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Non-invasive detection of macrophages in atheroma using a radiocontrast-loaded phosphatidylserine-containing liposomal contrast agent for computed tomography

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Abstract

Purpose—Macrophage plays an important role in plaque destabilization in atherosclerosis. By harnessing the affinity of macrophages to certain phospholipid species, a liposomal contrast agent containing phosphatidylserine (PS) and computed tomographic (CT) contrast agent was prepared and evaluated for CT imaging of plaque-associated macrophages in rabbit models of atherosclerosis.

Procedures—Liposomes containing PS and iodixanol were evaluated for their physicochemical characteristics, *in vitro* macrophage uptake, *in vivo* blood pool clearance and organ distribution. Plaque enhancement in the aorta was imaged with computed tomography (CT) in two atherosclerotic rabbit models.

Results—*In vitro* macrophage uptake of PS-liposomes increased with increasing amount of PS in the liposomes. Overall clearance of PS-liposomes was more rapid than control liposomes. Smaller PS-liposomes ($d = 112 \pm 4$ nm) were more effective than control liposomes of similar size or larger control and PS-liposomes ($d = 172 \pm 17$ nm) in enhancing aortic plaques in both rabbit models.

Conclusions—Proper liposomal surface modification and appropriate sizing are important determinant for CT-based molecular imaging of macrophages in atheroma.

Keywords

Atherosclerosis; Macrophage; Molecular Imaging; Computed Tomography; Apoptosis; Inflammation; Liposomes

Introduction

Macrophage plays an important role in the progression and destabilization of atherosclerotic plaques [1]. Activated macrophages produce a variety of pro-inflammatory cytokines and proteases that are implicative for the formation of necrotic lipid core, thin fibrous cap and rupture-prone atheroma [2]. The ability to detect the distribution, density and activities of plaque-associated macrophages may offer an opportunity to determine a patient's risks of

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Conflict of Interest

The authors have no conflict of interest to disclose.

developing major cardiovascular events in the near future. A number of imaging modalities and contrast agents have been evaluated for their utility in detecting plaque-associated macrophages. Since the ultimate goal of this imaging approach is to screen high-risk subjects for the presence of the vulnerable lesions prior to the declaration of the clinical events, non-invasive imaging modalities will be the preferred imaging technique, and magnetic resonance imaging (MRI), nuclear imaging, ultrasound imaging and computed tomography (CT) imaging belong to those imaging categories. Among those imaging modalities, CT imaging is an attractive option given the fact that CT angiography is an established clinical tool for detecting stenotic lesions in major vascular territories. Furthermore, CT imaging offers excellent spatial resolution and tissue penetration [3], and could potentially be useful in detecting deep-seated lesions in the coronary and cerebrovascular systems.

Given the favorable features of CT imaging, it is surprising to note that only a handful of CT contrast agents have been developed for molecular imaging of atherosclerosis but none are available for clinical use. The most prominent candidate is N1177 developed by NanoScan Imaging, LLC., an emulsified suspension of crystalline iodinated particles that appears to be taken up by macrophages, resulting in enhanced signals in the aorta of atherosclerotic rabbits for CT imaging [4–6]. Another CT contrast agent is a liposome-based agent containing iodixanol as the payload, which demonstrated a degree of contrast uptake by resident macrophages in the aortic arch of apoE^{-/-} mice [7]. Our group previously developed a self-assembled liposomal preparation that contained an iodinated phospholipid in the bilayer, leaving the liposomal core for drug encapsulation [8].

Biocompatibility is a major consideration when designing any contrast agent. Many synthetic and exogenous agents have been used to enhance interactions between the contrast agent and macrophages, however, those agents will need extensive testing prior to human use. In the current study, we utilize a naturally occurred phospholipid species, phosphatidylserine (PS), in the preparation of the liposomal shell to enhance macrophage uptake. Phosphatidylserine is externalized on the cell surface of senescent cells and is a potent target for macrophage recognition and their subsequent uptake [9, 10]. We hypothesize that the inclusion of PS, a naturally occurring and biocompatible phospholipid, in the liposomal CT contrast agent will enhance uptake and accumulation of CT contrast agents in plaque-associated macrophages for non-invasive CT imaging in atherosclerotic animal models.

