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Copper-64 labeled liposomes for imaging bone marrow☆

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ABSTRACT

Introduction: Bone marrow is the soft tissue compartment inside the bones made up of hematopoietic cells, adipocytes, stromal cells, phagocytic cells, stem cells, and sinusoids. While [^{18}F]-FLT has been utilized to image proliferative marrow, to date, there are no reports of particle based positron emission tomography (PET) imaging agents for imaging bone marrow. We have developed copper-64 labeled liposomal formulation that selectively targets bone marrow and therefore serves as an efficient PET probe for imaging bone marrow.
Methods: Optimized liposomal formulations were prepared with succinyl PE, DSPC, cholesterol, and mPEG-DSPE (69:39:1:10:0.1) with diameters of 90 and 140 nm, and were doped with DOTA-Bn-DSPE for stable ^{64}Cu incorporation into liposomes.
Results: PET imaging and biodistribution studies with ^{64}Cu-labeled liposomes indicate that accumulation in bone marrow was as high as 15.18 ± 3.69%ID/g for 90 nm liposomes and 7.01 ± 0.92%ID/g for 140 nm liposomes at 24 h post-administration. *In vivo* biodistribution studies in tumor-bearing mice indicate that the uptake of 90 nm particles is approximately 0.89 ± 0.48%ID/g in tumor and 14.22 ± 8.07%ID/g in bone marrow, but respective values for Doxil® like liposomes are 0.83 ± 0.49%ID/g and 2.23 ± 1.00%ID/g.
Conclusion: Our results indicate that our novel PET labeled liposomes target bone marrow with very high efficiency and therefore can function as efficient bone marrow imaging agents.

1. Introduction

Bone marrow is the soft tissue compartment found inside bones (both hollow and spongy compartments) and is estimated to account for 2%–5% of human body weight. Bone marrow comprises hematopoietic cells, adipocytes, stromal cells, phagocytic cells, stem cells, and sinusoids [1]. Regulation of hematopoiesis by resident hematopoietic stem cells is one of the essential functions of bone marrow, making it one of the most critical organs for survival. Bone marrow is dynamic in nature and its spatial distribution and composition change with age. However, bone marrow disorders such as leukemias, lymphomas, myeloproliferative diseases, and myelosuppressive diseases lead to abnormal distribution of bone marrow compartments. In addition, certain pharmacological interventions such as chemotherapy and/or radiotherapy during cancer treatment or antibiotic treatments can interfere with marrow function, leading to reversible or irreversible suppression of bone marrow [2]. These conditions can cause significant changes in bone marrow distribution; thus, imaging of bone marrow is helpful in distinguishing among different hematological disorders.

To understand the behavior of bone marrow, sampling by aspiration is the gold standard, as it can provide detailed information about the different cell types. However, biopsies are not always feasible and are highly invasive in nature. Magnetic resonance imaging (MRI)-based techniques have been developed that take into account the presence of fatty tissue that leads to fast T1 relaxation time [3]. Nuclear medicine-based approaches have taken advantage of the phagocytic compartment to develop bone marrow imaging agents. Technetium-99m-labeled sulfur/albumin colloids phagocytosed by mononuclear phagocyte system (MPS) post-intravenous administration have been developed and successfully used for imaging bone marrow

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compartments in clinical practice [4,5]. Though the functions of the hematopoietic system and phagocytic system are completely different, the distribution of these two systems is remarkably similar in bone marrow and thus utilizing either system to reflect overall bone marrow distribution has been highly successful for such applications [6,7]. However, SPECT imaging is not quantitative and therefore has some limitations.

Iron-52 ($t_{1/2}$ = 8.2 h; β^+ = 56%), a PET isotope, can be used for labeling erythrocytes by simple i.v. administration of [^{52}Fe]-Citrate to provide quantitative information about the erythropoietic compartment and thus imaging the bone marrow. However, the production of iron-52 (requiring ~70 MeV proton beam) is not feasible in routine biomedical cyclotrons and therefore limits its application in standard clinical settings. [^{18}F]-3′-Fluoro-3′-deoxythymidine ([^{18}F]-FLT) has been successfully used to image proliferating marrow and can aid in identifying hematological disorders that involve the disruption of proliferating bone marrow [8,9]. To date, there have been no reports of PET-labeled particulate systems that can target the phagocytic compartment of the bone marrow. In the current manuscript, we report on the development of a copper-64 labeled liposomal system that can selectively target bone marrow and other organs of the MPS and can therefore function as a bone marrow PET probe. In addition to their imaging capability, the liposomes can function as drug carriers and can thus potentially be used for selective delivery of drugs to the bone marrow.

Liposomes are closed artificial spherical vesicles made of lipid bilayers that can range in size from 50 to 1000 nm. For biological applications, the optimal size ranges from 50 to 500 nm and the liposomes can be employed for packaging and delivering different types of molecules, including small organics, peptides, RNA, DNA, and diagnostic and therapeutic agents [10,11]. Indeed, liposomes are commonly referred to as nanocarriers and have been developed to mitigate side effects, enhance delivery, and reduce non-target toxicity based on either active or passive targeting mechanisms. The most famous example is the FDA-approved drug Doxil® (the trade name of the liposome-encapsulated topoisomerase inhibitor Doxorubicin), which is used to treat certain breast and pediatric cancers [12] and can target tumors due to long circulation time and enhanced permeability and retention (EPR) effect [13]. Liposomes have been developed to target bone marrow to deliver drugs. The utility of such liposomes was demonstrated through SPECT imaging of technetium-99 m hexamethylpropyleneamine oxime ([^{99m}Tc]-HMPAO)-labeled liposomes by Phillips et al. [14]. However, to date, no PET-labeled liposomes that can image bone marrow have been reported.

