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Enhanced Tumor Visualization by γ-Scintigraphy with ^{111}In-Labeled Polychelating-Polymer-Containing Immunoliposomes

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Abstract: Here, we have prepared long-circulating PEGylated liposomes heavily loaded with ^{111}In via the liposome-incorporated polylysine-based (PLL-based) polychelating amphiphilic polymer (PAP) and additionally modified with the monoclonal antibody 2C5 (mAb 2C5) possessing the nucleosome-restricted (NS-restricted) specificity and capable of specific recognition of a broad variety of live cancer cells via the cancer cell surface bound NSs. These liposomes have been tested as a tumor-specific contrast agent for the γ-scintigraphic visualization of model tumors in mice. The tumor accumulation of mAb 2C5 modified liposomes prepared in this study was significantly (3-to-5-fold) higher than in the neighboring muscle tissue at all times after administration (6, 24, and 48 h) in mice bearing murine Lewis lung carcinoma (LLC) and human HT-29 tumors. The whole body direct γ-imaging of LLC tumor bearing mice at different times has demonstrated the superior in vivo tumor accumulation of the targeted mAb 2C5 modified PAP-containing PEGylated liposomes compared to nontargeted liposomal control formulations, which resulted in better and faster tumor imaging as shown with LLC-bearing mice.

Keywords: Tumor imaging; γ-scintigraphy; ^{111}In; polychelating amphiphilic polymer; liposome; monoclonal antibody 2C5

Introduction

Medical diagnostic imaging requires the sufficient intensity of a corresponding signal from an area of interest to be achieved in order to differentiate this area from surrounding tissues.[1] To enhance the imaging of different organs and tissues for early detection and localization of numerous pathologies, various contrast agents are used, specific for each imaging modality. The differing chemical nature of reporter moieties used in different modalities and the differing signal intensity (sensitivity and resolution) require various amounts of a diagnostic label to be delivered into the area of interest. Still, the main task is to accumulate a sufficient quantity of a contrast agent in the area of interest while keeping its presence in normal tissues and organs to a minimal level. In addition, this accumulation should be achieved as quickly as possible to minimize the time required for producing clinically useful images.

To enhance contrast agent accumulation in the required area, the use of various particulate carriers, such as liposomes capable of entrapping multiple reporter moieties within both the aqueous phase and the liposome membrane compartment, was suggested.[2−6] Thus, liposomes loaded with various heavy metals, such as ^{111}In or ^{99m}Tc for γ-imaging and Gd or Mn

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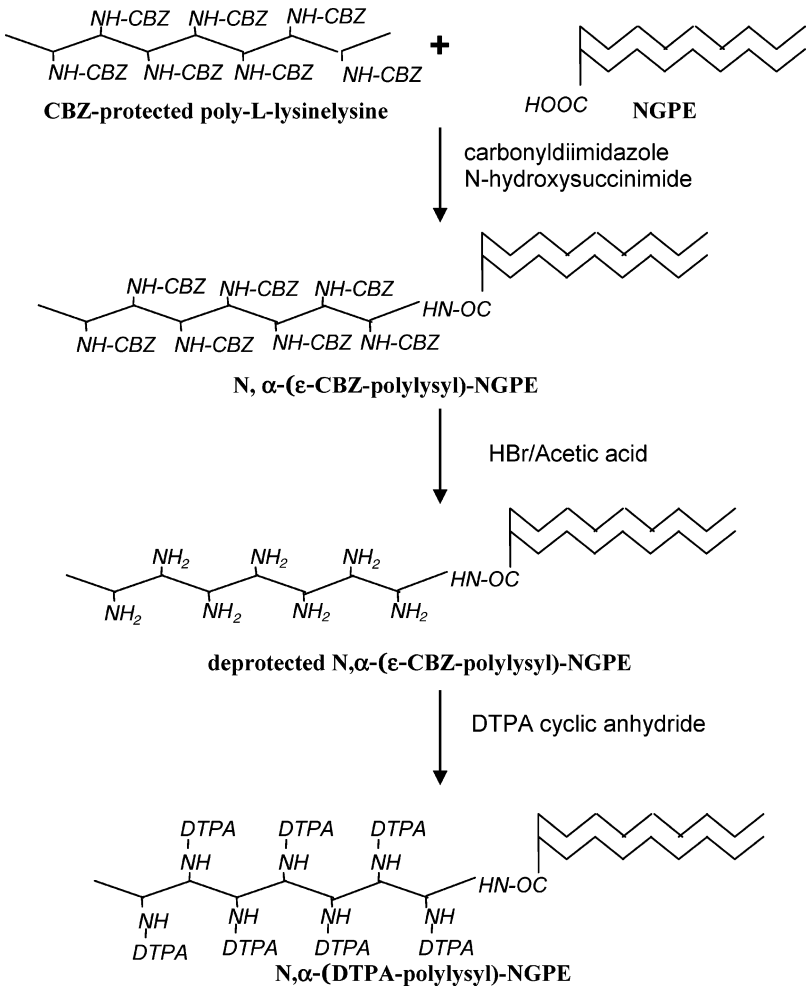