Materials and Methods

Materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (16:0 PEG2000 PE); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (16:0 LissRhod PE) and 200 nm and 80 nm nucleopore membranes were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphoserine (sodium salt) (DPPS); cholesterol were purchased from Echelon Lipids, Inc. (Salt Lake City, Utah, USA); Lipex TM extruder was purchased

from Northern Lipids, Inc. (Burnaby, BC, Canada). All solvents were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin, were purchased from Invitrogen (Carlsbad, CA, USA). MTT assay kit was purchased from Roche Applied Science (Indianapolis, IN, USA). Lab-Tek™ chamber slide system was purchased from Thermo Scientific/ NalgeNunc International (Rochester, NY, USA). Vectashield mounting medium with DAPI was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). MicroKros hollow fiber diafiltration modules were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). RAM-11 antibody was purchased from Dako Inc. (Carpinteria, CA, USA). Alexa 488 (green color) goat anti-mouse was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). New Zealand White (NZW) rabbits (2–3 kg) (n = 11) were purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA) and Watanabe Hereditary Hyperlipidemic (WHHL) rabbits (n = 16) were bred in-house at the University of Texas Health Science Center at Houston.

Preparation and characterization of liposomal preparations

Liposomes were prepared by dissolving lipids (molar ratios as indicated in Table 1) in anhydrous ethanol (10 vol%) at 60°C for 2h. For incorporation of fluorescent labels, rhodamine lipid was also added to the ethanol solution in the molar ratio as indicated in Table 1. The dissolved lipids in ethanol were added to iodixanol (500 mg iodine/ ml) in PBS buffer (1 wt%) maintained at 60°C and stirred for several hours until ethanol was completely evaporated. Next, liposomal suspension was subjected to several freeze thaw cycles and extruded using 200 nm to obtain ~ 200 nm size liposomes and further with 80 nm nucleopore membrane to obtain ~ 100 nm size liposomes using 10 mL Lipex™ extruder several cycles to ensure uniform size distribution of liposomes. Finally, they were purified using hollow fiber dialysis microkros membrane (Spectrum labs) 500 kD membrane cut off, to remove unencapsulated iodixanol and stored at 4°C until further use. The iodixanol standard curve and the corresponding amount of encapsulated iodixanol in liposomes were analyzed by measuring the X-ray attenuation of the samples with a General Electric (GE) eXplore Ultra flat panel CT scanner. The mean hydrodynamic diameter, polydispersity index and zeta potential charge of liposomes was measured using Zeta Nanosizer ZS (Malvern Instruments Ltd, Worcestershire, UK).

In vitro cellular uptake of liposomal preparations by macrophages

The cellular uptake and distribution of DPPC, PS liposomes (2, 8, 20 mol%) were observed by fluorescence microscopy. After the J774A.1 macrophage cells achieved 80% confluency, the cells were scraped and seeded onto 4 well chamber slides at a density of 4.0×10^3 per well (surface area of 1.7 cm² per well, 4-chamber slides) and incubated for 24 h at 37°C. The DPPC, PS (2, 8, 20 mol %) liposomes at 3 mM lipid concentration were added and incubated for 4 h at 37°C. After incubation, all reagents were removed. The cells were washed with PBS (pH 7.4) for at least 3 times and fixed with 4% formalin for 10 min and washed with PBS and mounted with mounting medium containing DAPI to stain the nuclei. The cells were observed by epifluorescence microscope (NIKON Eclipse) using separate filters for nuclei, DAPI filter (blue); for rhodamine, TRITC filter (red). The cellular uptake

