicin, linked to recombinant, humanized anti-CD33 antibody called Mylotarg (Gemtuzumab Ozogamicin), has demonstrated great clinical promises. Actually, Mylotarg is approved by the US Food and Drug Administration since 2000 as a single-agent therapy for CD33-positive AML in first-relapse patients who are not considered candidates for standard cytotoxic therapy.[19]

Although liposomal ara-C can be specifically delivered to target cells with mAb, its degradation in lysosomes after receptor-mediated endocytosis limits its intracellular bioavailability.[15,20,21] In this regard, pH-sensitive LP that are stable at physiological pH but undergo destabilization and release their content under acidic conditions constitute a hopeful approach. In our laboratory, promising *in vitro* results have been obtained with sterically stabilized LP composed of egg phosphatidylcholine (EPC), cholesterol (Chol) and a pH-sensitive copolymer made of dioctadecyl (DODA),

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N-isopropylacrylamide (NIPAM) and methacrylic acid (MAA). Indeed, these polymeric pH-sensitive LP were found to be stable in the presence of plasma proteins,[16,22,23] trigger the release of cargo entrapped cargo in acidic intracellular organelles,[16,24,25] target specifically the CD33 cell surface antigen when decorated with a mAb,[16] and induce cell death by delivering loaded active ara-C.[16,25] The present study is aimed at comparing immunoliposomes targeted *via* whole anti-CD33 mAb and its Fab′ fragment to conventional PEGylated-LP [PEG = poly(ethylene glycol)] in terms of their *in vivo* pharmacokinetic and biodistribution profiles in healthy and immnunodeficient mice inoculated with HL60 leukemic ($CD33^+$) cells. In a preliminary experiment, the anticancer efficacy of ara-C encapsulated in different pH-sensitive LP was also investigated.

2. Materials and Methods

2.1. Materials. EPC (760 g/mol), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy (DSPE)-PEG 2000 and 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), Sepharose CL-4B, Sephadex G-50, dithiotreitol (DTT), sodium *meta*-periodate, Ellman's reagent, ara-C, and mouse isotype control IgG_{1b} MOPC21 were procured from Sigma-Aldrich (St. Louis, MO). Purified anti-CD33 antibodies were from AbD Serotec (Raleigh, NC). 3-(2-Pyridyldithio)propionyl hydrazide (PDPH), bicinchoninic acid (BCA) protein kit and Immunopure IgG_1 Fab′ and $F(ab)_2$ preparation kit were bought from Pierce (Rockford, IL). Centricon-10 and -50 tubes were supplied by Millipore (Milford, MA). PD-10 desalting columns were from GE Healthcare Life Science (Uppsala, Sweden). [^{3}H]-Ara-C (15−30 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO). [^{14}C]-Cholesteryl oleate (52 mCi/mmol) and [^{3}H]-ara-C (33 Ci/mmol) were obtained from Perkin-Elmer (Waltham, MA). HL60 (human promyelocytic leukemia cells) were from the American Type Culture Collection (Rockville, MD). Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoate (cholesteryl-BODIPY FL C12), RPMI 1640, Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin G (100 U/mL), streptomycin (100 μg/mL) solution, and trypan blue were procured from Invitrogen (Burlington, ON, Canada). All products were used without further purification. Water was deionized with a Milli-Q purification system (Millipore, Bedford, MA).

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 %), as described elsewhere.[26] The composition of the synthesized polymers was confirmed by ^{1}H NMR spectroscopy. The weight-average molecular weight (M_w) of the copolymer was 11,000 [polydispersity index (PI) = 2.1]. This DODA-P(NIPAM-*co*-MAA) had a coil-to-globule phase transition at pH 5.6 at 37 °C.

2.3. Preparation of PEGylated pH-Sensitive LP. LP 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 and DODA-P(NIPAM-*co*-MAA) (0.12 w/w) were dissolved in chloroform and mixed with 0.3 mol % of the fluorescent probe cholesteryl-BODIPY FL C12. After solvent evaporation, the film was hydrated with a buffered solution of *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) (HBS, 20 mM, pH 7.2) and NaCl (144 mM), or a solution of ara-C (230 mM, 5 mM HEPES, pH 7.4, 340 mOsm). The LP were then extruded several times through polycarbonate membranes (400, 200, and 100 nm) in a LiposoFast extruder (Avestin, Ottawa, ON, Canada) to yield vesicles with diameters of ca. 140−160 nm (PI of 0.03−0.05) as determined by dynamic light scattering (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, U.K.). Excess polymer and unencapsulated ara-C were removed by gel filtration on a Sepharose CL-4B column.[16,23] LP concentration was quantified by phosphorus assay.[27]

2.4. Modification of Antibodies. Anti-CD33 antibodies (clone p67.6) (5−10 mg/mL) were oxidized at carbohydrate sites with cold sodium *meta*-periodate (final concentration of 15 mM) at 4 °C for 40 min in sodium acetate buffer (0.1 M, pH 5.5). After removing the excess by dialysis (cutoff 6−8,000 g/mol), the oxidized antibodies were reacted with PDPH (final concentration of 5 mM) for 5 h at room temperature under agitation. PDPH-antibodies were purified overnight by dialysis 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 a Sephadex G-50 column and eluted with HBS (20 mM, pH 7.2) under nitrogen flux. Twenty microliters of each collected fraction was treated with Ellman's reagent (4 mg/mL in PBS) to verify the removal of excess DTT. Fractions containing thiolated antibodies were pooled

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together under nitrogen atmosphere, and the final protein concentration was assayed with a BCA kit.