Figure 1. Synthesis of amphiphilic polychelate DTPA-PLL-NGPE consisting of hydrophilic DTPA-polylysyl (DTPA-PLL) moiety and hydrophobic *N*-glutaryl phosphatidyl ethanolamine (NGPE) moiety.

for magnetic resonance imaging, have been designed and successfully applied.[7−8] Usually, all listed metals are incorporated into particulate pharmaceutical nanocarriers, including liposomes, as firm complexes with various chelators, such as diethylene triamine pentaacetic acid, DTPA.[9−10] In order to increase the liposome load with reporter metals the use of polychelating amphiphilic polymers (PAP) has been suggested.[11−13] Such polymers consist of hydrophilic blocks carrying multiple chelating residues as side chains and hydrophobic (lipid-based) blocks capable of anchoring polychelating fragments into the liposome membrane and sharply increasing the number of chelated heavy metal atoms attached to a liposome via a single lipid anchor (see the schematics of the preparation of a typical PAP in Figure 1).

On the other hand, the accumulation of pharmaceutical nanocarriers loaded with various therapeutic and diagnostic

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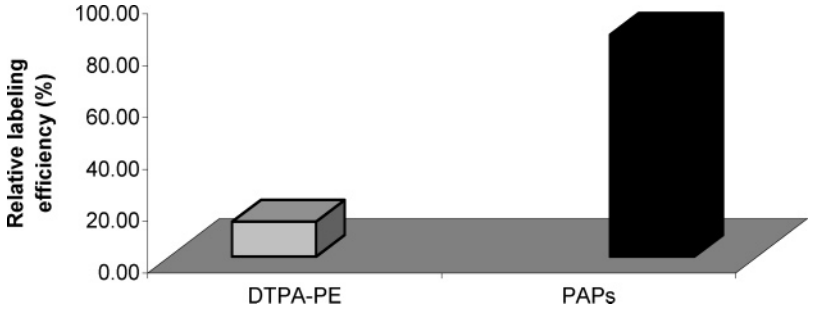

Figure 2. Labeling efficiency of liposomes containing the same molar fraction of PAP or DTPA−PE.

(imaging) agents in biological targets (such as tumors) can be significantly increased by targeting such carriers with various specific ligands, first of all, monoclonal antibodies.[14−16] In recent years, we have identified a subset of natural antinuclear autoantibodies (ANAs) capable of binding to the surface of variety of cancer cells but not normal cells.[17] These antibodies (including monoclonal 2C5 antibody, mAb 2C5) demonstrate the nucleosome-restricted specificity and recognize various live cancer cells via the cancer cell surface bound nucleosomes released from apoptotically dying cancer cells.[18] Nucleosomes are well presented on the surface of many cancer cells both in cell culture and in growing tumors.[17,18] Being attached to the surface of drug-loaded liposomes or micelles, mAb 2C5 dramatically increases their tumor accumulation and therapeutic efficacy.[19−21]

