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A novel method to label preformed liposomes with ^{64}Cu for positron emission tomography (PET) imaging

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Abstract

Radiolabeling of liposomes with ^{64}Cu ($t_{1/2}$ = 12.7 h) is attractive for molecular imaging and monitoring drug delivery. A simple chelation procedure, performed at a low temperature and under mild conditions, is required to radiolabel pre-loaded liposomes without lipid hydrolysis or the release of the encapsulated contents. Here we report a ^{64}Cu post-labeling method for liposomes. A ^{64}Cu-specific chelator, 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*',*N*",*N*"'-tetraacetic acid (BAT), was conjugated with an artificial lipid to form a BAT-PEG-lipid. After incorporation of 0.5% (mol/mol) BAT-PEG-lipid during the liposome formulation, liposomes were successfully labeled with ^{64}Cu in 0.1 M NH_4OAc pH 5 buffer, at 35 °C for 30~40 min with an incorporation yield as high as 95%. After 48 hour incubation of ^{64}Cu-liposomes in 50/50 serum/PBS solution, more than 88% of the ^{64}Cu label was still associated with liposomes. After injection of liposomal ^{64}Cu in a mouse model, 44 ± 6.9, 21 ± 2.7, 15 ± 2.5, and 7.4 ± 1.1 (n = 4) % of the injected dose per cubic centimeter remained within the blood pool at 30 min, 18, 28, and 48 hours, respectively. The biodistribution at 48 hours after injection verified that 7.0 ± 0.47 (n = 4), and 1.4 ± 0.58 (n = 3) % of the injected dose per gram of liposomal ^{64}Cu and free ^{64}Cu remained in the blood pool, respectively. Our results suggest that this fast and easy ^{64}Cu labeling of liposomes could be exploited in tracking liposomes *in vivo* for medical imaging and targeted delivery.

Keywords

liposome; Cu-64; PET; BAT

Introduction

Liposomes are widely used in preclinical and clinical applications as carriers of drugs, genes, or contrast agents (1,2). The incorporation of hydrophilic polyethylene glycol (PEG) on the surface of liposomes further improves their properties (3). To obtain the biodistribution of long-circulating liposomes and to obtain non-invasive images in animals and humans, past studies have utilized liposomes loaded or labeled with radionuclides such as indium-111 (4), technetium-99m (5-7), and gallium-67 (8) for single photon emission computed tomography

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(SPECT). Labeling with fluorine-18 (9,10) has been used for positron emission tomography (PET).

Three distinct approaches have been developed for the labeling or loading of liposomes with radioisotopes: (a) entrapping a soluble radiotracer inside the aqueous liposomal core, e.g., via chelating the pre-loaded polar ligand with ^{111}In (11), ^{67}Ga (12), or ^{99m}Tc-complex (5,7), or via extrusion with 2-[^{18}F]fluorodeoxyglucose (2-[^{18}F]FDG) (13); (b) inserting radiolabeled lipid into the liposome bilayer (9,10), and (c) attaching ^{99m}Tc onto the surface of liposomes via the chelation between ^{99m}Tc and a chelator-lipid conjugate (14,15). In contrast to the extensive studies using SPECT to track liposomes in vivo, reports of PET imaging of liposomes are limited, although PET has many potential advantages (16). Previous reports have focused on the use of fluorine-18 to label liposomes (9,10), but its short 110-min half-life makes extended studies difficult. In the treatment of diseases such as cancer, it is desirable to create stable particles that can circulate and accumulate in tumors over days to weeks (17,18). Because of the temperature dependence of liposome structures (19), these particles may not withstand exposure to high temperatures after formulation. Here, we report a gentle post-labeling method using ^{64}Cu (half-life 12.7 h) and its application to track liposomes over 48 hours. For the surface chelation method, the incorporation of a lipid-PEG-chelate conjugate (Scheme 1) in the liposome bilayer (Figure 1) was chosen, as used in previous nuclear medicine studies (14,20). Previous surface chelation methods used lipid-chelate conjugates such as octadecylamine diethylenetriaminepentaacetic acid (DTPA) (15), dipalmitoylphosphatidylethanolamine-diethylenetriaminetetraacetic acid (DPPE-DTTA) (20,21), and hydrazino nicotinamide (HYNIC) conjugated-DSPE (14). However, none of those reagents have been shown to bind copper stably under physiological conditions, nor would they be expected to, due to the kinetic properties of the copper ion. Among several effective copper(II) macrocyclic chelators, such as DOTA, NOTA, TETA and TE2A (22,23), we chose 6-[*p*-(bromoacetamido) benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N'*,*N"*,*N"'*-tetraacetic acid, BAT(24), as the chelating ligand. BAT, a derivative of 6-benzyl-TETA (Scheme 1), has been found to form stable (25,26) and highly selective complexes with copper(II) (27), and has been utilized for antibody labeling (28,29) for preclinical and clinical trials. Although the cross-bridged ligand TE2A has been shown to be very stable for radiolabeling small molecules (30), the heating step (> 70 °C) required for the incorporation of ^{64}Cu into TE2A exceeds the transition temperature of many liposomal formulations (thus releasing the contents) and could also alter lipid structure in other ways, such as hydrolysis.

EXPERIMENTAL PROCEDURES

Materials

Fmoc-Cys(Mmt)-OH, Fmoc-NH-PEG$_{28}$COOH, Fmoc-Lys(Fmoc)-OH, and coupling agent (HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) were purchased from EMD Biosciences (La Jolla, CA), and stearic acid from Sigma-Aldrich. Fmoc-PAL-PEG-PS resin (0.16-0.21 mmol/g) was from Applied Biosystems (Foster City, CA). Solvents and other agents were all of analytical purity and from Sigma-Aldrich (Milwaukee, WI) and VWR (Brisbane, CA). All lipids and a mini-extruder were purchased from Avanti Polar Lipids (Alabaster, AL). $^{64}CuCl_2$ was purchased from Trace life Science (Denton, TX, specific activity > 80,000 Ci/g for *in vitro* and *in vivo* studies) or Nordion (Ontario, Canada, specific activity > 5000 Ci/g for the feasibility study) under a protocol controlled by the University of California, Davis. High purity ammonium acetate (≥ 99.995%) was purchased from Sigma-Aldrich (St. Louis, MO). PBS was purchased from Invitrogen Corporation (Carlsbad, CA). Mouse serum was purchased from Innovative research (Novi, MI). All eppendorf tubes and glassware for labeling were pre-washed with absolute ethanol and acetone and dried in an oven before use.