2.5. Preparation of F(ab)$_2$ and Fab′ Fragments. F(ab)$_2$ fragments of antibody were produced with Immunopure IgG$_1$ Fab′ and F(ab)$_2$ preparation kits. In brief, anti-CD33 antibody was concentrated in Centricon-50 tubes (5−10 mg/mL), resuspended in 0.5 mL of phosphate-buffered saline (PBS) and added to 0.5 mL of Immunopure IgG$_1$ mild elution buffer from kits containing 1 mM of cysteine. The solution was then incubated with an immobilized ficin column for 35−40 h at 37 °C. It was eluted with 4 mL of Immunopure binding buffer, and fragments were separated on a protein A column, which retained Fc fragments and undigested IgG$_1$, whereas F(ab)$_2$ fragments were collected in 0.5 mL fractions. Fractions containing F(ab)$_2$ were analyzed from absorbance reading at 280 nm and pooled together. The F(ab)$_2$ fragments (110 kDa) were then concentrated in Centricon-50 tubes and resuspended in 1 mL of PBS.

F(ab)$_2$ fragments were incubated with a final concentration of 0.05 M of 2-mercaptoethylamine-HCl (MEA, pH 6.0) for 90 min at 37 °C under nitrogen atmosphere. MEA cleaves disulfide bridges between heavy chains but preserves disulfide linkages between heavy and light antibody chains. The solution (2.5 mL) was eluted on a PD-10 column (Sephadex G-25 Medium gel, 5 cm), and Fab′ fragments were collected in 0.5 mL fractions. Ellman's assay was performed to confirm the separation of MEA residue from the Fab′ fragments. Fractions containing Fab′ fragments (55 kDa) were determined from absorbance reading at 280 nm, pooled together and concentrated in Centricon-10 tubes under nitrogen atmosphere. A gel electrophoresis (SDS−PAGE) under nonreducing conditions using 10% acrylamide was conducted to verify the integrity of the antibodies following the modification process.

2.6. Coupling Reaction. Immediately after antibody modification (whole antibodies or Fab′ fragments), functionalized LP containing DSPE-PEG maleimide were coupled to thiolated antibodies or thiolated Fab′ fragments under nitrogen atmosphere at a ratio of 100 and 35 μg of proteins per μmol of lipids, respectively. The mixture was incubated for 30 min at room temperature, followed by overnight incubation at 4 °C on a rotating plate set at low speed. After the coupling period, all the formulations were incubated with a 5-fold excess of β-mercaptoethanol (from a 3 mM solution) relative to DSPE-PEG maleimide concentration for 20 min at room temperature to quench free maleimide groups.[13,28] The vesicles were then chromatographed over a Sepharose CL-4B column equilibrated with isotonic HBS (pH 7.2), to separate LP from excess β-mercaptoethanol and free antibodies/fragments.

2.7. Liposomal Lipid Extraction Procedure and Ara-C Assay. Lipids from the liposomal formulations were extracted before determining, by high performance liquid chromatography (HPLC), the ara-C concentration encapsulated into these vesicles. For lipid extraction, 200 μL of liposomal suspension was mixed with 250 μL of dichloromethane, followed by 500 μL of methanol. The mixture was then vortexed until a clear solution was obtained. Next, 250 μL of a 0.2 M NaOH solution and another 250 μL of dichloromethane were introduced and vortexed vigorously.[29] The sample, containing 2 phases, was centrifuged for 5 min (3000*g*) in glass tubes. Lipids were located in the lower phase, and ara-C was found in the upper water−methanol phase. Ara-C concentration in the supernatant was assayed with a Waters HPLC system equipped with a 1,525 binary pump, a 2,487 dual wavelength absorbance detector, and Breeze chromatography software (Waters, Milford, MA). The mobile phase consisted of 100 mM sodium acetate (pH 5.5) plus 1% (v/v) acetonitrile.[30] The column was a Waters Nova-Pack C18, 60 Å, 4 μm (3.9 × 300 mm). Flow rate, detection wavelength, temperature, and injection volume were set at 1 mL/min, 272 nm, 25 °C, and 40 μL, respectively.

2.8. Cell Culture and Internalization Assays. The human monocyte cell line HL60 (CD33$^+$) was grown as cultures suspended in RPMI 1640, supplemented with 20% (v/v) heat-inactivated FBS, 2 mM glutamine, 1% (v/v) penicillin G (100 U/mL) and streptomycin (100 μg/mL). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed on mycoplasma-free cell lines. Only cells in the exponential phase of growth were used.

Leukemic HL60 cells (5 × 10^5/tube) were incubated at 37 °C for 2 h with 0.2 μmol of different LP formulations labeled with the hydrophobic probe BODIPY FL C12. Competitive binding assays were conducted in the presence of 20-fold excess, free anti-CD33 antibody. At the end of the incubation period, LP that did not bind to the cells were removed by washing 3 times with cold, isotonic PBS. The cells were fixed with 1% (v/v) formalin/PBS for 10 min at 4 °C, and the mean fluorescence intensity of single cells in each sample was recorded with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were acquired and analyzed with CellQuest software (Becton Dickinson). Cell profiles were constructed according to parameters of side scatter and forward scatter. This region was gated in order to exclude dead cells and cell debris. Cholesteryl-BODIPY FL C12 excitation was obtained with an argon ion laser (488 nm), and 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 so that no more than 1% of events with autofluorescence controls (attributable to native cells) occurred in the positive region.