Here, in an attempt to facilitate γ-imaging of tumors in vivo, we combined in a single preparation of long-circulating liposomes an increased load with diagnostic radiometal by using liposome-incorporated PAP, and tumor specificity by additionally decorating liposomes with mAb 2C5. Earlier, specific delivery of increased quantities of a heavy metal to target cancer cells by such liposomes was clearly demonstrated in cell cultures in vitro.[22]

Materials and Methods

Materials. Egg phosphatidyl choline (PC), polyethylene glycol (MW 2000)-phosphatidyl ethanolamine conjugate (PEG_{2000}−PE), *N*-glutaryl-phosphatidyl ethanolamine (NGPE), and cholesterol were from Avanti Polar Lipids, Inc. (Alabaster, AL). *p*-Nitrophenylcarbonyl-PEG−PE (pNP-PEG_{3400}−PE) for attaching antibodies to liposomes was synthesized in our laboratory following the procedure described in ref 23. *N*,*N*′-Carbonyldiimidazole was purchased from Fluka Chemie GmbH (Buch, Switzerland). *N*-Hydroxysuccinimide and ϵ,*N*-carbobenzoxy-poly-L-lysine (CBZ-PLL, MW 5400) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and buffer solution components were analytical grade preparations.

Dulbecco's modified Eagle medium (DMEM), McCoy 10 medium, trypsin, and serum-free medium were from Mediatech, Inc. (Herndon, VA). Heat-inactivated fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Cancer-specific monoclonal antinucleosome 2C5 antibody (mAb 2C5) was prepared by Harlan Bioproducts for Science (Indianapolis, IN) using the hybridoma cell line from our laboratory. Cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD).

Synthesis of Polychelating Amphiphilic Polymer (PAP), DTPA-PLL-NGPE. DTPA−PLL−NGPE was synthesized as described before.[11−12] Briefly, NGPE (25 mg) was activated with *N*,*N*′-carbonyldiimidazole (20 mg) in the presence of *N*-hydroxysuccinimide (13 mg) for 16 h at room temperature (RT). At this time, CBZ-PLL (186 mg), triethylamine (5 μL), and chloroform (10 mL) were added to the initial mixture, and the reaction was allowed to proceed for

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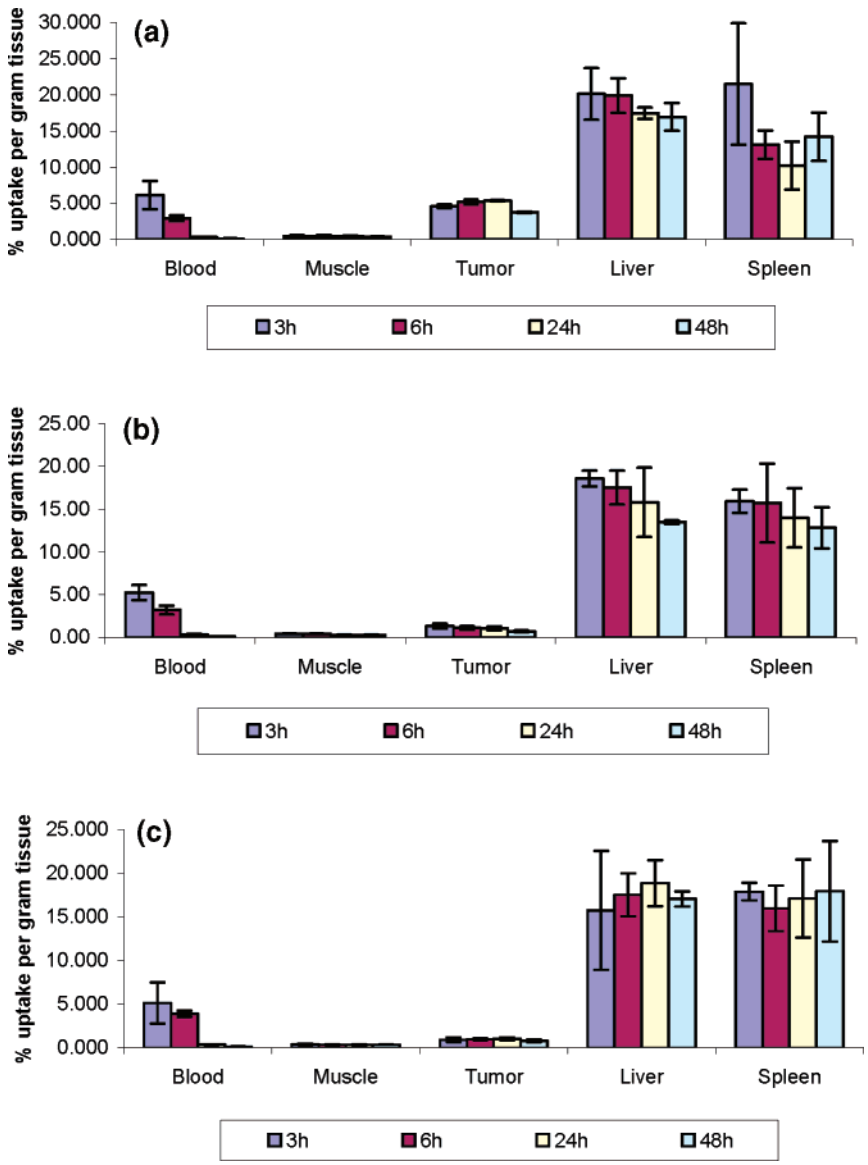