The synthesis of lipid-PEG-Cys(BAT) conjugate (7)

As shown in Scheme 1, **7** was synthesized by Fmoc solid phase synthesis. In brief, Fmoc-Cys (Mmt)-OH, Fmoc-PEG_{28}-COOH, Fmoc-Lys(Fmoc)-OH and stearic acid were coupled to the PAL-PEG-PS resin sequentially, to obtain **5**. After the protecting group, Mmt, was removed with 1% TFA/DCM, BAT (24) was coupled onto the resin in the solvent (DMF:DMSO:H_2O = 1:1:1, v/v/v), at pH = 8.0 ± 0.5, adjusted with DIPEA. The reaction was monitored with DTNP solution. The final product (**7** in Scheme 1) was cleaved from the resin by TFA/TIPS/H_2O mixture (TFA/TIPS/H_2O = 95:2.5:2.5, v/v/v) and purified by reversed-phase high performance liquid chromatography (HPLC), with the mass confirmed by MALDI-TOF (M $+H^+$, measured: 2662, calculated: 2663). Reversed-phase HPLC was performed using a Phenomenex Jupiter 4μ Proteo 90A (250 × 4.6 mm, analytical), and a Phenomenex Jupiter 10μ Proteo 90A (250 × 10.0 mm, semi-preparative) with a gradient from 10-90% B in 30 min (solvent A: 0.05% TFA, solvent B: acetonitrile) and a flow rate of 1.5 or 3.0 mL/min for the analytical/semi-preparative column. The retention time of the BAT-PEG-lipid conjugate was 35.5 min. MALDI was measured with ABI-4700 TOF-TOF (Applied Biosystems) using matrix sinapinnic acid with a 3-layer sample preparation method (31). The MALDI spectral results are shown in Figure 2.

BAT-liposome preparation

Lipids in chloroform were mixed in a test tube, and dried by gently blowing nitrogen gas with vortexing to create a thin film, which was further dried under vacuum overnight to completely remove chloroform. The dried lipid thin film was re-suspended by adding 0.1 M ammonium acetate buffer (pH 5) and incubated for 5-10 min at 60 °C. After 1 minute sonication, this lipid mixture was extruded by 21 passes through a 100 nm membrane filter (Whatman, NJ) at 60-62 °C on a heating block, to form 100 nm liposomes. The liposome solution was stored at 4 °C until needed for radiolabeling.

^{64}Cu labeling of BAT-PEG-lipids

BAT-PEG-lipids (20 μg, 7.5 nmol) in chloroform (20 μL) were added into a glass test tube and chloroform was evaporated by gently blowing nitrogen gas with vortexing. After overnight under high vacuum, citrate buffer (0.1 M, 0.4 mL, pH 5.5) was added to the glass test tube containing BAT-PET-lipids. Buffered $^{64}CuCl_2$ (5.31 mCi, 196.47 MBq) in ammonium citrate (pH 5.5, 0.1 M) was added to the lipid solution, which was transferred to a plastic tube. The mixture was incubated at 37 °C and monitored by radio TLC (eluant: chloroform:ethanol:H_2O = 50:40:60, v/v/v), which showed 97% labeling yield after 40 min incubation. After cooling to room temperature, the solution was passed through an activated C-18 sep-pak cartridge and washed with deionized water (5 mL) and anhydrous acetonitrile (8 mL). Radioactivity remaining in the cartridge was extracted by mixed ethanol and chloroform solution (1 mL, 1:1, v/v). After the solution was removed under a nitrogen stream, the activity was re-suspended with 7% ethanol/PBS for the *in vivo* study. Radiochemical yield at the end of synthesis (EOS) was 59% and specific activity was greater than 575 Ci/mmol. Radiochemical purity on both radio TLC and reversed-phase HPLC was 99% (C4 column, 250 × 4.6 mm, analytical, solvent A: 0.05 volume% trifluoroacetic acid in deionized water, solvent B: acetonitrile, flow rate: 1.5 mL/min, gradient from 10-90% B in 30 min and 90-10% B in 30-60 min, retention time: 29.6 min).

^{64}Cu labeling of liposomes

For the feasibility study—$^{64}CuCl_2$ (8.31 mCi, 307.47 MBq) in a volume of 35 μL (pH 1-2) was received and NH_4OAc solution (4 μL, 1 M, pH 5.0) was added to adjust the pH. This solution (5.08 mCi, 187.96 MBq, 25 μL) was added to 475 μL of 0.1 M NH_4OAc solution (pH 5.0) and the final concentration of ^{64}Cu was 10 mCi/mL. This ^{64}Cu solution (50 μL) was added

to the liposome solutions (12 mg/300 μL, total lipid mol = 12.7 μmol, DPPC:DSPE-PEG2000:BAT-PEG-lipid = 90-x:10.0:x, mol/mol/mol, x = 0, 0.1, and 1.0). The concentration of BAT-PEG-lipids in 12 mg of lipids was 0, 12.7, and 127 nmol, respectively. After vortexing, the mixture was incubated for 1 h at room temperature. Then, EDTA solution (0.1 M, 35 μL) was added (final EDTA concentration 10 mM) and the mixture was incubated for 20 min at room temperature. Liposomes were separated by a G-75 column (GE Healthcare, NJ) in PBS, and fractions were collected in 250 μL tubes. The radioactivity was measured with a calibrated Cu-64 mode of CRC-15 DualPET (Capintec, NJ) with non-correction of Cu-67, and the incorporation yield was determined by equation *(1)* as decay corrected yield.

$$Incorporation \quad yield = \frac{Fl}{Fa} \tag{1}$$

(*Fl*: radioactivity in liposome fractions, *Fa*: sum of all radioactive fractions)

***For the in vitro* study—**liposomes (10 mg) containing 0.5 mol% of BAT-PEG-lipid (HSPC:cholesterol:DSPE-PEG2000: BAT-PEG-lipid = 55.5:39:5.0:0.5, mol/mol/mol/mol, the number of BAT-PEG-lipids: 68.7 nmol) and without BAT-PEG-lipid (HSPC:cholesterol:DSPE-PEG200 = 56:39:5.0, mol/mol/mol) were prepared by the procedure above. Both liposome formulations were incubated with $^{64}CuCl_2$ (2.15 mCi) at 35 °C for 40 min. The purification continued as above and the labeling yield was determined by equation *(1)*.