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2.9. *In Vivo* Pharmacokinetics and Biodistribution. *In vivo* studies were undertaken in naive female Balb/c mice (~20 g), and CB17 immunodepressed (SCID) mice 6−8 weeks old (Charles River, St-Constant, QC, Canada) inoculated with 1.2×10^7 leukemic HL60 cells *via* the tail vein. They were approved by the Animal Welfare and Ethics Committee of the University of Montreal in accordance with Canadian Council on Animal Care guidelines. Ara-C-loaded, pH-sensitive LP labeled with [^{14}C]-cholesteryl oleate and [^{3}H]-ara-C were prepared, as described in section 2.3.

The Balb/c mice were divided into 4 groups (4 mice/group). The first group received a solution of ara-C spiked with [^{3}H]-ara-C, whereas the second, third and fourth groups were injected with ara-C-loaded, pH-sensitive LP, pH-sensitive mAb-LP and pH-sensitive Fab′-LP, respectively. The formulations (160 μL) were given *via* the tail vein with 40 μmol/kg of lipids, corresponding to 3.4 mg/kg ara-C, 15 μCi/kg [^{3}H]-ara-C and 35 μCi/kg [^{14}C]-cholesteryl oleate. For experiments with SCID mice, ara-C-loaded, pH-sensitive LP and pH-sensitive Fab′-LP were injected 7 days postinoculation of the HL60 cells, at the same dose as the Balb/c mice. All the formulations were administered in animals less than 1 week after their preparation to avoid premature drug leakage, aggregation or loss of specificity.

Blood samples (400 μL) and major organs (*i.e.*, heart, lungs, spleen, liver, muscle, bone marrow, kidneys) were excised from individual euthanized animals at selected time points, *i.e.* 30 min and 1, 2, 4, 8, 12, and 24 h postinjection of the formulations. The mice were perfused with saline prior to harvesting the organs. Blood and tissues were weighed and treated with Solvable (Perkin-Elmer, Waltham, MA). After digestion, blood samples were bleached by successive additions of hydrogen peroxide (30% v/v). The samples were left to stand in the dark overnight at 4 °C after the addition of Hionic Fluor scintillation cocktail (Perkin-Elmer). Radioactivity was measured by scintillation counter in dual mode (^{3}H/^{14}C). The mean area under the blood concentrations vs time curve (AUC_{0-24h}), blood clearance (CL) and volume of distribution (V_d) parameters were determined in a noncompartmental model. The apparent half-life of the β-elimination phase ($t_{1/2\beta}$) of all the formulations injected was calculated by linear regression of the 4 last time points of the pharmacokinetic data. The data were analyzed statistically by one-way analysis of variance followed by the Tukey test. Differences were considered significant at $p < 0.05$.

2.10. *In Vivo* Survival Experiment. SCID mice were inoculated with 1.2×10^7 HL60 cells *via* the tail vein. At 24 h postinoculation, they were injected iv with saline (control) or a 8 mg/kg dose of free ara-C, ara-C-loaded Fab′-LP or pH-sensitive Fab′-LP. A second injection (8 mg/kg) of free and encapsulated ara-C was given to the mice 3 days after inoculation of the cells. They were monitored, weighed daily up to 79 days by Mispro Biotech Services Inc. (Montreal, QC, Canada). The mice were euthanized when tumor size exceeded 1,500 mm^3 or when they developed hind-leg paralysis. The significance of differences between the experimental groups ($n = 8$ mice/group) in the survival experiment was determined by Kaplan−Meier curves with the log-rank (Mantel−Cox) test using GraphPad Prism software version 5.0 (San Diego, CA). These findings were considered significant at $p < 0.05$.

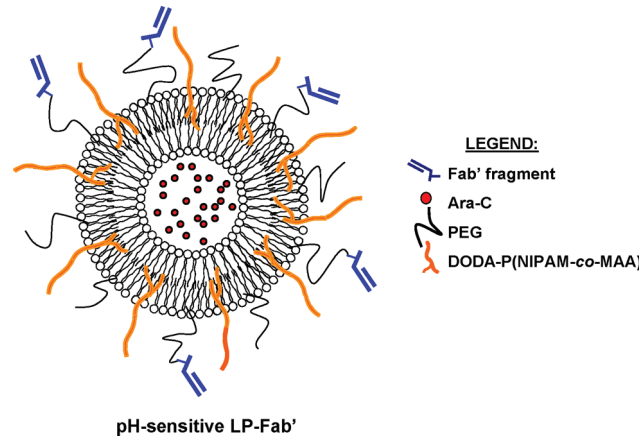

Figure 1. Schematic representation of pH-sensitive anti-CD33 Fab′-LP.

