
Preclinical Assessment of ^{99m}Tc -Labeled Liposome Agents as an Effective Tracer in Nuclear Medicine

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This study assessed the use of ^{99m}Tc -liposome agents for nuclear medicine purposes. **Methods:** A variety of ^{99m}Tc -liposome formulations were compared with common lymphoscintigraphic agents, including ^{99m}Tc -labeled regular sulfur colloid and ^{99m}Tc -labeled human serum albumin, besides assisting the use of positively charged liposomes in rabbits. Ten male rabbits (2–2.5 kg) were anesthetized with ketamine and xylazine intramuscularly. Then, they were injected with different ^{99m}Tc -liposome agents subcutaneously in the dorsum of each hind foot over the region of the metatarsals at the midline, as well as intravenously. Dynamic (1 min) scintigraphic γ -camera images were acquired in a $256 \times 1,024$ matrix both early and 60 min after injection. Afterward, the tissue biodistribution was determined by calculating the percentage injected dose per organ for each ^{99m}Tc agent and studying the heart-to-liver, heart-to-bone marrow, and heart-to-kidney ratios. **Results:** All agents demonstrated good migration from the injection site to the blood pool but not to lymphatic drainage. Agents were starting to clear out rapidly after 60 min. **Conclusion:** ^{99m}Tc -liposome imaging can be used to develop novel liposome compositions with improved cardiac diagnostic and drug delivery characteristics.

Key Words: liposomes; nuclear medicine; nanotechnology; nanocolloid; human serum albumin; tracer

J Nucl Med Technol 2020; 48:269–273

DOI: 10.2967/jnmt.119.239756

Liposomes have properties that promote their use as a drug delivery system, particularly in targeted administration, for enhancing the efficiency and reducing the related toxicity of the drugs and for other therapeutic purposes (1). However, few studies have examined the lymphoscintigraphic agents added to liposomes, although animal-stage research has supported the use of liposomes for the administration of lymphoscintigraphic agents. The reason behind this study was not only to detect the lymph nodes but also to discover other findings that can be illustrated using dif-

ferent lymphoscintigraphic agents (2). Liposomes are an ideal vehicle for drug administration because they allow certain agents to concentrate into specific targeted cells, as confirmed by much research; several drugs have shown decreased toxicity when delivered in a liposome-encapsulated formulation (3). A liposome consists of an aqueous solution enclosed within a hydrophobic membrane. Hydrophobic chemicals can easily be dissolved into the lipid membranes; in this way, liposomes are able to carry both hydrophilic and hydrophobic molecules. However, the extent of localization of the drug will depend on its physiochemical characteristics and the composition of the lipid. To deliver the necessary drug molecules to the site of action, the lipid bilayers fuse with other bilayers of the cell (cell membranes) to release the liposomal content. Liposomes are ideal structures for delivering therapeutic agents to lymph nodes. Their ideal features are their size, which prevents their direct absorption into the blood; the large amount of drugs and other therapeutic agents that they can carry; and their biocompatibility. For medications with a diagnostic or therapeutic index, a drug carrier is used to help in drug delivery to increase the safety and efficacy of administration. Phillips studied the delivery of γ -imaging agents by liposomes in 1999 (4). He described how the liposome structure is able to deliver γ -imaging agents and how the ability to modify the surface of liposomes permits customization of liposome formulations for each particular diagnostic use. This research aimed to study the lymph node delivery system by adding liposomes to different lymphoscintigraphic tracers such as ^{99m}Tc -human serum albumin (HSA) and ^{99m}Tc -sulfur colloid. He also studied the effect of positively charged liposomes on lymphatic imaging. In the present study, subcutaneous and intravenous injections were used to investigate the biodistribution of other combinations of liposomes on lymphoscintigraphy.

MATERIALS AND METHODS

Liposome Preparation

Neutral multilamellar vesicles of liposomes were prepared by the thin-film hydration method as described previously (5,6). Briefly, L- α -dipalmitoyl phosphatidylcholine was dissolved in ethanol in a round-bottom flask. The solution was shaken well for a few minutes and then was vigorously stirred in a vortex mixer to ensure complete solvation. The organic solution was removed gradually using a rotary

Received Nov. 23, 2019; revision accepted Apr. 5, 2020.

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Published online Jun. 9, 2020.