***For the in vivo* study—**>Liposomes (1 mg, HSPC:cholesterol:DSPE-PEG2000: BAT-PEG-lipid = 55.5:39:5.0:0.5, mol/mol/mol/mol, the number of BAT-PEG-lipid: 6.85 nmol) were prepared by the procedure above. $^{64}CuCl_2$ (2.75 mCi) solution was added to 300 μL (0.1 M NH_4OAc, pH 5) of BAT-liposome solution and incubated at 37 °C for 40 min. Purification continued as above. After the radioactivity decayed, size and zeta potential were measured with a NICOMP^{TM} 380 ZLS (Particle Sizing Systems, CA).

In vitro stability test

Separated ^{64}Cu-labeled liposomes (~ 1.0 mg lipids) in PBS buffer (pH 7.4, 0.5 mL) were added to 0.5 mL of mouse serum and albumin solution (final concentration: 8 mg/mL). The mixture was kept in a shaker (300 rpm) at 37 °C , a sample of 100 μL was taken from the solution at 48 hours and hand-loaded onto a sephachryl-300HR (GE Healthcare, NJ) packed column (15 mm I.D, 250 mm height), which was eluted with PBS under a pressure of 2 psi. Fractions (1.5 mL per fraction) were collected into test tubes, and radioactivity was measured with the 1470 Automatic Gamma Counter (Perkin Elmer Life Sciences, MA). After the radioactive decay, the absorbance of all fractions was measured at 280 nm. *Radio TLC assay*: Each aliquot (10 μL) of six solutions described below was dissolved into 50% ethanol solution (100 μL) after 20 and 48 hour incubation at room temperature: $^{64}CuCl_2$ in 0.1 M NH_4OAc solution, $^{64}CuCl_2$ in serum solution (PBS:serum = 1:1, v/v), $^{64}CuCl_2$ in albumin solution, ^{64}Cu-labeled liposomes in PBS buffer, ^{64}Cu-labeled liposomes in albumin solution and ^{64}Cu-labeled liposomes in serum solution (PBS:serum = 1:1, v/v). TLC was run on aluminum-backed silica gel sheets (silica gel 60 F_{254}, EMD, NJ), developed with chloroform:methanol:H_2O (50:40:6, v/v/v). The radio TLC was recorded by a radio-TLC Imaging Scanner (Bioscan, NW).

Biodistribution study

All animal studies were conducted under a protocol approved by the University of California, Davis Animal Use and Care Committee. A total of 10 animals (male FVB mice, 8-13 weeks, 25-30 g, Charles River, MA) were examined over the course of this study. For each image, four or three mice per group were anesthetized with 3.5% isofluorane and maintained at 2.0–2.5% and catheterized to ensure proper tail vein injection, after which bolus injections of ^{64}Cu-BAT-PEG-lipids (0.263 ± 0.013 mCi (9.73 ± 0.48 MBq) per animal), ^{64}Cu-labeled liposomes (4.6-18 μmol/kg, 0.17-0.21 mCi (6.29-7.77 MBq) per animal) and $^{64}CuCl_2$ (carrier free, 0.15-0.4 mCi (5.55-14.7 MBq)) in PBS (pH 7.2) were administered as PET scans were initiated, using a manually-controlled injection that was timed for uniform administration over 15 seconds. After the 48-h PET scans, the mice were euthanized by cervical dislocation and organs of interest were harvested and weighed. Radioactivity was measured using a 1470 Automatic Gamma Counter.

PET scans and Time–activity curves (TAC)

PET scans were conducted with the microPET Focus (Concorde Microsystems, Inc., TN) over 60 minutes. Maximum a posteriori (MAP) files were created with ASIPro software (CTI Molecular Imaging) and used to obtain quantitative activity levels in each organ of interest as a function of time. TACs were obtained with region-of-interest (ROI) analysis using ASIPro software and expressed as percentage of injected dose per cubic centimeter (%ID/cc).

RESULTS AND DISCUSSION

Fmoc solid phase synthesis was used to create the lipid-PEG-chelate conjugate to minimize hydrolysis of the ester group in the lipid conjugate and maximize purity of the final conjugate (**7** in Scheme 1) (32). A short PEG spacer (28 repeat units, MW ~ 1232) was introduced to facilitate accessibility of ^{64}Cu to the chelator by minimizing the steric effect of neighboring lipid head groups, while burying the chelator within the longer PEG brush (45 repeat units, MW ~ 1980) of the surrounding lipid molecules. In brief, Fmoc-Cys(Mmt)-OH, Fmoc-PEG_{28}-COOH, Fmoc-Lys(Fmoc)-OH and stearic acid were coupled to the PAL-PEG-PS resin sequentially, to obtain **5** in scheme 1. After the protecting group, Mmt, was removed with 1% TFA/DCM, BAT was coupled to the resin in the mixture solvent (DMF:DMSO:H_2O = 1:1:1, v/v/v), at pH = 8.0 ± 0.5, adjusted with DIPEA. The reaction was monitored with DTNP solution. The final product (**7** in scheme 1) was cleaved from the resin by a TFA/TIPS/H_2O mixture (TFA:TIPS:H_2O = 95:2.5:2.5, v/v/v) and purified by HPLC, with the mass confirmed by MALDI ($M+H^+$, measured: 2662, calculated: 2663, Figure 2).

To check the feasibility of chelating ^{64}Cu onto BAT liposomes, the chelation yields were compared for three temperature sensitive liposome formulations (33,34), with 0, 0.1, and 1.0 mol% BAT-PEG-lipid, respectively (Figure 3). Similar to the labeling protocol developed for antibodies (25), chelation of ^{64}Cu onto liposomes was performed in 0.1 M NH_4OAc pH 5 buffer at room temperature for 60 min. Unchelated ^{64}Cu was removed by mixing the liposome solution with 1/10 (v/v) 0.1 M EDTA solution for 20 min. ^{64}Cu-labeled liposomes and ^{64}Cu-EDTA complexes were separated with a Sephadex-G75 column with the normalized radioactivity of each fraction shown in Figure 3. The ^{64}Cu chelation yields for 0, 0.1, and 1.0 mol% BAT liposomes were 0%, 83%, and 89%, respectively. Specific activity of the chelator was determined as 29.1 μCi (0.1 mol%) and 31.3 μCi (1.0 mol%) per 1 mg of total lipids and as 27.5 and 2.96 Ci per 1 mmol BAT-PEG-lipids. The number of BAT-PEG-lipid molecules in the 0.1 mol% BAT liposomes was 11 times more than the molecules of added $^{64}CuCl_2$ (0.42 mCi).