3. Results and Discussion

3.1. Preparation and Characterization of pH-Sensitive Immunoliposomes. Ara-C, a potent inhibitor of DNA synthesis, has been employed clinically as an antitumor agent for the treatment of AML.[2,5] However, in plasma, tissues and cells, it is easily deaminated by deoxycytidine deaminase into its uracil analogue, thereby losing its antitumor activity. The purpose of this study was to characterize the pharmacokinetics and biodistribution of ara-C encapsulated in pH-sensitive LP that target leukemia cells and rapidly release their cargo upon uptake. Drug release is triggered by destabilization of the bilayer membrane after the phase transition of membrane-anchored DODA-P(NIPAM-*co*-MAA). As opposed to most other pH-sensitive LP described in the literature,[31] these liposomal formulations are relatively stable in the presence of blood proteins and exhibit long circulation times *in vivo*.[16,25,32] To ensure better selectivity, such pH-sensitive LP can be further decorated with a targeting ligand. Recently, we reported the conjugation of anti-CD33 mAb to the surface of ara-C-loaded, pH-sensitive LP.[16] These nanocarriers showed specific cellular uptake and improved ara-C cytotoxicity against HL60 leukemia cells. The whole mAb was attached to the LP surface after the oxidation of carbohydrate sites from the Fc fragment, derivatization with PDPH and conjugation with DSPE-PEG maleimide.[16] As for Fab′-decorated LP (Figure 1), the fragments were prepared by first cleaving the whole mAb into F(ab)$_2$ on an immobilized ficin column in the presence of cysteine as activator.[33] Fab′ was then obtained from F(ab)$_2$

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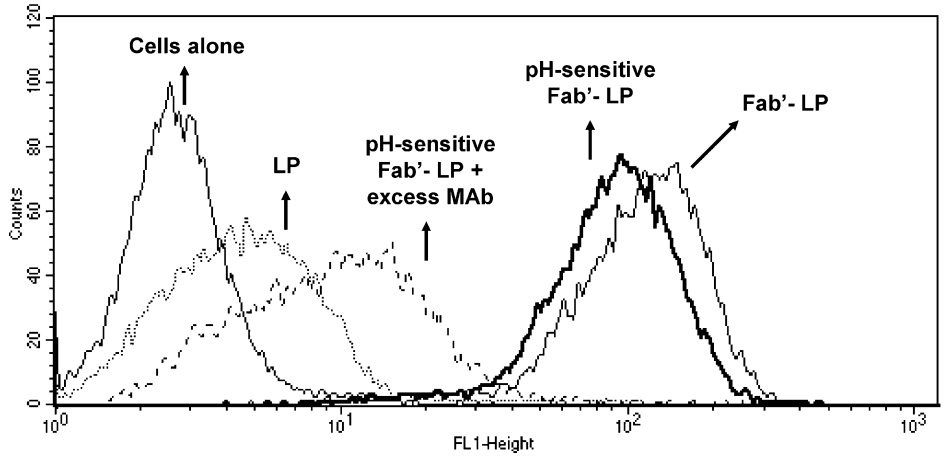

Figure 2. Fluorescent labeling of HL60 ($CD33^+$) cells after 2 h incubation of different LP formulations labeled with BODIPY FL C12 at 37 °C, as determined by flow cytometry. The *x*-axis represents the logarithm of green florescence signal, and the *y*-axis is the cell count. The first plain line corresponds to basal cellular fluorescence (without any probe). The striped line represents competitive binding assays of pH-sensitive LP-Fab′ performed in the presence of a 20-fold excess of free anti-CD33 antibody in the medium.

after incubation with MEA-HCl which cleaves disulfide bridges between the heavy chains (yield ca. 100%). Thiolated Fab′ was reacted with DSPE-PEG maleimide, as described for the whole mAb.[34] Coupling efficiency for both proteins on the vesicles was in the 15−30% range (data not shown). This percentage lies within the normal range for this coupling technique.[35,36] To graft the same number of mAb and Fab′ targeting moieties on the LP surface, the feed ratios of fresh thiolated proteins were fixed at 100 and 35 μg per μmol of lipids, respectively. With this coupling technique, it was estimated that 30−40 monoclonal antibody molecules were attached per individual liposome. Targeted vesicles, with a mean diameter of ∼140−160 nm (PI = 0.03−0.05) were obtained.

To determine whether attachment of anti-CD33 Fab′ to pH-sensitive LP would target HL60 ($CD33^+$) cells *in vitro*, the formulations were fluorescently labeled with cholesteryl-BODIPY FL C12, and cellular association (binding + internalization) was monitored by flow cytometry (Figure 2), as reported previously for LP decorated with the full mAb.[16] As expected, a relatively low amount of nontargeted LP was taken up by HL60 cells after 2 h. Coating the LP with anti-CD33 Fab′ resulted in a 4.5-fold increase in fluorescence intensity uptake, where 99% of the cells incubated with Fab′-LP were positively stained vs only 5% for control LP. There was a slight difference between the uptake of pH-sensitive Fab′-LP and Fab′-LP devoid of DODA-P(NIPAM-*co*-MAA). This phenomenon can be ascribed to the steric hindrance created by DODA-P(NIPAM-*co*-MAA) which possesses a M_w of 11,000 Da, largely superior to that of DSPE-PEG-maleimide (3400 Da). Similar results were obtained previously with the coupling of whole mAb.[16] A competition experiment performed with 20-fold excess of free anti-CD33 mAb resulted in decreased cell association of the targeted Fab′-LP (Figure 2). These data confirmed the binding specificity of anti-CD33 Fab′-LP.