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evaporator (Re-2010; Lanphan Zhengzhou) under a vacuum (produced by a circulating water-aspiration vacuum pump [SHB-III; USA Lab Equipment]) in a warm water bath at a temperature above the phase-transition temperature of the suspended lipid (50°C) at 60 rpm to produce a uniform thin film of lipid on the inner wall of the flask. The flask was then left under a vacuum for 12 h to ensure evaporation of all traces of ethanol. The lipid film was hydrated with 10 mL of tris buffer (pH 7.4 at 37°C) in a water bath at 50°C for 15 min at 60 rpm to form multilamellar vesicles serving as control (empty) liposomes. The flask was mechanically shaken for 1 h at 50°C. Then, a nitrogen stream was flashed through the flask, and the flask was immediately closed. Parallel to the control L- α -dipalmitoyl phosphatidylcholine, HSA-loaded liposomes were prepared following the same method as described using only aliquots of mass HSA at a 2:7 molar-to-lipid ratio. Cationic HSA liposomes were prepared by the addition of 1 mg of stearyl amine to the lipid composition to introduce a net positive charge at a molar ratio of 1:7.

^{99m}Tc Labeling of Liposomes. All kits were checked for contamination using paper chromatography to ensure the absence of free technetium pertechnetate impurities, and the labeling efficiency was more than 85% (7). Sterile apyrogenic vacuumed vials were used. All filled vials included stannous chloride and had been lyophilized. The solution was purged with sterile nitrogen gas for 20–30 min, and the vials were then stoppered and kept in a refrigerator until use. All liposome agents were labeled with ^{99m}Tc. After generator elution, 814 MBq of ^{99m}Tc-pertechnetate was diluted with 3 mL of saline. Aliquots of each ^{99m}Tc-liposome formulation (0.3 mL, 81.4 MBq) were prepared for injection into each rabbit.

^{99m}Tc Labeling of Positively Charged Liposomes. After preparing positively charged liposomes, ^{99m}Tc-pertechnetate was added. The kit volume after addition of saline was 1.5 mL, with activity equaling 444 MBq. Aliquots of ^{99m}Tc-labeled positively charged liposomes for each injection were 0.15 mL (44.4 MBq).

^{99m}Tc Labeling of Liposomes and Sulfur Colloid. A commercial kit of sulfur colloid and liposomes labeled with regular ^{99m}Tc was prepared for injection into 1 group of rabbits. It contained 777 MBq in 2 mL of saline. Aliquots for each injection were 0.2 mL (77.7 MBq).

^{99m}Tc Labeling of Liposomes and HSA. The ^{99m}Tc was added to HSA and a liposome kit (555 MBq, 1.5 mL of saline). Aliquots of ^{99m}Tc HSA and liposome (0.15 mL, 55.5 MBq) were prepared for injection (8).

Imaging Studies

All proposed animal work was reviewed and approved by the Institutional Animal Care and Use Committee. Animal experiments were conducted on 10 male New Zealand White rabbits (2–2.5 kg), which were anesthetized by injection of a cocktail of ketamine (10 mg/kg) and xylazine (10 mg/kg) in the brachial muscle. An aliquot (0.3–1.5 mL) of each ^{99m}Tc agent was injected subcutaneously in the dorsum of each foot over the region of the metatarsals at the midline. Dynamic (1 min) scintigraphic images were acquired in a 256 × 1,024 matrix using a Symbia γ -camera. The subcutaneous bleb was massaged gently for 5 min. At 60 min after injection, the dynamic acquisition was halted.

Biodistribution

A biodistribution study was performed for all rabbits in the imaging study; thus, 1-h-postinjection imaging was acquired. Another imaging session was involved for 2 rabbits, which were injected intravenously in the marginal ear vein. After imaging, the

percentage injected dose per organ for each ^{99m}Tc agent was calculated by comparison with a standard aliquot of the respective ^{99m}Tc agent.

Image Analysis

For every rabbit, images were acquired at 1 and 60 min after injection. All images were corrected for background activity and analyzed. The baseline image was specified to be the image acquired at 1 min after injection, which was the first image in the dynamic acquisition, and represented the distribution from 0 to 1 min after injection. This image was also compared with the image that was acquired with ^{99m}Tc and the manufactured kit of albumin nanocolloid for each rabbit. For all images, the regions of interest, after being corrected for decay, were drawn to calculate the percentage injected dose (%ID) per gram of tissue or organ. The %ID in the injection site of each rabbit foot was considered to be 100%. Also, the %ID in the injection-site region of interest at 60 min was calculated.

Statistical Analysis

Values are reported as mean \pm SEM. Other calculations were used to compare the heart-to-kidney ratios, heart-to-liver ratios, and heart-to-bone marrow ratios. Also, bladder activity was examined for each agent at a given time. Blood clearance and injection site curves were fitted to an exponential model with consideration of radiopharmaceutical half-life. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

All studies were performed on 10 male rabbits. Whole-body images were acquired immediately after injection and 60 min after injection as shown in Figure 1.