To demonstrate that this technique can successfully label multiple liposomal formulations and to demonstrate stability over 48 hours, *in vitro* and *in vivo* assays were performed with long circulating liposomes (35). The *in vitro* stability of ^{64}Cu-labeled liposomes was also tested with 0.5 mol% BAT-PEG-lipids in a non-temperature sensitive formulation. Incubation of both liposomes with $^{64}CuCl_2$ at 35 °C for 45 min, followed by treatment with EDTA solution for 15 min, gave an estimated 95% labeling yield with 0.5 mol% BAT liposomes (Figure 4a). Specific activity was measured as 0.194 mCi per 1 mg of total lipids or 28.2 Ci per 1 mmol BAT-PEG-lipids.

^{64}Cu was not detected on liposomes lacking the BAT-lipid. Labeled liposomes in PBS (pH 7.4) were mixed with albumin (10 mg/mL) and mouse serum (PBS:serum = 1:1, v:v). Mixtures were allowed to stand at 37 °C and the stability was monitored by radio TLC (Figure 4b) and chromatography with a size exclusion column (Figure 4c-d). As shown in the TLC study (Figure 4b), radioactivity in the form of $^{64}CuCl_2$ (lane 1), $^{64}CuCl_2$ associated with albumin (lane 3) and $^{64}CuCl_2$ associated with serum (lane 5) were retained at the origin. However, the relatively lipophilic ^{64}Cu-liposomes (lane 2) (^{64}Cu-BAT-PEG-lipid on silica plate), shifted to R_f 0.8, including 99% of the radioactivity. ^{64}Cu-liposomes incubated with albumin (lane 4) for 48 hours also showed 99% of the radioactivity in the R_f 0.8 region. This suggests that ^{64}Cu-liposomes are inert to albumin. With ^{64}Cu-liposomes incubated with mouse serum, 6% and 8% of the activity was found in R_f 0.0 after 20 and 48 hour incubation, respectively. The chemical form of the copper was not investigated further.

To further characterize the effect of serum on chelation stability, an analysis was performed using size exclusion chromatography, with aliquots of solution loaded into the size exclusion column after incubation with albumin and serum for 1 and 48 hours. The radioactivity was measured with a gamma-counter and absorbance of the serum component read at 280 nm. The size exclusion chromatogram of radioactivity from the albumin mixture (Figure 4c) showed that fractions (f1-30) contain more than 99% of the total radioactivity. Liposome fractions in the main peak (f7-15) and minor broad peak (f16-22) contained 95% and 4.1% of the total radioactivity, respectively. Thus, the labeled liposomes (radioactivity) and albumin (280 nm) could be separated and albumin, the major component of serum, did not affect the stability of the labeled liposomes.

Size exclusion chromatography (Figure 4d) following serum incubation also showed that fractions (f1-30) contain more than 99% of the total radioactivity. As shown in Figure 4d, three peaks, with fractions f9-15, f16-22, and f23-30, were found to have 88%, 9.1%, and 2.7% of the radioactivity, respectively. To elucidate whether the second peak (f16-22) contains ^{64}Cu-BAT-PEG-lipid or dissociated ^{64}Cu, radio TLC of one fraction was performed. TLC showed three peaks (Figure 4e) corresponding to R_f equal to 0 (7%), 0.35 (22%) and 0.8 (71%). If the result is compared with lane 2 in Figure 4b, the major peak (R_f of 0.8) corresponds with the ^{64}Cu-liposomes. Radioactivity after the 14th fraction in Figure 4d is assumed to represent ^{64}Cu-BAT-PEG-lipids which associated with serum during incubation. Therefore, we estimate that more than 88% of the ^{64}Cu remained associated with the liposomes after 48 h in serum. Prior work with ^{67}Cu-antibodies labeled using BAT showed a dissociation of copper from the chelator of approximately 1% per day under physiological conditions in serum (25). The properties of liposomes, including possible exchange of lipids with other serum components, make it difficult to specifically measure the rate of copper dissociation for the system studied here.

The *in vivo* performance of ^{64}Cu-labeled liposomes was studied, with the *in vivo* results compared with previously reported ^{99m}Tc-labeled liposome studies (14,36). Liposomes with 0.5 mol% BAT-PEG-lipid were labeled with $^{64}CuCl_2$ (3.0~4.0 mCi/mg lipids) at 37 °C for 40 min. The labeling yield was 62 ± 13% (n = 2) and the specific activity was 1.65 ± 0.29 (n = 2)

mCi per 1 mg of total lipids or 242 ± 42 (n = 2) Ci per 1 mmol BAT-PEG-lipids. The size and zeta potential of liposomes before and after labeling are listed in Table 1. The liposomes were reduced in size by about 10 nm after labeling, likely due to a difference in osmolarity before (0.1 M ammonium acetate buffer, ~ 240 mOsm, pH 5.0) and after labeling (10 mM PBS buffer, ~ 300 mOsm, pH 7.4). Zeta potential was not significantly changed before (−40.8 mV) and after (−41.2 mV) labeling.

In vivo imaging with ^{64}Cu-labeled liposomes was then performed in FVB mice with microPET and compared with free ^{64}Cu ($^{64}CuCl_2$). All animal studies were conducted under a protocol approved by the University of California, Davis Animal Use and Care Committee (Davis, CA). Figure 5 shows four images from 30-min scans at 0, 18, 28, 48 h after i.v. injection, for both a free ^{64}Cu injection (left) and a liposomal ^{64}Cu injection (right). Free ^{64}Cu rapidly accumulated in the liver (Figure 5a-d) and accumulation was highest in the liver throughout the scan as in (37). In contrast, liposomal ^{64}Cu remained in the blood pool (Figure 5a-d) through 18, 28, and 48 h. Uptake of the liposomes by the reticulo-endothelial system is expected with previous SPECT studies showing similar accumulation to that observed here (14). The accumulation of ^{64}Cu in the liver and other organs can result from intracellular processes via metabolism of liposomes (38) and transchelation of ^{64}Cu to ceruloplasmin (26), superoxide dismutase (SOD) (38,39), or copper chaperone for SOD (40); however, comparing our results with previous liposomal SPECT studies using Tc-99m, these effects do not appear to be substantial.

Time activity curves (TAC) corresponding to blood and liver are shown in Figure 6. Decay-corrected radioactivity in the blood pool decreased to 44 ± 6.9, 21 ± 2.7, 15 ± 2.5, and 7.4 ± 1.1 (n = 4) %ID/cc at 30 min, 18, 28, and 48 h, respectively (Figure 6). To compare our TAC data with (14) , the maximum %ID/cc (50 ± 8.9) at 2 min was fixed as the initial blood pool activity and the TAC estimates at 30 min, 18, 28, and 48 h were converted to 88%, 42%, 30%, and 15% of the original radioactivity in the blood pool. These values are similar to those observed by Laverman in a study of rats with ^{99m}Tc-HYNIC liposomes, which showed 40% of the initial blood-pool radioactivity remaining at 24 hours (14). Similarly, 38% of initial activity was retained in the blood pool at 20 hours following injection of labeled liposomes into rats in (36). A solution of ^{64}Cu-BAT-PET-lipid (specific activity: 575 Ci/mmol) was also injected into the mouse tail vein (n = 3). As a result, radioactivity accumulated in the liver within 30 min and cleared in a manner similar to the injected $^{64}CuCl_2$ (Figure 6).