3.2. Pharmacokinetics and Biodistribution in Balb/c Mice. The pharmacokinetics and biodistribution profiles of nontargeted or targeted pH-sensitive LP loaded with ara-C were first examined in naive Balb/c mice, where distribution was not affected by the presence of tumor cells (Figures 3A, 5A and S1 in the Supporting Information, Table 1). As reported previously by our group, nontargeted, PEGylated, pH-sensitive LP exhibited relatively long circulation times,[23] although slightly lower than pH-insensitive LP.[37] As illustrated in Figure 3A, in Balb/c mice, less than 16% of the total injected dose was still present in blood 12 h postinjection, which was approximately 2-fold lower than that observed in rat.[23] Compared to other PEGylated-LP formulations administered intravenously to Balb/c mice, the circulation time of our pH-sensitive LP was shorter. Maruyama et al.[38] showed that approximately 80 and 30% of the injected dose of PEGylated LP prepared with similar lipids (EPC/Chol, 1:2 molar ratio) circulated at 30 min and 12 h

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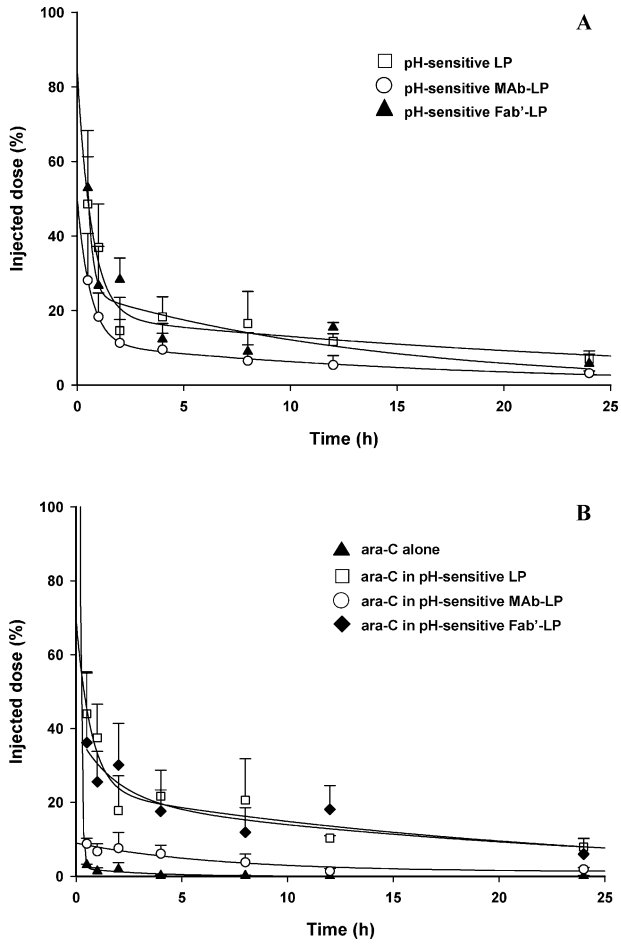

Figure 3. Blood concentration−time profiles of pH-sensitive LP and pH-sensitive LP bearing whole anti-CD33 mAb or the Fab′ fragment (A) and of free and encapsulated ara-C (B) in naive Balb/c mice. Each mouse received iv 3.4 mg/kg ara-C and 40 μmol/kg of lipids. Values represent the mean (±SD) obtained for $n = 4$ animals per group per time point.

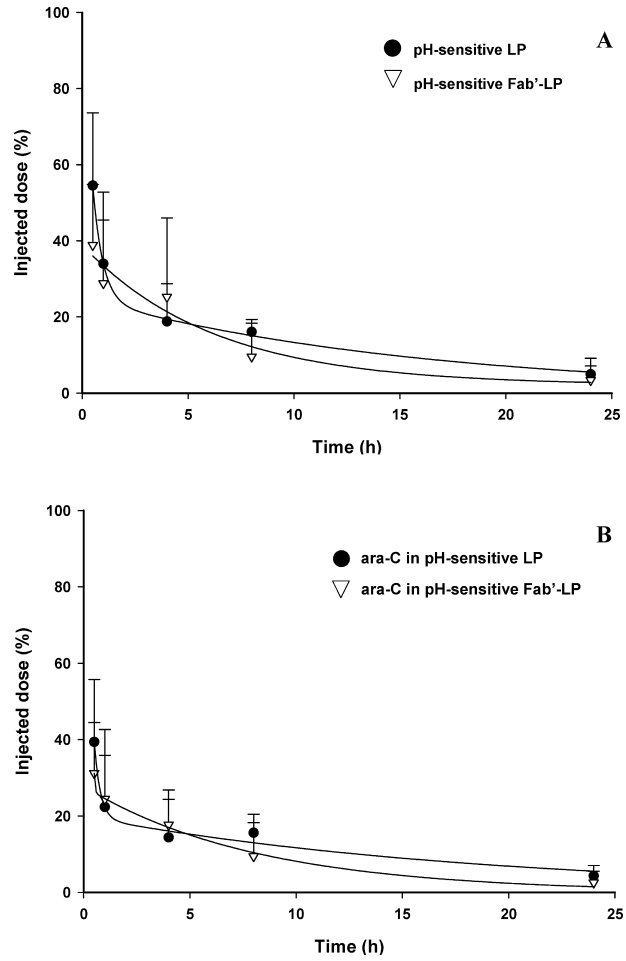

Figure 4. Blood concentration−time profiles of pH-sensitive LP and pH-sensitive anti-CD33 LP bearing the Fab′ fragment (A) and of encapsulated ara-C (B) in SCID-HL60 mice. Each mouse received iv 3.4 mg/kg ara-C and 40 μmol/kg of lipids. Values represent the mean (±SD) obtained for $n = 4$ animals per group per time point.