efficiency was also semi-quantitated by flow cytometry. After the macrophage cells obtained 80% confluency, the cells were detached by scraper and seeded onto a 6-well plate with a density of 6×10^4 cells per well and incubated overnight. The culture medium was removed and DPPC and PS (2, 8, 20 mol %) liposomes at 30 mM lipid concentration were added and incubated for 4 h at 37°C. After incubation, all reagents were removed and cells were washed with PBS at least 3 times. After washing with PBS, cells were scraped and the supernatant was carefully removed. PBS buffer containing 2% (v/v) FBS was added to the cell pellet and resuspended. The cells were analyzed using a FACS Calibur fluorescence-activated cell sorter (FACS™) equipped with Cell Quest software (Becton Dickinson Biosciences, San Jose, CA, USA). The cytotoxicity of liposomes toward the macrophage cells was evaluated using colorimetric MTT assay kit. After the macrophages achieved 80% confluency, the cells were scraped and seeded onto 96-well plates at a density of 1.5×10^4 cells per well. After 24 h of incubation, the cell culture medium was removed. Cytotoxicity was measured after 24 h of incubation with 0.86 mM final lipid concentration DPPC and PS liposomes (2, 8, 20% PS) at 37°C under a 5% CO₂ atmosphere. After the incubation period, the cells were treated with 10 µL/well MTT reagent at 37°C for 4 h according to the manufacturer's protocol. The absorbance was read at a wave length of 550 nm with a spectra max micro plate reader (Molecular Devices, Sunnyvale, CA, USA). The assay was run in triplicate.

In vivo pharmacokinetic parameters and organ distribution of liposomal preparations

The animal protocol was approved by the animal welfare committee, Center for Laboratory Animal Medicine and Care (CLAMC) at University of Texas Health Science Center at Houston. All applicable institutional and/or national guidelines for the care and use of animals were followed. Blood pool enhancement and plasma clearance of intravenously injected iodixanol-loaded PC- and PS-liposomes were evaluated in NZW and WHHL rabbits using a flat panel CT scanner over 48 hours. Organ bio distribution of iodixanol-loaded PC- and PS-liposomes in C57BL/6 mice were serially imaged with a flat panel CT scanner for up to 2 months.

Plaque-associated contrast enhancement by liposomal preparations in WHHL and cholesterol-fed balloon-denuded NZW rabbits

Two atherosclerotic rabbit models were studied, the WHHL rabbits and balloon-denuded cholesterol-fed NZW rabbits. A stable colony of WHHL rabbits is maintained at the University of Texas Health Science Center at Houston. These animals are homozygous for the low-density lipoprotein receptor and spontaneously develop hyperlipidemia (plasma total cholesterol > 600 mg/dL) on a normal chow diet and extensive atheroma in the aorta. The balloon-injured cholesterol-fed NZW rabbits were created by a single balloon denudation technique. NZW rabbits (2–3 kg) were pre-fed with 1–2 weeks of an atherogenic rabbit chow diet containing 0.2% cholesterol and 4% coconut oil, followed by surgical denudation of the infra-diaphragmatic aorta using a 4F Fogarty catheter under fluoroscopic guidance. The animals were continued on the atherogenic diet for 3–6 months for the development of neointimal hyperplasia.

WHHL rabbits aged between 9 months and 24 months and NZW rabbits were sedated with a subcutaneous cocktail of ketamine/ acepromazine and injected IV via a marginal ear vein with PC- or PS-liposomes (200–350 mg iodine/ kg body weight). The iodixanol concentration in the liposomal preparations range between 52 mg/ml and 86 mg/ml. Group allocation was as follows: Control NZW + large PC liposomes (n = 3), Control NZW + large PS liposomes (n = 3), WHHL + large PC liposomes (n = 5), WHHL + large PS liposomes (n = 5), WHHL + small PC liposomes (n = 1), WHHL + small PS liposomes (n = 5), balloon-denuded NZW + small PC liposomes (n = 1), balloon-denuded NZW + small PS liposomes (n = 4). The animals were imaged at baseline prior to and over 48 hours after contrast agent administration. The final CT imaging was performed immediately after euthanizing the animal to ensure no motion artifacts will interfere with image acquisition. The animals were imaged in a supine position in a GE eXplore Ultra flat panel CT scanner with no respiratory or cardiac gating with the following imaging parameters: Tube voltage 90 kVp, tube current 22 mA with 16 rotations/ exposure, section thickness = 0.2 mm. The region of interest (ROI) covered the aortic arch to the infra-renal abdominal aorta. Images were post-processed with OsiriX using cuvilinear reconstruction and pseudo-colored with the window level and width at 100 HU and 404 HU. Reconstructed images are displayed using maximum intensity projection at 1.0 mm. All experiments were approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston.