The biodistribution at 48 hours shows that activity in the urine, blood, and spleen of liposomal ^{64}Cu was significantly higher than that of free ^{64}Cu or ^{64}Cu-BAT-PET-lipid (Figure 7). Subsequent studies have indicated that the activity within the spleen is dependent on the lipid dose injected, decreasing with larger amounts of lipid (data not shown).

CONCLUSIONS

We describe a novel ^{64}Cu labeling method for liposomes. By solid phase synthesis, a BAT-PEG-lipid conjugate was prepared, and by incorporating BAT-PEG-lipid into liposomes and incubating the resulting liposomes with $^{64}CuCl_2$, ^{64}Cu liposomes were obtained with a labeling yield as high as 95%. The liposomal ^{64}Cu was stable in mouse serum *in vitro* and was long-circulating *in vivo*. These results suggest that this novel labeling method (^{64}Cu-BAT-liposomes) can be further exploited for tracking liposomes with PET *in vivo*.

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1 Abbreviations

BAT, 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N'*,*N"*,*N"'*-tetraacetic acid
BMEDA, *N*,*N*-bis(2-mercaptoethyl)-*N'*,*N"*-diethylethylenediamine
Cys, cysteine
DCM, dichloromethane
DIPEA, diisopropylethylamine
DMF, dimethylformamide
DMSO, dimethylsulfoxide
DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N'*,*N"*,*N"'*-tetraacetic acid
NOTA, 1,4,7-triazacyclononane-N,N',N"-triacetic acid
DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPE-PEG-2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000]
DTNP, 2,2′-dithio bis-(5-nitropyridine)
EDTA, ethylenediaminetetraacetic acid
FDG, fluorodeoxyglucose
Fmoc, 9-fluorenylmethyloxycarbonyl
HSPC, hydrogenated soy phosphatidylcholine
HYNIC, hydrazino nicotinamide
MALDI, matrix-assisted laser desorption/ionization
Mmt, 4-monomethoxytrityl
PBS, phosphate buffered saline
PEG, polyethylene glycol
PET, positron emission tomography
ROI, region of interest
SOD, superoxide dismutase
SPECT, single photon emission computed tomography
TAC, time activity curve
TETA, 1,4,8,11-tetraazacyclotetradecane-*N*,*N'*,*N"*,*N"'*-tetraacetic acid
TFA, trifluoroacetic acid
TIPS, triisopropylsilane

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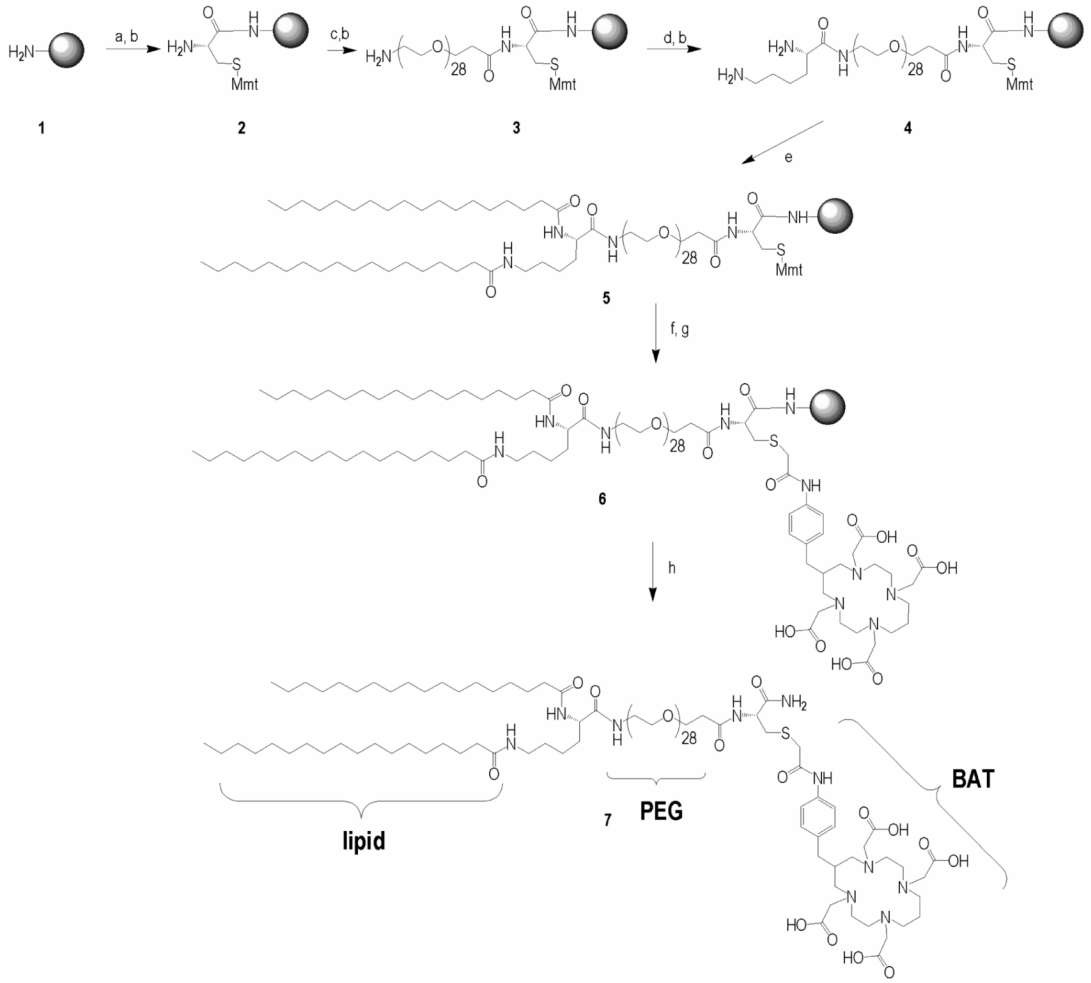

Scheme 1.