Clearance

^{99m}Tc-liposome agents have significant clearance from the bladder (Fig. 2) and injection site (Fig. 3) after 60 min. Also, ^{99m}Tc-liposome agents were only faintly visualized and had poor retention in the popliteal node. Thus, there was low uptake of ^{99m}Tc-liposomes by the popliteal node on early and 60-min images. Injection-site and bladder images were acquired early and after 60 min for all ^{99m}Tc agents, and %ID was analyzed. Overall, for all ^{99m}Tc-liposome formations, the activity at the injection site at 60 min was reduced compared with the early images. Obviously, clearance of ^{99m}Tc-positive liposome HSA was the greatest. Clearance of ^{99m}Tc-liposome from the injection site was significantly different in early images than in 60-min images. The %ID in 60-min images was 10.8% \pm 0.17%, leading to 89% clearance at the injection site.

It was interesting to find a significant difference in injection-site clearance of ^{99m}Tc-labeled positively charged liposome HSA between early and 60-min images. The %ID (13.5% \pm 0.2%) after 60 min resulted in clearance of 86%. Additionally, bladder clearance of ^{99m}Tc-labeled positively charged liposome HSA on early images was significantly different from that on 60-min images. The clearance was 65% on 60-min images.

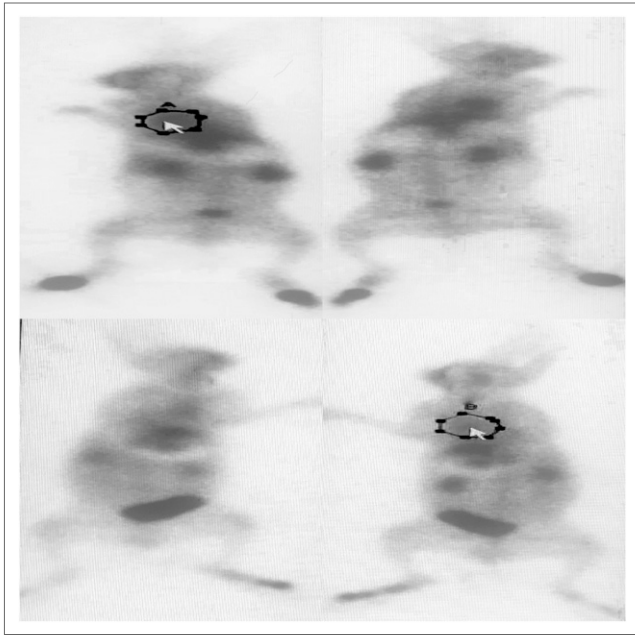


FIGURE 1. Dynamic scintigraphic images using a Symbia γ -camera. (A) Anteroposterior early images of rabbit injected with ^{99m}Tc -labeled positively charged liposome HSA. (B) Anteroposterior 60-min-postinjection images of rabbit injected with ^{99m}Tc -labeled positively charged liposome HSA. Arrows point out testing the count rate of heart and surrounding organs.

Cardiac Activity Retention

To assist the cardiac ejection fraction efficiency, a relationship was applied comparing the heart count rate to the count rate in other organs, such as liver, kidneys, and bone marrow, to determine the cardiac function using different agents. All images using different agents combined with liposomes did not show any lung or lymph node activity. The ratio between the heart and the other blood-pool organs, using different types of liposome formulations, was at least 1.

The ratio for early images of ^{99m}Tc -labeled positively charged liposomes was greater and significantly different from that for early images of ^{99m}Tc -labeled liposome HSA. Generally, the heart-to-liver ratio was the highest for early

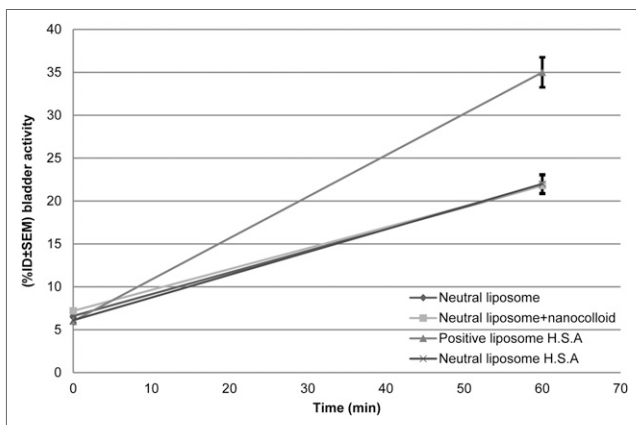


FIGURE 2. Bladder clearance according to injection time.