Figure 3. Biodistribution data for ^{111}In-labeled (a) mAb 2C5 modified, (b) nonspecific IgG-modified, and (c) unmodified PAP-containing PEGylated liposomes, in LLC tumor bearing C57BL mice ($n = 5$, mean $\pm$ SD).

Table 1. Tumor/Muscle Ratios for LLC and HT-29 Tumor Bearing Mice at Different Time Points

time (h)	2C5−PAP-liposomes		IgG-PAP-liposomes		PAP-liposomes	
	LLC	HT-29	LLC	HT-29	LLC	HT-29
3	10.66 ± 3.93		3.02 ± 0.61		2.33 ± 0.55	
6	11.30 ± 1.22	3.79 ± 0.19	2.54 ± 0.48		2.76 ± 0.65	1.84 ± 0.54
24	13.91 ± 4.53	7.21± 0.71	4.31 ± 1.25		3.00 ± 0.25	1.96 ± 0.39
48	9.50 ± 1.68		2.73 ± 0.61		2.19 ± 0.17	

another 5 h at RT with stirring. The product was precipitated with water, centrifuged, washed 3 times with water, and freeze-dried to yield 168 mg of *N*,α-(ϵ-CBZ-PLL)-NGPE. The *N*,α-(ϵ-CBZ-PLL)-NGPE obtained was dissolved in 8 mL of a 30% solution of HBr in glacial acetic acid, and the reaction mixture was stirred for 2 h at RT to deprotect *N*,α-(ϵ-CBZ-PLL)-NGPE by removing CBZ groups. Deprotected *N*,α-PLL-NGPE was precipitated with dry ether, washed with the same solvent, and dried under vacuum, yielding 128 mg of *N*,α-PLL-NGPE. Then, *N*,α-PLL-NGPE was suspended in 2 mL of chloroform:methanol (1:1 v/v) and treated with DTPA anhydride (478 mg in 2 mL of dimethyl sulfoxide) in the presence of 185 μL of triethylamine for 16 h at RT with stirring. Succinic anhydride (415 mg in 1 mL of dimethyl sulfoxide) was added to block unmodified amino groups in PLL block, and the reaction mixture was stirred