postinjection, respectively. As shown in Figure 3A, 49 ± 13% and 12 ± 2% of our pH-sensitive formulation was found in the blood compartment at the same times points. Apart from the presence of the pH-sensitive polymer, the main difference between both formulations is the LP size which was slightly larger in our case. The larger diameter may have in part contributed to the observed shorter half-life.[39]

Compared to the nontargeted formulation, the decoration of pH-sensitive LP with whole anti-CD33 mAb resulted in a substantial decrease of liposomal blood levels. For example, 1 h after administration, 37% of injected pH-sensitive LP were still circulating versus only 18% of pH-sensitive mAb-LP. Overall, CL increased by more than 2-fold (Table 1). In contrast, the effect of anti-CD33 Fab′ on the LP pharmacokinetic profile was relatively modest, with no noticeable impact on CL, the $t_{1/2\beta}$ and AUC_{0-24h} (1842 vs 1743 nmol h/mL). The lower influence of antibody fragments on the circulation times of PEGylated LP was in accordance with the data published by other groups.[40−42] The biodistribution profiles also reflected this difference between the mAb and Fab′-coated formulations (Figure 5). For example, at 1 h postadministration, 17% of mAb-LP was deposited in the

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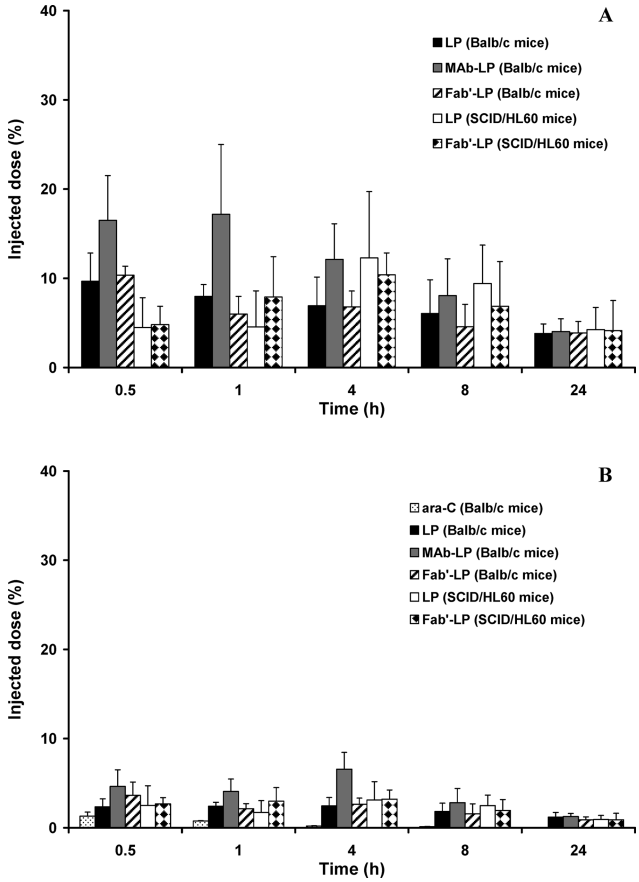

Figure 5. Liver distribution of different pH-sensitive LP formulations (A), and of free or encapsulated ara-C (B) in Balb/c and SCID/HL60 mice. Each mouse received iv 3.4 mg/kg ara-C and 40 μmol/kg of lipids. Values represent the mean ($\pm$SD) obtained for $n = 4$ animals per group per time point.

liver compared to only 8 and 6% of LP and Fab′-LP, respectively ($p < 0.05$ between mAb-LP and Fab′-LP). It has been shown that the Fc region of mAb can lead to enhanced removal of immunoliposomes by the mononuclear phagocyte system (MPS), mainly *via* Fc receptors on macrophages.[43] The coupling method employed in this work is supposed to position the Fc fragment toward the bilayer while exposing the Fab′ domain on the surface.[44,45] However, some mAb may not be well orientated on the vesicles and present their Fc portion to macrophages as similar data were obtained for immunoliposomes graphing the mAb directly on the PEG chain without using any spacer arm.[38] Indeed, in Balb/c mice, immunoliposomes (90−130 nm of diameter) composed of EPC/Chol (2:1 molar ratio) and DSPE-PEG_{2200} (6 mol %) exhibited blood concentrations of approximately 17−32% and 8−28% of the injected dose at 30 min and 6 h postinjection, respectively. The circulation times depended on the nature of the antibody and on the number of antibody molecules per liposome (30 or 15). For our pH-sensitive liposomal formulation exposing the whole monoclonal antibody (mAb-LP, 30−40 antibody molecules by LP), 28 and 7% of the administered vesicles were still circulating in blood at 30 min and 8 h, respectively (Figure 3A). Although this procedure has demonstrated superior circulation time *in vivo* compared to approaches relying on the random grafting of whole mAb, the CL of LP still remains rapid in the presence of the Fc fragment.[44] Another factor potentially at play is ligand density, with faster clearance observed at high mAb densities (>50 μg/μmol phospholipids).[46] However, this should not be the case for our pH-sensitive mAb-LP considering the relatively low proportion of grafted mAb (<20 μg/μmol phospholipids).