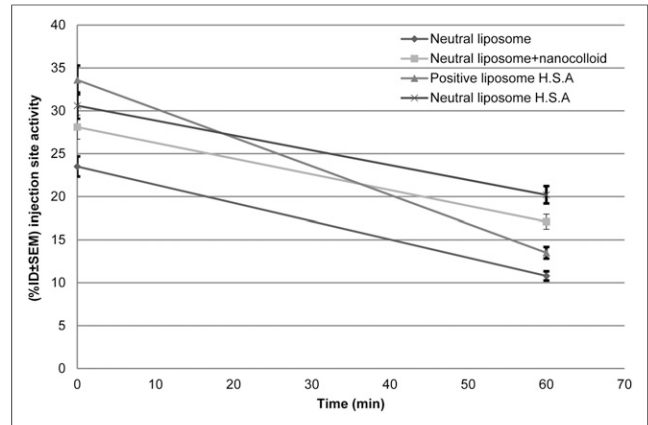


FIGURE 3. Injection site clearance according to injection time.

images of ^{99m}Tc -labeled positively charged liposome HSA, compared with all other ^{99m}Tc -liposome agents (Table 1).

Early images of heart-to-kidney ratio were uppermost for ^{99m}Tc -labeled liposomes. In early images of ^{99m}Tc -labeled positively charged liposome HSA, there was a significant difference from ^{99m}Tc -labeled liposome HSA and ^{99m}Tc -labeled liposomes. Delayed images of ^{99m}Tc -labeled liposome HSA and early images of ^{99m}Tc -labeled liposome nanocolloid showed no significant difference between each other ($P < 0.05$) (Table 2).

Heart-to-bone marrow ratio showed no significant difference between delayed images of ^{99m}Tc -labeled liposome HSA and early images of ^{99m}Tc -labeled positively charged liposome HSA. However, there was a major significant difference between early and delayed images for all ^{99m}Tc -liposome agents. Early images of ^{99m}Tc -labeled positively charged liposome HSA had the highest ratio, whereas ^{99m}Tc -labeled liposome HSA was the lowest, although still greater than 1 (Table 3).

DISCUSSION

In this study, ^{99m}Tc -liposome, ^{99m}Tc -liposome nanocolloid, ^{99m}Tc -labeled positively charged liposome HSA, and ^{99m}Tc -liposome HSA were investigated. First, the labeling efficiency for each ^{99m}Tc -liposome agent was determined by instant thin-layer chromatography. Particularly interesting were the remarkable results for clearance of activity from the bloodstream. Furthermore, there were significant differences in injection site and bladder activity between early images and 60-min images. Although ^{99m}Tc -liposome nanocolloid had the lowest bladder clearance, it was significantly different from early image and an hour after the injection. It was noticeable that ^{99m}Tc -labeled positively charged liposome HSA had the highest bladder activity clearance of all ^{99m}Tc -liposome agents. When bladder clearance was compared between ^{99m}Tc -labeled positively charged liposome HSA and ^{99m}Tc -liposome HSA, the positively charged liposome was clearly the reason for the high blood-pool clearance. Consequent retention of activity in the blood is increased, because the bladder clearance is also increased. This result will lead to better safety for the patient

TABLE 1
Heart-to-Liver Ratio

Time	Liposome	Liposome, nanocolloid	Positive liposome, HSA	Liposome, HSA
Early	1.15 ± 0.3*	1.08 ± 0.3	1.4 ± 0.2	1.01 ± 0.2 [†]
60 min	0.72 ± 0.1 [‡]	0.78 ± 0.1*	0.7 ± 0.2*	0.76 ± 0.2 ^{†‡}

**P* < 0.05 vs. liposome.
[†]*P* < 0.05 vs. liposome HSA.
[‡]*P* < 0.01 vs. liposome HSA.

and for those exposed to the patient, because when the radiopharmaceutical clears quickly from the patient's body, the remaining dose will be less.

A further advantage is that preparing ^{99m}Tc-liposome was not difficult. It is widely available and affordable and can be prepared before the patient arrives. Long-term storage of the liposome by lyophilization is another positive feature. HSA and nanocolloid are easy and safe to use, because they come in well-packed, sterile, ready-to-use kits. It is believed that the higher heart-to-liver ratio for ^{99m}Tc-labeled positively charged liposome HSA than for ^{99m}Tc-liposome HAS was because of the presence of positively charged liposome. Obviously, the highest range of all agents was for the heart-to-liver and heart-to-bone marrow ratios for ^{99m}Tc-labeled positively charged liposome HSA.