Solid phase synthesis of BAT-PEG-lipid Reagents: a) Fmoc-cysteine (2 eq.), HBTU (O-benzotriazole-*N*,*N*,*N*’,*N*’-tetramethyl-uronium-hexafluoro-phosphate) (2 eq.), DIPEA (4 eq.), 60 min; b) 20% piperidine in DMF, 2 × 10 min, 10 min; c) Fmoc-PEG28-COOH (1.1 eq.), HBTU (1.1 eq.), DIPEA (2.2 eq.), 60 min; d) Fmoc-Lys(Fmoc)-OH (2 eq.), HBTU (2 eq.), DIPEA (4 eq.), 60 min; e) stearic acid (4 eq.), HBTU(4 eq.), DIEA(8 eq.), 60 min; f) 1% TFA/DCM, 5 × 3 min; g) BAT(1.5 eq.), DIPEA (10 eq.), 4 hours; h) TFA/TIPS/H_2O (95/2.5/2.5), 3 hours.

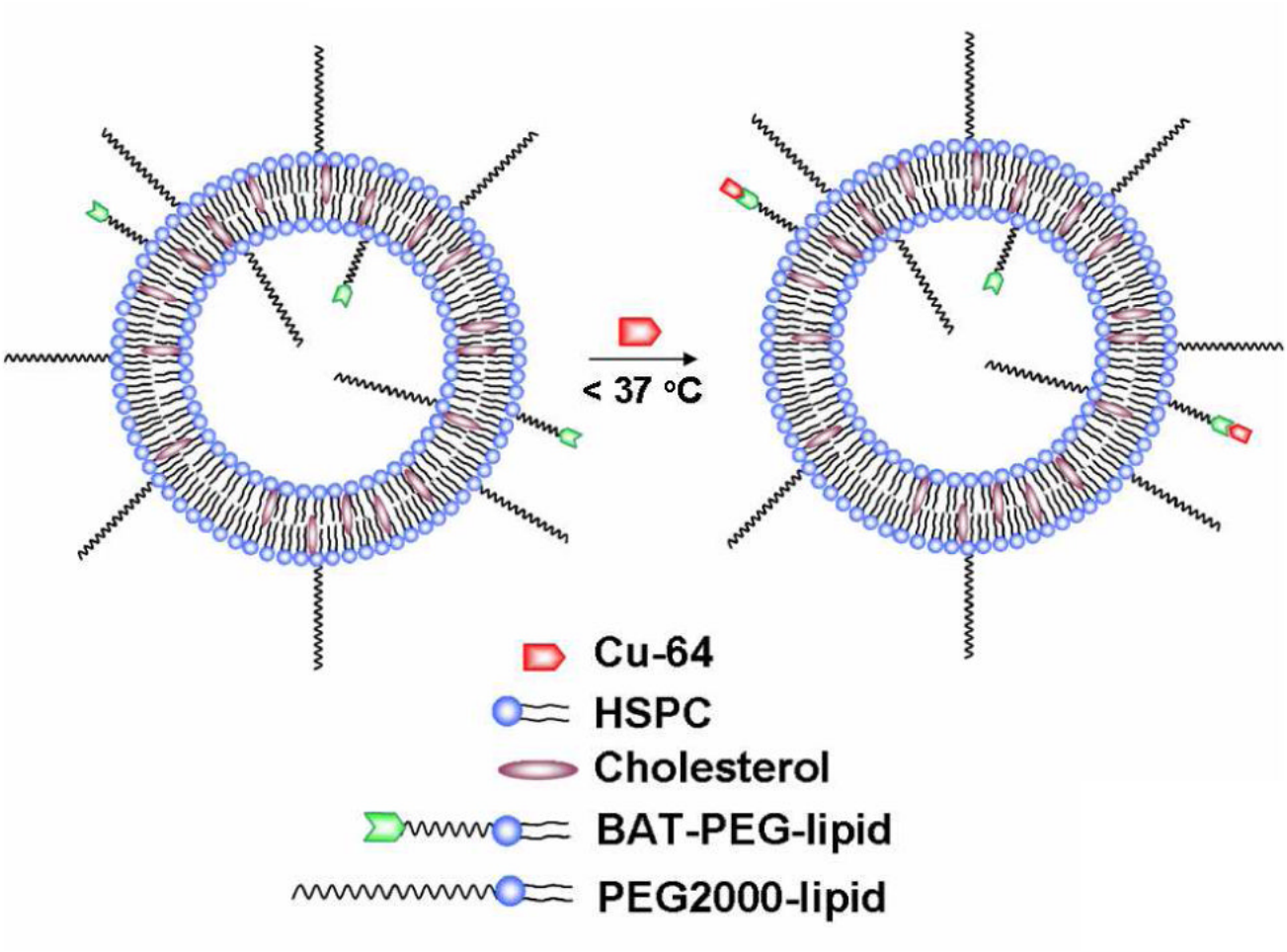

Figure 1.
Surface chelation model of ^{64}Cu-labeled long circulating liposomes, where labeling was performed under mild conditions (< 37 °C) to preserve liposome stability and loading during labeling.

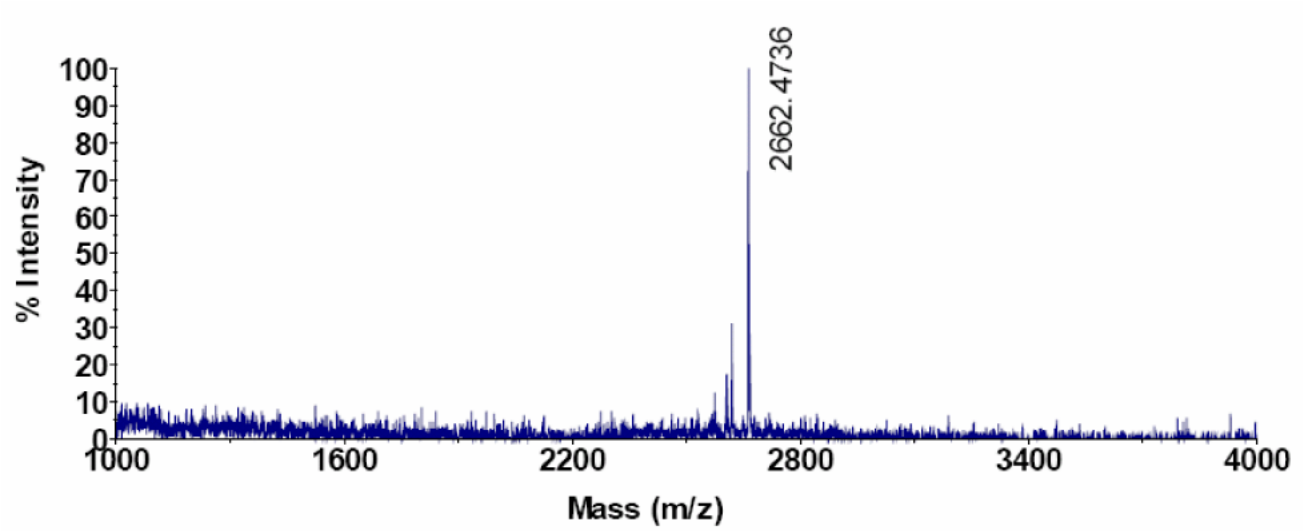

Figure 2.
Matrix-assisted laser desorption/ionization (MALDI) spectrum of conjugated BAT-PEG-lipid.