The pharmacokinetic and biodistribution patterns of encapsulated ara-C followed a similar trend (Figures 3B, 5B and S1 in the Supporting Information, Table 1). While free ara-C was cleared from the blood within minutes, its encapsulation resulted in an increase in circulation time. However, in the case of pH-sensitive mAb-LP, the gain was relatively modest compared to nontargeted and Fab′-LP (7- vs 33-fold increase in the AUC, respectively). Such an impact of mAb coating on ara-C blood levels could be explained by (i) the greater clearance of pH-sensitive mAb-LP, as discussed above, and (ii) a higher leakage rate from these LP compared to nontargeted or Fab′-LP. Although rapid ara-C release in plasma was not observed in our previous *in vitro* investigations,[16] increased permeability may occur *in vivo* upon activation of the innate immune system. Exposure of Fc fragments may lead to complement activation and subsequent destabilization of the LP membrane. In the case of pH-sensitive LP and pH-sensitive Fab′-LP formulations, the proportion of LP circulating in blood at different time points roughly corresponded to the percentage of circulating ara-C (the calculated drug-to-lipids ratios were not inferior to 1, compare Figures 3A and 3B). These data suggest that the drug remained mainly encapsulated in circulating pH-sensitive LP during the time course of the study. Moreover, these two formulations exhibited similar V_d and CL values for encapsulated ara-C (Table 1). Liposomal ara-C accumulated mainly in the liver (Figure 5B) and spleen (Figure S1 in the Supporting Information). The higher concentration in the liver occurred with the pH-sensitive mAb LP formulation. Deposition into lungs, heart, kidneys, muscles, bone marrow and spleen was less than 3% of the injected dose for all formulations (data not shown).

It has been repeatedly demonstrated that ara-C encapsulation into LP can improve its AUC, mainly by slowing down

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Table 1. Comparison of the Pharmacokinetic Parameters of Different pH-Sensitive LP Formulations and of Encapsulated Ara-C in Naive Balb/c and SCID-HL60 Mice

	ara-C				vectors			
formulations	AUC (0→24 h) (μg·h/mL)	V_d (mL)	$t_{1/2\beta}$ (h)	CL (mL/h)	AUC (0→24 h) (nmol·h/mL)	V_d (mL)	$t_{1/2\beta}$ (h)	CL (mL/h)
			Naive Balb/c Mice					
ara-C alone	5	31.1	1.5	14.3				
pH-sensitive LP	166	3.0	7.6	0.2	1842	2.5	6.1	0.2
pH-sensitive mAb-LP	36	13.8	7.8	0.9	913	4.0	4.3	0.5
pH-sensitive Fab′-LP	166	3.2	7.4	0.2	1743	1.9	5.3	0.2
			SCID-HL60 Mice					
pH-sensitive LP	143	2.6	4.4	0.3	1983	2.0	3.4	0.3
pH-sensitive Fab′-LP	111	4.0	3.6	0.5	1563	3.0	3.5	0.4

its renal clearance and rapid deamination.[11,12,47] Here, we show that adequate pharmacokinetic profiles can be maintained by using pH-sensitive LP decorated with Fab′. This is an important issue as improved efficacy of liposomal formulations in leukemia models has been correlated with increased circulation times.[40] In the next step, it was therefore important to verify whether the pharmacokinetic profile would be altered in SCID mice inoculated with leukemic HL60 cells.

3.3. Pharmacokinetics and Biodistribution in SCID/HL60 Mice. Since the first successful report of human cancer xenograft in SCID mice in 1987, they have become a popular host for growing human tumors and have been used to evaluate a variety of therapeutic strategies.[40,48] Most inoculated animals were found to have a survival rate of 7−10 weeks after the injection of HL60 cells when no treatment was given.[49,50] Surprisingly, it has been reported that $CD33^+$ cells in bone marrow, spleen, and peripheral blood were detected at 10 weeks, although the presence of HL60 cells could be ascertained by chromosome analysis. The steady loss of cell surface markers over a 8−10-week period could make this model inadequate for the *in vivo* evaluation of anti-CD33 LP at later time points. Therefore, we decided to characterize the pharmacokinetics of ara-C-loaded LP, 7 days after the inoculation of HL60 cells.

HL60-bearing SCID mice were injected with pH-sensitive LP and pH-sensitive Fab′-LP. The mAb formulation was not retained due to its inadequate pharmacokinetic profile. As shown in Figures 4A and 4B, both formulations exhibited nearly superimposed blood profiles. The pharmacokinetic parameters of the nontargeted and Fab′-decorated LP were almost similar for LP and encapsulated ara-C (Table 1). Slightly faster clearance of Fab′-LP relative to LP was observed for the lipids and drugs. The overall AUC was about 20% lower for the Fab′-decorated formulation. Here again, the drug and carriers mostly localized in the liver, and no difference was noticeable between pH-sensitive LP and Fab′-LP formulations (Figure 5). Uptake into the lungs, heart, kidneys, muscle and bone marrow (data not shown) was similar for both formulations and was less than 2% of the injected dose. Compared to the Balb/c model, SCID/HL60 mice exhibited significantly less LP deposition in the spleen ($p < 0.05$ for all time points) (Figure S1 in the Supporting Information). This could be explained by depleted immune cells in the SCID model.[51] Preferential accumulation of Fab′-LP in bone marrow was expected but, surprisingly, not observed. The lack of accumulation of the specific anti-CD33 formulation in bone marrow could be due to the low concentration of HL60 cells that may have reached the bone marrow 7 days after their inoculation. Indeed, Xu and Scheinberg[49] reported that only 17% of injected HL60 cells (3×10^7) reached the bone marrow after 6 weeks.

