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^{99m}Tc-labeled Therapeutic Inhaled Amikacin Loaded Liposomes

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Abstract

The radiolabeling of the liposome surface can be a useful tool for *in vivo* tracking of therapeutic drug loaded liposomes. We investigated radiolabeling therapeutic drug (i.e., an antibiotic, amikacin) loaded liposomes with ^{99m}Tc, nebulization properties of ^{99m}Tc-labeled liposomal amikacin for inhalation (^{99m}Tc-LAI), and its stability by size exclusion low pressure liquid chromatography (LPLC). LAI was reacted with ^{99m}Tc using $SnCl_2$ dissolved in ascorbic acid as a reducing agent for 10 min at room temperature. The labeled products were then purified by anion exchange resin. The purified ^{99m}Tc-LAI in 1.5% NaCl solution was incubated at 4°C to assess its stability by LPLC. The purified ^{99m}Tc-LAI was subjected to studies with a clinically used nebulizer (PARI eFlow®) and the Anderson Cascade Impactor (ACI). The use of ascorbic acid at 0.91 mM resulted in a quantitative labeling efficiency. The LPLC profile showed that the liposomal peak of LAI detected by a UV monitor at both 200 nm and 254 nm overlapped with the radioactivity peak of ^{99m}Tc-LAI, indicating that ^{99m}Tc-LAI is suitable for tracing LAI. The ACI study demonstrated that the aerosol droplet size distribution determined gravimetrically was similar to that determined by radioactivity. The liposome surface labeling method using $SnCl_2$ in 0.91mM ascorbic acid produced ^{99m}Tc-LAI with a high labeling efficiency and stability that are adequate to evaluate the deposition and clearance of inhaled LAI in the lung by gamma scintigraphy.

Keywords

^{99m}Tc-labeled liposome-encapsulated amikacin; radiolabeling; size exclusion analysis

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Introduction

Liposomes have regained a lot of attention as nano-scale drug carriers due to their ability to accommodate high drug loading and manufacturing versatility regarding types of drugs that can be loaded, introducing targeting moieties and imaging modalities into a nanoparticle platform (Torchilin, 2005, Malam et al., 2009). *In vivo* tracking of liposomal drug becomes a more important issue to get information on the distribution and uptake of the liposomal drug at disease sites and its efficacy in treatment (Liu et al., 2012). To facilitate tracking, radiolabeling of liposomes with a tiny amount of radionuclides is often considered because powerful imaging tools such as PET and SPECT can be used. In this study we investigated the radiolabeling of therapeutic drug loaded liposomes with ^{99m}Tc and impaction properties of ^{99m}Tc-labeled liposomal amikacin for inhalation (^{99m}Tc-LAI).

Liposome encapsulated aminoglycosides have been developed recently for inhalation delivery to treat lung infection (Minic et al., 2010, Weers et al., 2009, Antos et al., 1995, Mugabe et al., 2005, Marier et al., 2003, Omri et al., 1994, Okusanya et al., 2009). LAI, an aminoglycoside antibiotic (amikacin,70mg/ml) encapsulated within phospholipid cholesterol liposomes, is an investigational drug developed to provide a sustained benefit in the lungs with once-daily dosing (Minic et al., 2010, Geller, 2009). LAI is being evaluated in clinical trials to treat *Pseudomonas aeruginosa* (Pa) infections in cystic fibrosis (CF) patients and patients with non-tuberculous mycobacteria (NTM) infections. The pulmonary inhalation drug product may improve patient compliance and treatment outcomes with its reduced dosing frequency, improved drug targeting to the lung infection site, its increased penetration of drug into the *Pseudomonas aeruginosa* (Pa) biofilm interior, and localization into NTM infected alveolar macrophages (Okusanya et al., 2009, Moss, 2002, Weers et al., 2010, Marier et al., 2003, Omri et al., 1994, Geller, 2009). Previous studies showed many advantages in preclinical animal models and CF patients with Pa infections, including extended drug benefit and improved efficacy(Minic et al., 2010, Weers et al., 2010, Okusanya et al., 2009).