Immunochemical and immunofluorescent analysis

Aortas were embedded in optimal cutting temperature (OCT) compound and 5 μ m thick sections were cut. The sections were washed with PBS, permeabilized with 0.3% Triton X-100, blocked with goat serum, and incubated with monoclonal mouse anti-rabbit macrophage clone RAM-11 primary antibody (1:200 dilution) overnight. Next, slides were washed with PBS and incubated with Alexa 488 goat anti-mouse IgG secondary antibody (1:200 dilution) for one hour. Following washing, the slides were mounted with DAPI containing mounting medium and imaged with a NIKON fluorescence microscope. For DAB immunostaining, biotinylated goat anti-mouse secondary antibody was used and the vectastain DAB kit procedure was followed and slides were imaged with a light microscope installed with a CCD camera.

Results

Physicochemical characterization of liposomal preparations

The hydrodynamic diameter of the liposomal preparations is dependent on the pore size of the polycarbonate membrane (Table 2). The final lipid concentration of the liposome formulations was approximately 150 mM (total lipid concentration). Standard DPPC and PS liposomal preparations extruded with the 200 nm pore size polycarbonate membrane had an average hydrodynamic diameter of 173 ± 9 nm and 171 ± 24 nm, respectively. When extruded with the 80 nm pore size polycarbonate membrane, the hydrodynamic diameter of standard DPPC and PS liposomal preparations were reduced to 111 ± 5 nm and 113 ± 5 nm, respectively. The polydispersity index of the preparations ranges from 0.019 to 0.08. Predictably, the zeta potential of standard DPPC liposomes was higher at -17 mV than that

of PS liposomes at -60 mV. After diafiltration, the concentration of iodixanol was 61 ± 14 mg/ml in the larger liposomes and 35 ± 5 mg/ml in the smaller liposomes.

In vitro uptake of liposomal preparations by macrophage cell culture

Both fluorescent microscopy and flow cytometry revealed an increased amount of uptake of rhodamine-labeled liposomes by macrophages with increasing amount of PS in the liposomal shell (Fig. 1). However, flow cytometry suggests that the amount of liposome uptake by macrophages was heterogeneous when the %mol of PS increased to 20%, as indicated by the broader shift in the fluorescence signal. No detectable cellular toxicity was found with all standard and PS liposomal preparations. Given the stability and consistent macrophage uptake profile of 8% PS-liposomes, that preparation was used in subsequent *in vivo* experiments.

In vivo pharmacokinetic parameters and organ distribution of liposomal preparations

The predictable relationship between the iodixanol concentrations and x-ray attenuation values (HU) enabled non-destructive determination of iodixanol clearance in the blood pool and organ distribution in live animals. After intravenous administration of various iodixanol-loaded liposomal preparations into rabbits, peak blood pool enhancement reached a similar level at the earlier time points (Fig. 2a). However, the overall clearance was more rapid with decreasing liposome size and the presence of PS in the liposomal shell. Long-term bio distribution study was also performed in mice. The clearance of the contrast agent from the blood pool was sequestered in the liver and spleen and persisted in the reticulo endothelial system for up to 2 months (Fig. 2b).