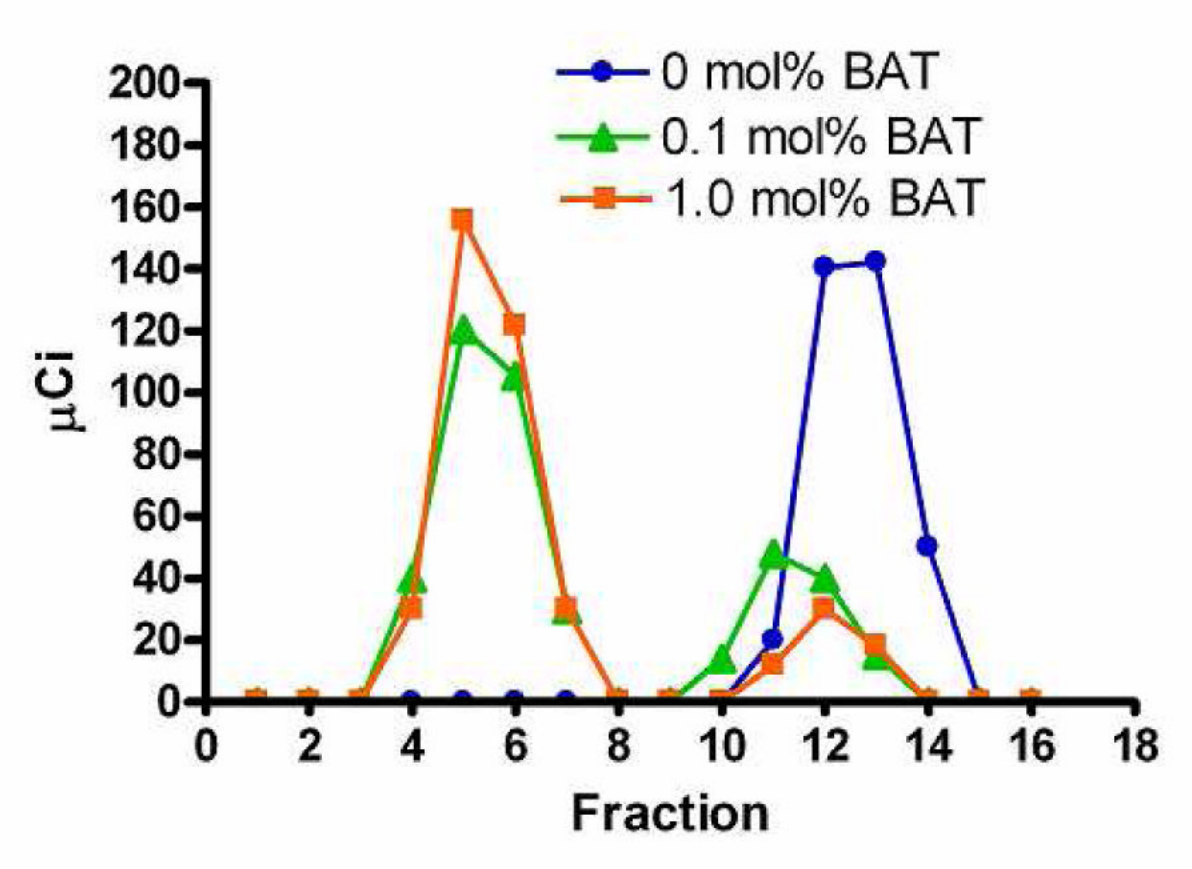

Figure 3.
Elution profiles of ^{64}Cu-labeled liposomes after incubation with $^{64}CuCl_2$ for 1 hour and EDTA for 20 min at room temperature in 0.1 M ammonium acetate buffer (pH 5.0). The molar ratios of lipid-PEG-BAT are: 0 mol% (circle), 0.1 mol% (triangle) and 1.0 mol% (square), and are combined with 90 % DPPC and 10% DSPE-PEG2000 (mol/mol).

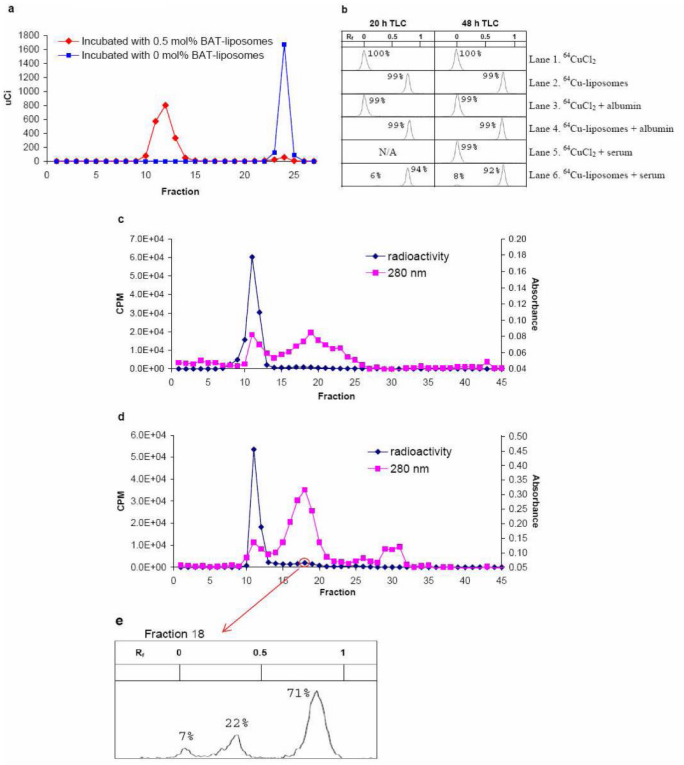

Figure 4.