Our major goal is to develop a liposomal formulation that can be radiolabeled with a suitable PET isotope under mild conditions and can selectively target bone marrow with optimal pharmacokinetic behavior. The distribution of liposomes *in vivo* is highly dependent on the size, composition, and charge of the liposome. To achieve maximal bone marrow targeting (BMT) efficiency while minimizing circulation time, we incorporated three features in the liposomal design: 1) lower the polyethylene glycol (PEG) load to <1% to minimize circulation time; 2) increase negative charge on the particle to facilitate phagocytic uptake in the bone marrow; [15,16] and 3) minimize the particle size to reduce the trapping in the hepatic sinusoids [17]. Copper-64 (^{64}Cu) was chosen as the isotope of choice because of its ideal 12.7 h half-life that matches the liposomal half-life, and since it is easy to chelate under mild conditions, forms stable complexes with chelators such as DOTA or 1,4,7-triazacyclononane-triacetic acid (NOTA), and exhibits little to no osseophilicity [18]. Additionally, studies from other groups have demonstrated that copper-64 labeled liposomes have good *in vivo* properties and accumulate in tumors by EPR effect [19,20].

Toward this goal, we have synthesized 90 nm DOTA-functionalized liposomes with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(succinyl) (succinyl DPPE), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG2000-DSPE) and DOTA-Bn-DSPE (DSPE functionalized with bifunctional chelator DOTA) in the ratio of (60:30:10:1:0.1) and labeled them with ^{64}Cu. The current manuscript describes the synthesis, evaluation, and biological characterization, including imaging and biodistribution studies, of ^{64}Cu-radiolabeled bone marrow-targeting liposomes.

2. Materials and methods

2.1. Materials

All chemicals were used as received without further purification. DSPC, 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine (DSPE), Succinyl-DPPE, and mPEG2000-DSPE, along with cholesterol, were purchased from Avanti Polar Lipids (Alabaster, AL). The S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (p-SCN-Bn-DOTA) was purchased from Macrocyclics (Dallas, TX), and the ^{64}Cu was purchased from Washington University (St. Louis, MO). PD-10 column was purchased from GE Healthcare Life Sciences (Pittsburgh, PA), and athymic male nude mice were purchased from Harlan Laboratories (Indianapolis, IN). Matrigel was purchased from BD Biosciences (San Jose, *CA*, USA). ITLC-SG chromatography paper was purchased from Agilent Technologies (SG10001, Santa Clara, *CA*).

2.2. Synthesis of DOTA-Bn-DSPE

Twelve micromoles of p-SCN-Bn-DOTA was dissolved in 1 mL of chloroform–methanol–water (65:35:8) mixture and 22 μmol of DSPE was dissolved in 1 mL of the chloroform–methanol–water mixture. After mixing two solutions, 48 μmol of triethylamine was added. The mixture was heated to 40 °C and stirred for 2 h, followed by stirring at room temperature for 16 h. The reaction progress was monitored using silica gel-coated TLC plates and product formation was confirmed by mass spectrum.

2.3. Preparation of liposomes

The major component of bone marrow-targeting liposomes is composed of DSPC and cholesterol in a molar ratio of 6:4. Depending on the formulation, the initial lipid mixture was supplemented with 1 or 2.5 mol% of mPEG2000-DSPE and/or 10% succinyl-DPPE. Doxil® like liposomes were composed of DSPC, cholesterol, succinyl-DPPE, and mPEG2000-DSPE in a molar ratio of 60:40:10:5. Additional 0.1 mol% DOTA-Bn-DSPE was added to all lipid composition for facilitating subsequent radiolabeling with copper-64 or other radiometals. All lipids were dissolved in chloroform and the solvent was evaporated under flowing nitrogen gas at 37 °C. Residual solvent was removed under reduced pressure (0.2 Torr) overnight. Lipid film was hydrated in PBS at 65 °C for 1 h and the crude lipid dispersion was extruded 11 times as specified in manufacturer's manual through either 0.1 μm or 0.03 μm pore size Whatman® Polycarbonate Membrane Filter using a mini-extruder system (Avanti Polar Lipids, Alabaster, AL) at 65 °C. After extrusion, the liposomes were purified using a PD-10 column (GE Life Sciences, Marlborough, MA) to remove unincorporated liposomal lipids and salts.

2.4. Characterization of liposomes

Liposome size distribution and zeta potential at 25 °C in PBS pH 7.4 were determined by dynamic light scattering using Zetasizer Nano-ZS from Malvern Instruments (Malvern, Worcestershire, UK). Liposome particle concentration was measured at room temperature in PBS by light scattering using NanoSight NS500 from Malvern Instruments. Liposome stability under serum was determined after incubation in 50% FCS in PBS for 24 h. Long-term liposome stability at 4 °C was tested for one year by analyzing size distribution (Fig. S5). DSPC concentrations in liposomes were determined by phopholipase D activity to hydrolyze the ester bond between the phosphate and the choline. The assay was