3.4. Preliminary *in Vivo* Efficacy Experiment. To examine the effects of treatment with different formulations of ara-C, a survival study was performed in HL60-bearing SCID mice at a dose of 8 mg of ara-C/kg injected twice (days 1 and 3 after cell injection) (Figure 6). The survival time of the leukemic mice treated with free ara-C was not significantly longer than that of untreated animals (*i.e.* injection of saline). The lack of antitumor activity of iv-administered free ara-C can be explained by its large V_d and fast CL (Table 1). Indeed, it has been shown previously that free ara-C could achieve extensive survival times only when the drug was given in a more intensive treatment schedule,

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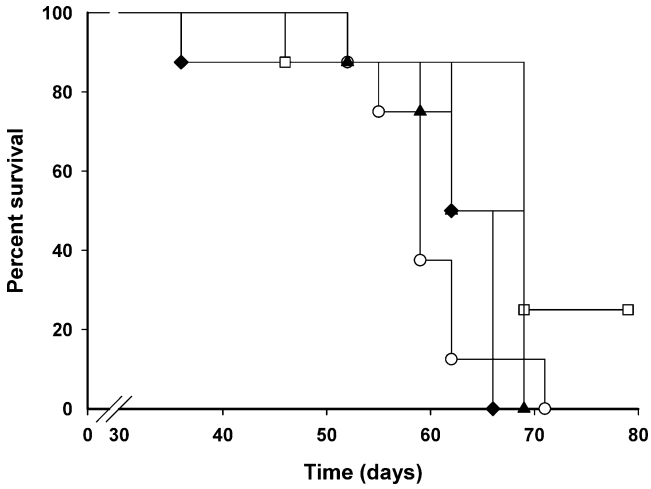

Figure 6. Kaplan−Meier plot for HL60-bearing SCID mice treated with various ara-C formulations. SCID mice (n = 8 mice/group) were injected with 1.2 × 10^7 HL60 cells (day 0) and were treated 24 h later with either saline (○), free ara-C (◆), ara-C-loaded Fab′-LP (□), and ara-C-loaded, pH-sensitive Fab′-LP loaded with ara-C (▲), at a dose of 8 mg of ara-C/kg. A second injection of saline or ara-C formulations (8 mg of ara-C/kg) was repeated at day 3. Mice were monitored daily and were euthanized when tumor size exceeded 1,500 mm^3 or when hind-leg paralysis appeared.

as an infusion of 3 h on 4 consecutive days.[52,53] Ara-C encapsulation of ara-C into pH-insensitive Fab′-LP was found to improve its antitumor activity by significantly prolonging the lifespan of HL60/SCID mice by more than 10 days (mean survival time) compared to the controls (saline and free drug, $p < 0.05$). Ara-C encapsulation into nanocarriers such as LP can protect the drug against degradation in blood and prolong its circulation time, leading to improved antitumor efficacy.[11,21,50,51] Unfortunately, despite promising *in vitro* cytotoxicity data reported earlier by our group,[16] the mean survival time of leukemic mice treated with pH-sensitive Fab′-LP was shorter than that of animals treated with pH-insensitive formulations. The reason for the lack of antitumor efficacy of the pH-sensitive formulation is unknown. It cannot be attributed to a lack of stability of the drug in the pH-sensitive formulation (see section 2.7). *In vitro*, the efficacy of ara-C was indeed shown to be improved after incorporation into pH-sensitive DODA-P(NIPAM-*co*-MAA)-coated LP.[16,25] It is unlikely that the pH-sensitive formulation is inactivated *in vivo*. This formulation has been shown to keep its pH-sensitivity after exposure to human serum and plasma[16,22,32] and to remain fully anchored in the LP bilayers *in vivo* over 48 h.[23] Drug leakage from the pH-responsive LP can also be ruled out since the drug/lipid ratios remained close to 1 during the time course of the experiment (see section 3.2). Difference in circulation time between the pH-sensitive and insensitive LP is the more likely explanation for the moderate *in vivo* efficacy of the pH-responsive LP.[37] The pH-responsive PEGylated LP tend to exhibit a slightly shorter half-life compared to the insensitive one. In the case of ara-C, it is possible that the drug's efficacy depends more on the overall exposure than on the intracellular release pattern. These preliminary results suggest that ara-C may not be the optimal drug to be delivered *via* these pH-sensitive immunoliposomes and/or that the anticancer activity of ara-C depends more on its circulation time than on its rapid release in the endosomal compartment.

4. Conclusion

This work showed that targeting pH-sensitive LP with an anti-CD33 Fab′ fragment was at least as effective as whole mAb-LP in recognizing leukemia cells expressing CD33 receptors. However, Fab′-decorated LP exhibited longer circulation times *in vivo* in normal mice and provided higher ara-C blood levels. This could be explained by the lower uptake of Fab′-decorated pH-sensitive LP by the MPS compared to mAb-LP, but also by a lower leakage rate of the encapsulated drug. Moreover, the long-circulating properties of pH-sensitive Fab′-LP and encapsulated drugs were largely preserved in immunodeficient mice inoculated with leukemic cells. Unfortunately, although ara-C-loaded Fab′-LP were able to prolong the survival of leukemic mice compared to the free drug, the addition of pH-sensitive polymer did not add any benefit to the formulation. Further work is required to establish whether this pH-sensitive LP would be advantageous with other drugs or animal models.

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Supporting Information Available: Figure depicting spleen distribution of different pH-sensitive LP formulations and of free or encapsulated ara-C in Balb/c amd SCID/HL60 mice. This material is available free of charge via the Internet at http://pubs.acs.org.

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