Liposome-encapsulated drugs have different biodistributions and toxicities as compared to ‘free’ drugs (e.g., amikacin)(Omri et al., 1994, Shah and Misra, 2004, Saari et al., 1999, Vidgren et al., 1995, Barker et al., 1994, Saari et al., 1998). Therefore, the radiolabeling of preformed liposomes such as ^{99m}Tc labeling using stannous chloride as a reducing agent is a good approach to track liposomal biodistribution in the body (Richardson et al., 1979). ^{99m}Tc radiolabeling of liposomes can be performed using different methods such as ^{99m}Tc radiolabeling on the outer leaflet of liposomes and encapsulation of ^{99m}Tc labeled drug molecule during liposome preparation or by a pH gradient loading method (see Figure 1) (Phillips, 1999). The encapsulation of ^{99m}Tc radiolabeled drug during liposome preparation showed less than 5% encapsulation efficiency, especially for the unilamellar vesicles (e.g., LUV (large unilamellar vesicle) and SUV (small unilamellar vesicle)) (Phillips, 1999). Moreover, it requires adulteration of the drug manufacturing process, which may be impractical, and may result in a labeled species that differs from the actual liposomal drug. In contrast, ^{99m}Tc radiolabeling on the liposome surface can be a preferable method because it can be performed on the final pharmaceutical drug product and allow

encapsulated drugs to remain inert inside the liposome even though it may change *in vivo* behavior of liposomes due to altered surface properties.

Gamma scintigraphy has been used to evaluate the deposition and clearance of these inhaled ^{99m}Tc-labeled liposomes in the lungs (Richardson et al., 1979, Weers et al., 2009, Conway, 2012, Elbayoumi and Torchilin, 2006, Phillips, 1999). ^{99m}Tc-labeled LAI in the preclinical study indicated no change of *in vivo* behavior of LAI unlike other studies (Weers et al., 2009, Farr et al., 1985), Although ^{99m}Tc radiolabeling of the liposome surface can be a preferable method and a dialysis analysis method demonstrated the radiolabeling efficiency of ^{99m}Tc-labeled LAI was successful (Weers et al., 2009), this dialysis method takes a relatively long time (about 10 hrs) to report radiolabeling efficiency and it cannot differentiate ^{99m}Tc-labeled LAI and tin-colloid particles. Considering the short decay time of ^{99m}Tc (~6 hrs), a quick and robust assessment of ^{99m}Tc-labeled LAI is a key step toward successful clinical trials. Furthermore, the analysis reports on ^{99m}Tc radiolabeling on the liposome surface have shown inconsistent results (Baljosevic et al., 2002), so a reliable analytical method is critical to assess the labeling efficiency.

In this study, we optimized ^{99m}Tc labeling parameters to yield a high labeling efficiency without a significant impact on the liposome size and stability with drug encapsulated liposome formulation, LAI. The radiolabeling efficiency and the stability of ^{99m}Tc-LAI were investigated by size exclusion low pressure liquid chromatography (LPLC). With this analysis method, both ^{99m}Tc binding and release from the liposome outer leaflet, and encapsulated drug leakage are shown simultaneously in this study. Finally, the purified ^{99m}Tc-LAI was subjected to studies using a nebulizer instrument (PARI eFlow®) and the Anderson Cascade Impactor (ACI) to assess aerosol droplet size, an important physical parameter for the deposition and clearance of this inhaled drug.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. LAI for this study was provided by Insmed Incorporated. Oasis®Max Plus syringe cartridge was obtained from Waters corporations (Lot No. 186003517, Milford, MA, USA).

Liposomal amikacin for inhalation (LAI) preparation

LAI was prepared aseptically with dipalmitoylphosphatidylcholine (DPPC) and cholesterol in a 2:1 weight ratio using a two-stream mixing process where streams of lipid in ethanol solvent and amikacin sulfate in an aqueous solution were combined. Unencapsulated amikacin and residual solvent were removed by diafiltration to be replaced with 1.5% NaCl solution. Amikacin and total lipid concentrations in the final drug formulation were approximately 70 mg/ml and 49 mg/ml, respectively. Prior to nebulization, greater than 98% of the amikacin was encapsulated within the liposomes.