CT detection of aortic wall enhancement by iodixanol-loaded liposomal preparations in atherosclerotic rabbit models

Initial *in vivo* imaging studies were performed using standard and PS liposomes extruded with the 200 nm polycarbonate membrane ($d = 172 \pm 17$ nm) based on the favorable *in vitro* macrophage uptake studies and previous studies suggesting liposomes in that size range have prolonged blood pool circulation [11]. After intravenous administration, peak blood pool enhancement reached about 150 HU and maintained for about 2 hours with both standard and PS liposomes (Fig. 2a). Forty-eight hours after contrast agent administration, blood pool enhancement approached baseline levels, and specific imaging for aortic wall enhancement was performed. No matter which liposomal preparation was injected, no enhancement was seen in the aortic wall in both the control NZW ($n = 6$) and the atherosclerotic WHHL rabbits ($n = 10$). Further studies were performed to elucidate whether the lack of aortic wall enhancement was due to the size of the contrast agent or the selected atherosclerotic animal model. Smaller standard and PS liposomes ($d = 112 \pm 4$ nm) were prepared using the 80 nm polycarbonate membrane. In addition to the WHHL rabbits, another atherosclerosis model was created in NZW rabbits with combined balloon-denudation and cholesterol feeding to determine if macrophages in the two atherosclerotic animal models may be qualitatively different in terms of their phagocytic capacity, which explains the lack of uptake in the initial experiments. In both atherosclerotic animal models, the smaller PS liposomes resulted in focal enhancement in the aortic regions corresponding

to plaque formation in both the balloon-denuded cholesterol-fed rabbits (n = 4) and WHHL rabbits (n = 5) (Fig. 3b & d). In contrast, control liposomes produced no enhancement in the aorta in both animal models (Fig. 3a & c).

Validation of specific contrast uptake in ex vivo aortic specimens and immunofluorescence microscopy

Immunohistochemical staining confirmed abundant macrophage infiltration in the lesions of both models (Fig. 4). Immunofluorescence microscopy revealed the pattern of liposome uptake in the aortic wall with respect to different liposomal preparations (Fig. 4). In animals injected with the smaller standard liposomes, the rhodamine signal was evenly distributed throughout the aortic wall with no evidence of co-localization with the resident macrophages. In WHHL and cholesterol-fed NZW rabbits, the smaller PS liposomes specifically co-localized with macrophages in the aortic wall. In contrast, no rhodamine signal was detected in the aortic wall when the larger standard and PS liposomes were injected in both atherosclerotic animal models.

Discussion

CT angiography is a clinically established imaging modality to identify the presence of stenotic lesions in various vascular territories. By harnessing the phagocytic activities of macrophages of apoptotic bodies such as PS-liposomes and subsequent retention of CT contrast agent, this may provide additional complimentary pathologic information on plaque vulnerability in the coronary and carotid arteries as well as the thoracic and abdominal aortas. In this study, we have demonstrated that liposome uptake by macrophages in vivo is highly dependent on the size and surface modifications of liposomes. In vitro studies demonstrated that macrophage uptake of liposomes increased with increasing amount of PS in the liposomal shell. However, cell culture experiments do not take into account of other physiological barriers that might impede liposomal entry into the arterial wall. The initial consideration in our liposome design was to maximize the interactions between the liposomes and intended targets by prolonging blood pool circulation of the liposomes. Based on previous studies, that goal could be achieved with liposomes with an average diameter of around 200 nm [11]. Additional surface modification with pegylation was also included in the liposomes to reduce sequestration by the reticulo endothelial system. One concern with pegylation is the steric hindrance of PEG for macrophage uptake of liposomes. In agreement with previous studies [12, 13], our macrophage culture experiments demonstrated that a small amount of pegylation did not appear to compete with PS for macrophage uptake but substantially reduced the uptake of control liposomes. We initially hypothesized that prolonged blood pool circulation of PS-liposomes was vital for increased interactions between PS-liposomes and plaque-associated macrophages prior to their clearance. We were surprised to note that no matter whether control or PS-liposomes in the size range of around 172 ± 17 nm were administered in our WHHL rabbits, we could not detect any contrast enhancement with CT imaging or any evidence of macrophage uptake with fluorescence microscopy.