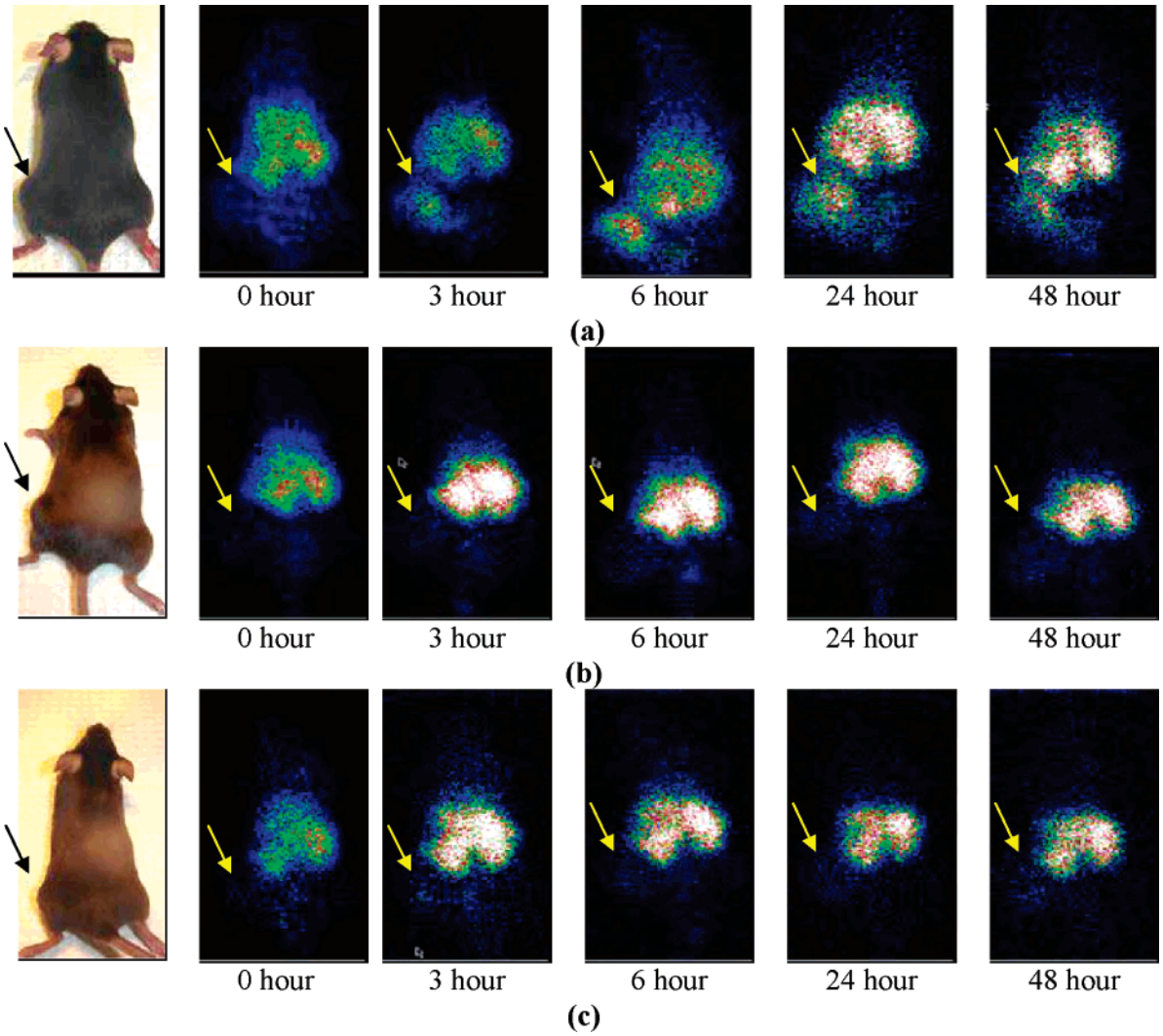

Figure 4. Whole body imaging of LLC tumor bearing mice at different time points after the injection of ^{111}In-labeled PAP-containing PEGylated liposomes: (a) 2C5-modified, (b) control IgG-modified, and (c) unmodified. Arrows indicate tumor locations.

for 1 h at RT and purified by dialysis against deionized water. The yield of PAP, DTPA-PLL-NGPE, after drying was 137 mg.