In vitro assay of ^{64}Cu-labeled liposomes: a) Elution profiles of liposomes with 0.5 mol% BAT-PEG-lipid (HSPC:cholesterol:DSPE-PEG2000:BAT-PEG-lipid = 55.5:39:5.0:0.5, mol/mol/mol/mol, red diamond) and 0 mol% BAT-PEG-lipid (HSPC:cholesterol:DSPE-PEG2000 = 56:39:5.0:0.5, mol/mol/mol, blue square) after incubation with $^{64}CuCl_2$ for 40 min and EDTA for 15 min at 35 °C in 0.1 M ammonium acetate buffer (pH 5.0). Liposomes were separated by a size exclusion column. With 0.5 mol% BAT-PEG-lipid, ^{64}Cu was associated with liposomes; without BAT-PEG-lipid ^{64}Cu was not detected in the liposomal fraction. b) Radio TLC on silica plate was developed with eluant (chloroform:methanol:H_2O = 50:40:6, vol/vol/vol). Retention factor (R_f) of ^{64}Cu-BAT-PEG-lipid was 0.8 and that of $^{64}CuCl_2$ was 0. Lanes 1-6 were measured at 20 and 48 hours as indicated in figure 3b. c) Chromatogram of the mixture of ^{64}Cu-liposomes and albumin (8 mg/mL) in PBS after 48 h incubation at 37 °C. The mixture was separated by a size exclusion column Elution of liposomes and serum proteins was measured by gamma-counter (diamonds) and UV absorbance at 280 nm (squares). d) Chromatogram of the mixture of ^{64}Cu-liposomes and serum in PBS (PBS:serum = 1:1, vol/vol) after 48 h incubation at 37 °C. e) Radio TLC of fraction 18 performed as in b). The peak (R_f 0.8) represents ^{64}Cu-BAT-PEG-lipid. Small peak near 0 indicates free ^{64}Cu and middle peak has not been characterized.

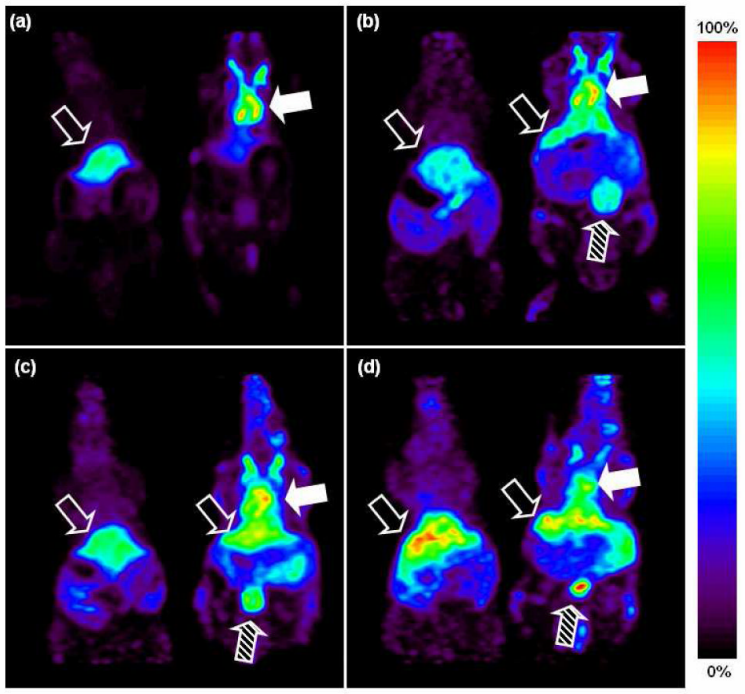

Figure 5.
Coronal view of microPET image with two mice (left: $^{64}CuCl_2$ injected, right: ^{64}Cu-liposomes) at four time points ((a) 0, (b) 18, (c) 28, (d) 48 hrs after i.v. injection). Relative scale (the brightest spot is maximum) was applied for images. Filled arrow indicates blood pool, striped arrow indicates bladder, and unfilled arrow indicates liver.

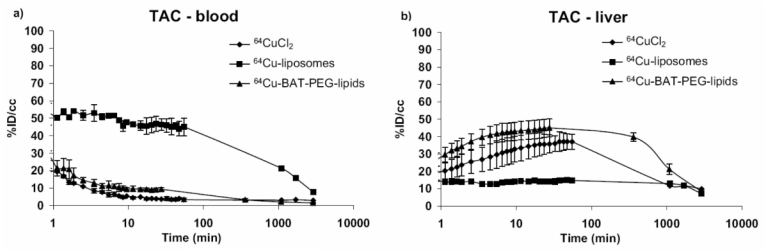

Figure 6.

Time activity curves in blood and liver of mice (n = 3~4 per group) after bolus injections of $^{64}CuCl_2$, ^{64}Cu-BAT-PEG-lipid, and ^{64}Cu-labeled liposomes. TACs for heart and liver (a and b) were obtained from microPET in each organ of interest as a function of time by drawing regions of interest (ROI).

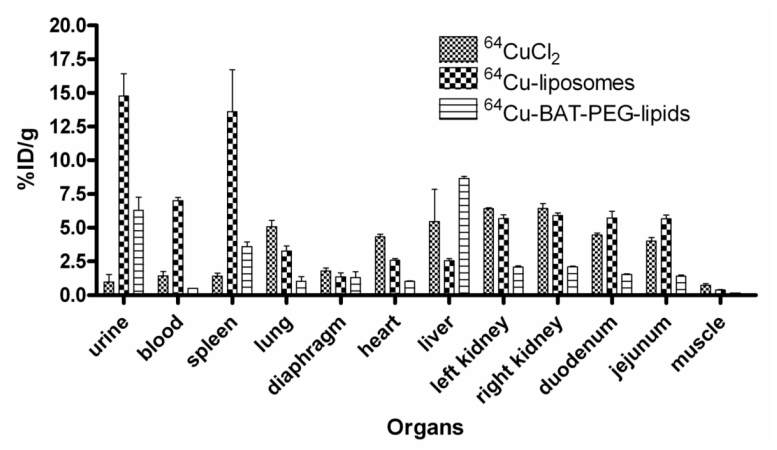

Figure 7.
Biodistribution of liposomal ^{64}Cu (n = 4), ^{64}Cu-BAT-PEG-lipid (n = 3), and free ^{64}Cu (n = 3) in mice at 48 hours after injection.

Table 1

Size (nm) and Zeta potential (mV) of 0.5 mol% BAT-liposomes (n = 2).

Liposome	Size (nm)		Zeta potential (mV)	
	before	after	before	after
BAT-liposome	108.8 ± 5.5	97.2 ± 3.5	-40.8 ± 4.3	-41.2 ± 3.2