Thus, we explored two possible scenarios relating to the atherosclerotic animal model and the size of the liposomes. Our lab is fortunate to have access to an excellent model of atherosclerosis, the Watanabe Hereditary Hyperlipidemic rabbits. Other research groups rely on the creation of atheroma-like lesions/ neointimal hyperplasia by a combination of a hyperlipidemic diet and endothelial injury by balloon denudation. The complexity of macrophage biology has been extensively studied and it has become apparent that plaque-associated macrophages can be either pro- and anti-inflammatory and their phagocytic capacity can vary depending on their subtype and metabolic integrity [14, 15]. Indeed, phagocytic capacity of macrophages can be drastically reduced when they undergo apoptosis, a phenomenon known as defective efferocytosis, which is not uncommonly seen in advanced lesions [15]. This also raises the question whether there is a direct relationship between macrophage contents in the plaques and the amount of contrast that can potentially be retained by the resident macrophages for diagnostic and imaging applications. Would a more inflammatory model of atherosclerosis with endothelial dysfunction [16] such as one created by a combination of hyperlipidemic diet and endothelial injury generate macrophages with more active phagocytic activities and an endothelium that is more permeable to contrast agent penetration? Our findings did not support this notion. When we evaluated the two atherosclerotic rabbit models with the larger control and PS-liposomes, we did not detect any liposome uptake in the aortic wall regardless of the type of liposomes being used.

Thus, we decided to consider whether the size of the liposomes could affect efficient contrast uptake by macrophages in the plaques. Apart from the potentially favorable size for long circulation, larger liposomes can encapsulate more contrast materials to compensate for the relative insensitivity of x-ray imaging. As the size of the liposomes is reduced, the amount of encapsulated payload decreases exponentially. In our case, when reducing the diameter of liposomes from 172 ± 17 nm to 112 ± 4 nm, the concentration of encapsulated iodixanol reduced from 61 ± 14 mg/ml to 35 ± 5 mg/ml. Despite the lower encapsulated payload, sufficient focal CT contrast enhancement was seen in the aortic wall in both atherosclerotic rabbit models after injected with the smaller PS-liposomes, but not with the smaller control liposomes. Size-dependent contrast uptake by plaque-associated macrophages remains a contentious topic. A comprehensive study evaluated three forms of gadolinium: unencapsulated form (Gd-HP-DO3A), in association with micelles (~15 nm) and encapsulated in liposomes (~125 nm) in atherosclerotic mice [17]. The unencapsulated form rapidly entered the aortic wall but washed out within an hour and the micelles were retained within the aortic wall for up to 24 hours. Similar to our findings, the liposomal form of gadolinium was not detected in the aortic wall at all. One study reported control liposomes with diameters up to 200 nm were taken up by macrophages in the atheroma [7] but another study demonstrated macrophage uptake was seen with smaller paramagnetic liposomes (d ~ 90 nm) [18]. It remains to be determined if unmodified liposomes were retained by “enhanced permeability and retention” effect or being retained within macrophages. The presence of PS in liposomes clearly targets the phagocytic activities of plaque-associated macrophages. PS-liposomes with diameters up to 230 nm appeared to be taken up by macrophages in the aorta [19] but the uptake of the relatively smaller liposomes (d ~ 100 nm) was higher than the larger liposomes in vivo [20]. Both smaller contrast agents