Preparation of PAP-Containing PEGylated Liposomes. The liposomes were prepared from PC, PEG_{2000}−PE, cholesterol, and DTPA-PLL-NGPE by the lipid film solvation and ultrasonication protocol followed by dialysis. Briefly, a homogeneous chloroform solution of the liposome components (PC:Chol 2:1 molar ratio, 5 mol % of PEG_{2000}−PE, and 1.75 mol % of DTPA-PLL-NGPE) was evaporated, and the lipid film formed was dried under vacuum overnight. A nonionic detergent, octyl glucoside (Sigma) in HEPES-buffered saline (HBS, pH 7.4), was added to the film, and the mixture was vortexed and sonicated in an ice bath under the nitrogen for 1 h. The suspension formed was dialyzed for 2 days against HBS to yield liposomes.[22]

Preparation of PAP-Containing PEGylated Immunoliposomes. To prepare the cancer-specific targeted long-circulating immunoliposomes, a 10−40 molar excess of pNP-PEG_{3400}−PE dispersed in a 10 mg/mL solution of octyl glucoside in 5 mM Na citrate, 150 mM NaCl, pH 5.0, was added to an equal volume of a 1 mg/mL solution of an antibody (mAb 2C5 or control nonspecific IgG) in Tris-buffered saline (TBS), pH 8.5. The mixture was incubated at pH 8.5 overnight at 4 °C. Then, micelles of PEG_{3400}−PE-modified mAb 2C5 or IgG were incorporated into DTPA-PLL-NGPE-containing PEG-liposomes by the overnight coincubation, and the mixture was dialyzed against 10 mM HBS (pH 7.4) using cellulose ester membranes with a cutoff size of 300 kDa for 2 days (Spectrum Medical Industries).[22]

Loading of PAP-Containing Liposomes with ^{111}In. To load liposomal preparation with ^{111}In, PAP-containing liposomes were added to an equal volume of citrate buffer (pH 3.0) and then supplemented with the required quantity of $^{111}InCl_3$ in 0.1 N HCl. After 30 min incubation, free ^{111}In was separated from labeled liposome dispersions by overnight dialysis. After removal of free ^{111}In, the activity of the sample was measured by γ-counter.

Tumor Inoculation into Mice. The experiments were performed in female C57BL/6J and nude mice (Charles River Laboratories, Wilmington, MA) following a protocol approved by Northeastern University Institutional Animal Care

and Use Committee in accordance with "Principles of Laboratory Animal Care" (NIH Publication No. 85-23, revised in 1985). The animals were allowed free access to food and water. Solid murine Lewis lung carcinoma (LLC) and human colon adenocarcinoma (HT-29) were initiated in C57BL/6J and nude mice by a local subcutaneous injection of tumor cells, respectively. Cells, 2×10^5 (LLC cells) and 10×10^6 (HT-29 cells) in 0.2 cm^3 of Hanks buffered salt solution, were injected into each animal (in the left rear flank). Tumors were allowed to develop for 2 weeks until the tumor was detectable by direct palpation.

Biodistribution of ^{111}In-Labeled Liposomes in Tumor-Bearing Mice. For biodistribution studies, mice with tumors reaching 5−8 mm in diameter were injected with 5 mCi (185 MBq) of ^{111}In−PAP-containing PEGylated liposomal formulations via the lateral tail vein. At 3, 6, 24, and 48 h postinjection, blood was collected using a Pasteur pipet from the retro-orbital plexus of the eye, and then, the mice were euthanized by cervical dislocation followed by excision of the tumor and surrounding muscle excision in addition to liver, spleen, kidney and lung. The amount of radioactivity was quantified as CPM using a Beckman 5500B γ-counter. The amount of the accumulated radioactivity per gram of tissue was calculated.