According to a study of liposome distribution that compared ^{99m}Tc-HMPAO-labeled polyethylene glycol-coated liposomes with ^{99m}Tc-hydrazinonicotinyl-hydrazinonicotinamide-labeled polyethylene glycol-coated liposomes (9), researchers concluded that ^{99m}Tc-hydrazinonicotinamide liposome uptake was higher in abscesses but lower in the kidneys. They clarified that the reason behind low liposome tissue retention was the liposome surface charge.

In this trial, the same tracer (HSA) was used for both ^{99m}Tc-labeled neutral and positively charged liposomes. However, the heart-to-liver ratio in ^{99m}Tc-labeled neutral liposomes was not showing a significant difference (*P* < 0.05) as well as the ratio for heart to kidneys. Thus, the clearance was slower compared with ^{99m}Tc-labeled positively charged liposome HSA; accordingly, that is another proof of the effectiveness of positively charged liposomes in cardiac studies. In this study, two of the rabbits were injected with ^{99m}Tc-labeled positively charged liposome

HSA intravenously. A promising result was the significant activity retention in the cardiac muscle; accordingly, ^{99m}Tc-labeled positively charged liposome could be indicated for quantitative gated blood-pool imaging. Overall in this study, it was noticeable that the heart ratios for the early images were higher than for the 60-min-postinjection images; also, for all ^{99m}Tc-liposome agents, the ratios were greater than 1. These results indicate rapid clearance from the blood pool for the ^{99m}Tc-liposome agents. For this reason, ^{99m}Tc-liposome can play an important role in myocardial perfusion and in qualitative and quantitative assessment of function.

Another benefit is that liposomes are not interacting with the patient's other medications. This research found that ^{99m}Tc-liposome agents circulate in the blood even when subcutaneous injection is used, as they do with intravenous injection. As a result, these agents could be recommended for patients who have a lengthy hospital stay requiring multiple intravenous injections, resulting in damaged vessels or vascular trauma. All tested ^{99m}Tc-liposome formulations were poorly retained by the lymph nodes and, for this reason, would not be useful for sentinel node detection in lymphoscintigraphy imaging. However, lack of lymph node retention could be ideal for detecting lymph node tumors by administering liposomes subcutaneously. This feature will allow specific tumor receptors to be attached with liposomes in a particular lymph node that could obscure these tumors by using common ^{99m}Tc tracers such as sulfur colloid because of the lymph node retention.

Moreover, the liposome is a phospholipid just like the Myoview tracer, tetrofosmin, thus it passes the cardiac cell membrane and causes activity retention (10). The early washout of the liposomes is another advantage. Accordingly, 1-d myocardial

TABLE 2
Heart-to-Kidney Ratio

Time	Liposome	Liposome, nanocolloid	Positive liposome, HSA	Liposome, HSA
Early	1.54 ± 0.5	0.77 ± 0.2*	1.2 ± 0.1	0.74 ± 0.1 [†]
60 min	1.08 ± 0.3	0.87 ± 0.3	0.87 ± 0.4*	0.58 ± 0.09 ^{*†}

**P* < 0.01 vs. liposome HSA.
[†]*P* < 0.05 vs. liposome HSA.

TABLE 3
Heart-to-Bone Marrow Ratio

Time	Liposome	Liposome, nanocolloid	Positive liposome, HSA	Liposome, HSA
Early	2.6 ± 0.8	2.07 ± 0.6	3.1 ± 0.5*	1.85 ± 0.3
60 (min)	1.92 ± 0.6	1.8 ± 0.6	1.4 ± 0.4	1.56 ± 0.3*

* $P < 0.01$ vs. liposome HSA.

perfusion protocol using positively charged liposomes will be appropriate and more reliable over other tracers. In addition, liposomes cost less than sestamibi or pyrophosphate, and liposome agents could be useful for detecting gastrointestinal bleeding because of the rapid clearance of bowel activity. In view of the current circumstances surrounding the coronavirus disease 2019 outbreak, future studies could fruitfully explore this issue further using monoclonal antibodies in addition to positively charged liposomes to develop specific antiviral agents against this newly emergent pathogen.

Further testing would be needed to develop ^{99m}Tc -labeled positively charged liposomes for more effective tracers; to evaluate efficiency, safety, and toxicity in different animal models before clinical trials begin; and to comply with good manufacturing practices.

CONCLUSION

^{99m}Tc -liposome imaging can be used to develop novel liposome compositions with improved cardiac diagnostic and drug delivery characteristics.

DISCLOSURE

No potential conflict of interest relevant to this article was reported.

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