($d < 50\text{nm}$) such as ultra small super paramagnetic iron oxide [21–23] and larger crystalline iodinated particles ($d \sim 153\text{--}408\text{ nm}$) [4, 5] appear to be taken up by macrophages in the aorta. However, significant uptake of the iodinated nanoparticles, N1177, was only seen in ruptured plaques, suggesting the penetration of larger contrast agents ($d > 200\text{ nm}$) into intact plaques is size-dependent. The reliance on phagocytic capacity of macrophages may offer an incomplete assessment of the inflammatory activities in the plaques. The effective dose of contrast agent uptake by macrophages will be dependent on factors such as the efficiency of macrophages in phagocytosing specific contrast agent, the rate of blood pool elimination of contrast agent, and penetration of the contrast agent into the macrophage-laden regions in the plaques. Furthermore, macrophage biology is complex and can pose a challenge to accurate assessment of macrophage contents in association with atherosclerosis. The exact relationship between proinflammatory activities and phagocytic activities among macrophages is yet to be determined; namely whether phagocytic activities and macrophage contents peak in the most advanced form of atherosclerosis or display a bimodal pattern in which low density of macrophages in the early stage and defective macrophages in the late stage of the disease result in low phagocytic activities in both the early and late stages of atherosclerosis. In fact, it is fascinating to note that the iodinated nanoparticles, N1177, did not enhance rupture-prone plaques despite large amount of macrophages in the plaque characterized by a large necrotic core with a thin overlying intact fibrous cap [6].

Calcification is frequently encountered in atherosclerotic arteries and conventional CT imaging cannot differentiate between endogenous calcium and iodinated contrast agents. We also observed aortic calcification in both atherosclerotic rabbit models. We cannot exclude the presence of our contrast agent within the calcified segment given the detection of fluorescently-labeled liposomes in histological sections. However, when interpreting the CT findings, we compared the calcification pattern at baseline and 48 hours after contrast injection and only registered definite enhancement in the uncalcified segments in the aorta. New CT techniques with dual-energy and “multi-color” CT imaging may resolve this issue [7, 24].

The encapsulation of iodixanol in liposomes shifted the metabolism of the payload from a renal route to the reticulo endothelial system. Pharmacokinetic analysis also indicated that PS-liposomes are cleared more rapidly from the blood pool than control liposomes. This is consistent with previous studies that indicated the presence of PEG on liposomes reduced the uptake of liposomes by the Kupffer cells in the liver, but increased their chance of clearance by the spleen [11]. While the intention of encapsulating a contrast agent in liposomes made up of naturally occurring phospholipid is to maximize biocompatibility, the unintended consequence of prolonged splenic retention of contrast agent will pose a universal issue with patient safety for all liposomally-loaded contrast agent. In fact, PEG cannot completely antagonize the effect of PS for uptake by the reticulo endothelial system and contrast retention in the spleen was observed for up to 2 months after administration in our model. To realize the full potential of macrophage targeting with PS, the formulation will need to be modified to reduce splenic retention, in turn, improving its safety profile and increasing its availability for macrophage targeting.

Another limitation of the current study relates to the technique how blood pool clearance of various liposomal preparations was measured. Most studies radio labeled the lipid components of the liposomes and measure the decline in radioactivity in serial blood sampling [25]. In the current study, the radiocontrast in the blood pool was serially measured by CT imaging and the accuracy of this approach is highly dependent on the stability of payload encapsulation in various liposomal preparations. The size and lipid composition can obviously affect how liposomes interact with various organs *in vivo* and the resultant payload stability. Based on the objective of the current study to evaluate contrast uptake by macrophages, this approach seems most appropriate because any free iodixanol would be rapidly cleared from the blood pool and only the encapsulated iodixanol within the liposomes would interact with macrophages in the arterial wall and generate contrast that could be detected by CT imaging.

Conclusions

PS-liposome serves as a biocompatible vehicle for targeting plaque-associated macrophages and has the potential to deliver a range of contrast agents for non-invasive imaging of the macrophage-enriched atheroma. The clinical applicability of this mode of imaging will be dependent on proper sizing of liposomes, surface modification to reduce retention by the reticulo endothelial system, and selection of contrast payload to maximize sensitivity for detection. Clearly, better understanding of macrophage biology will also be needed to validate this imaging strategy for assessing plaque vulnerability.

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Figure 1.