γ-Radioscintigraphy of LLC-Bearing Mice. When the tumors were 5−10 mm in diameter, the mice were injected via the lateral tail vein with 50 mCi (1850 MBq) of ^{111}In-PAP-containing PEGylated liposome formulations. Accumulation of ^{111}In-PAP-containing PEGylated liposomes in tumors was visualized using an Ohio Nuclear 400 radioisotope camera (Ohio-Nuclear Inc., Solon, OH) equipped with a high energy collimator and Nu Mac computer (ONES medical, MA) at 3, 6, 24, and 48 h postinjection after the mice were anesthetized by injection of a mixture of xylazine and ketamine intraperitoneally. Digital pictures of the tumor-bearing mouse were taken using a Kodak digital camera (Eastman Kodak Company, Rochester, NY). Images were analyzed, and tumor-to-normal ratios were calculated.

Results and Discussion

To confirm that our preparations, similarly to the preparations synthesized in earlier studies, are capable of carrying higher amounts of radiotracer than similar liposomes loaded with a monomeric chelate, DTPA−PE, at the same molar fraction of DTPA−PE and PAP in the liposomal composition, we have investigated the labeling efficiency of these two types of ^{111}In-labeled liposomes. The data presented in Figure 2 clearly shows that liposomes loaded with ^{111}In via the liposome-incorporated PAP demonstrate significantly higher labeling efficiency than liposomes loaded with ^{111}In via the monomeric DTPA−PE at the same molar fraction of the chelate.

Biodistribution studies were conducted to determine the uptake of mAb 2C5 targeted and nontargeted PAP-containing liposomes by various tissues in tumor-bearing mice and tumor-to-nontumor (tumor-to-muscle, T/M) radioactivity ratio reflecting the specificity of this uptake. The mean tumor uptake of mAb 2C5 modified ^{111}In-PAP-containing PEGylated liposomes was approximately 5 times higher compared to the tumor uptake of IgG (control nonspecific antibody) modified or nontargeted PAP-containing liposomes (see full data for the LLC-bearing mice in Figure 3). As one could expect, the liver and spleen of experimental animals have also demonstrated high uptake of the liposomal preparations as well as kidneys, and this uptake by the organs other than tumors was not much influenced by the presence of the specific antibody on the liposome surface. The total radioactivity balance could be obtained when the activity associated with kidneys, lungs, urine, and feces is also accounted for (not shown).

T/M ratios for mAb 2C5 modified ^{111}In-PAP-containing PEGylated liposomes for both tested tumors at all times were also significantly ($P < 0.05$) higher than those for control ^{111}In-PAP-containing PEGylated liposomes, suggesting the active targeting by tumor-specific antibodies (Table 1). The T/M ratio for mAb 2C5 modified ^{111}In-PAP-containing PEGylated liposomes 24 h postinjection was sufficiently high for both tumors (ca. 14 for the LLC tumor and ca. 7 for the HT-29 tumor) to allow for the tumor detection by the planar γ-scintigraphy.

Finally, we have demonstrated that such preferential tumor accumulation of specific antibody-targeted liposomes together with heavy load of liposomes with the reporter metal results in an accelerated and enhanced tumor imaging by γ-scintigraphy. The direct whole body γ-imaging of tumor-bearing mice at different times after the injection of ^{111}In-2C5-modified and unmodified PAP-containing PEGylated liposomes is shown in Figure 4. Though the target delineation was achieved as early as 3 h postinjection of 2C5-liposome-based preparation, sharper and clearer images were obtained after 6 and 24 h. These images clearly demonstrated the better and faster in vivo imaging of tumors in mice with the targeted mAb 2C5 modified ^{111}In-PAP-containing PEGylated liposomes compared to the nontargeted control formulations in the LLC model.

Conclusion

Liposomes loaded with a radioactive tracer via the liposome-incorporated polychelating amphiphilic polymers and modified with cancer-specific monoclonal 2C5 antibodies may represent promising targeted contrast agents for tumor visualization.

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