In vitro cellular uptake of control (DPPC) and various preparations of PS-containing liposomes by J774A.1 macrophages. Liposomes were labeled with rhodamine lipid and incubated with J774A.1 cells for 24 h. (a) Qualitative uptake of fluorescently-labeled liposomes were observed by fluorescence microscopy which demonstrated increasing amount of rhodamine signals within the cells. (b) Flow cytometry showed a rightward shift in the rhodamine signal with increasing %mol PS, suggesting increased cellular uptake. However, the distribution appeared broader with 20% PS, suggesting more heterogeneous cellular uptake of that preparation.

Figure 2.

Plasma clearance of various liposomal preparations and long-term organ distribution of PS-liposomes in vivo. (a) Peak blood pool enhancement (HU ~ 180–220) was achieved within 5–10 mins after intravenous injection of various iodixanol-loaded liposomal preparations into rabbits and maintained at a detectable level (HU ~ 120–160) for 120 mins after administration. The presence of PS in liposomes and the smaller liposomes demonstrated faster clearance than the larger PC liposomal preparation. (b) Radiocontrast enhancement in various organs as a surrogate of bio distribution was monitored in mice. The excretion of the

radiocontrast was delayed and detectable amount of contrast agent was observed in the liver and spleen up to 2 months after administration. Percentages of injected doses in the liver and spleen at various time points are listed.

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Figure 3.

(Top) Aortic wall enhancement by smaller liposomal contrast agent ($d \sim 112$ nm) in Watanabe Hereditary Hyperlipidemic (WHHL) rabbits (a & b) and balloon-denuded cholesterol-fed New Zealand White (NZW) rabbits (c & d). Animals were injected intravenously with control liposomes (PC-liposomes) (a & c) or PS-liposomes (b & d). Imaging was performed with a GE eXplore flat-panel CT scanner at baseline and 48 hours after contrast injection. Section thickness was 0.2 mm and reconstructed images were displayed with a maximum intensity projection of 1.0 mm. Specific contrast enhancement in the aorta was determined by comparing images obtained at baseline and 48 hours after injection. Focal enhancement was detected in the atherosclerotic segments in the aortas in both animal models injected with PS-liposomes (yellow arrows) but not with control

liposomes. (Bottom) CT enhancement of highlighted regions at baseline and 48 hours after injection of various liposomal preparations in both animal models.

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Figure 4. Macrophage infiltration in rabbit models of atherosclerosis and specific macrophage uptake of rhodamine labeled liposomal contrast agents. (a) The distribution of rhodamine-labeled PC-liposomes (red) was widespread throughout the thickness of the aortic wall. (b& c) Specific co localization between rhodamine-labeled PS-liposomes (red) and FITC-labeled macrophages (green) was observed in the aorta of both WHHL and balloon-denuded cholesterol-fed NZW rabbits. (d& e) Qualitatively, RAM-11 staining (mouse anti-rabbit macrophage antibody) demonstrated abundant macrophage infiltration in the aortic wall in both atherosclerotic animal models.

Table 1

Preparation of control liposomes and liposomes containing different amount of phosphatidylserine

Preparation	DPPC (mol%)	PS (mol%)	Cholesterol (mol%)	mPEG (mol%)	Rhodamine- labeled lipid (mol%)
Control DPPC liposomes	54.7	0	40	5	0.3
2% PS liposomes	52.7	2	40	5	0.3
8% PS liposomes	46.7	8	40	5	0.3
20% PS liposomes	34.7	20	40	5	0.3

Abbreviations: DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; PS: 1,2-dipalmitoyl-sn-glycero-3-phosphoserine; mPEG: polyethylene glycol 2kD

Table 2

Size and polydispersity index (PDI) of liposomal preparations.

	Size by intensity (nm)	PDI
Large-PC	173 ± 9	0.07
Large-PS	172 ± 24	0.08
Small-PC	111 ± 5	0.077
Small- PS	113 ± 5	0.034

All measurement values are an average of three runs of each sample.