Radiolabeling of LAI

LAI was radiolabeled using sodium pertechnetate $Na^+[^{99m}TcO_4^-]$ as described previously (Farr et al., 1985, Weers et al., 2009, Koizumi et al., 1992). Briefly, 0.2ml of

$Na^+[^{99m}TcO_4^-]$ (30mCi)) in 1.5% NaCl (Technelite® $^{99}Mo/^{99m}Tc$ generator from Lantheus Medical Imaging, Inc.) was added to a sterile vial containing LAI (4 ml at 70 mg/ml amikacin) and the contents were shaken for approximately 30 sec. A volume of 0.2 ml of 2 mM $SnCl_2$ (Lot No.452335) solution prepared in deoxygenated 20 mM ascorbic acid or 20mM HCl and filtered through a 0.22 μm sterilizing filter (MillexGV filter unit, Millipore, Billerica, MA, USA) was then added into the vial. The vial was gently mixed for approximately 30 sec and incubated at room temperature for 10 min. An aliquot of known volume was withdrawn for determination of the specific activity (mCi/mg) of the radiolabeled LAI. The reaction mixture was neutralized with 0.2 ml of sterilized 16 mM sodium carbonate and passed through a sterilized anion exchange Oasis®Max syringe cartridge to remove unlabeled ^{99m}Tc pertechnetate and potential tin-colloid from ^{99m}Tc-LAI. The specific radioactivity concentration was 5.6±0.43mCi/mL (n=3). A further aliquot of known volume was withdrawn to determine the radiochemical purity. The purified ^{99m}Tc-LAI was mixed with unlabeled LAI (i.e., mixed dose = ^{99m}Tc-LAI+ unlabeled LAI) to make a patient dose containing a total of 560 mg amikacin with 7 mCi of ^{99m}Tc in a total volume of 8.5 ml for the ACI, stability, sterility and *Limulus amebocyte lysate* (LAL) tests. For the sterility and LAL tests, small aliquots of the patient doses (n=3) were diluted 20 times and 10 times, respectively with sterile 1.5% NaCl solution. The sterility and LAL tests were performed at Department of Laboratory Medicine, Clinical Center, NIH and Clinical Services Program, SAIC, National Cancer Institute, respectively. The remaining product solutions were used for other studies including stability, inertial impaction and impinger studies.

Liposome analysis

Liposome size distributions were determined by dynamic light scattering (DLS) measurements using a Malvern instrument (NANO ZS, Malvern Instruments, CA, USA). The Z-average particle size was measured in triplicate. The integrity of the radiolabeled liposomes was determined by several analytical methods. The samples were assessed by size exclusion LPLC equipped with a size exclusion Sepharose CL 6B column (25×300 mm, GE healthcare, 1.5% NaCl, pH 6.8; 0.8ml/min) (modified for pressure pump use), a UV monitor, and an on-line flow radioactivity detector (Bioscan Inc., Washington, DC, USA). The radiochemical purity of ^{99m}Tc-LAI was also evaluated by ascending ITLC (Gelman Sciences, Inc., Ann Arbor, MI,USA) using 100% acetone as the solvent phase (Arulsudar et al., 2003).

Evaluation of aerosolized radiolabeled LAI

Inertial impaction tests were performed to characterize the aerosol output using an Andersen cascade impactor (ACI, Copley Instruments, UK) operated at a flow rate of 28.3 L/min under 25ºC, the relative humidity (%RH) <10% and an inlet throat from a ten stage impactor (Glaxo Type, Copley Instruments, UK). An eFlow electronic nebulizer (PARI, Germany) adapted specifically for use with LAI was used to aerosolize the radiolabeled LAI. The nebulizer was operated continuously for 6 min. Deposition of the liposome preparation was determined by gravimetric assessment of the collection plates, and by dissolving the recovered stage contents in a known volume of water and counting activity with a gamma counter (Mini Gamma Counter 1275 LKB Wallac, Gaithersburg, MD). Also, samples of

radiolabeled liposomes recovered from the ACI were analyzed by LPLC to determine the radiochemical and amikacin-association stability of ^{99m}Tc-LAI. Similarly, an impinger study was performed to determine the stability of ^{99m}Tc-LAI, following aerosolization of the radiolabeled LAI with eFlow electronic nebulizer. The sample collected in an ice-water bath was stored in a refrigerator at 4°C and analyzed by LPLC at different time points. The eFlow nebulizer was equipped with an exhalation filter for these studies and future patient studies to capture exhaled radioactivity.

Results and discussion

LAI analysis

LAI was analyzed with dynamic light scattering before ^{99m}Tc radiolabeling. The size was determined to be 238.2±11.5 nm. LAI was subjected to analysis with LPLC equipped with a Sepharose CL 6B resin column. For LAI, LPLC results show that the liposome peaks were detected by a UV monitor set at 254 nm and 200 nm due to its turbidity and drug encapsulation (Figure 2). Less than 2% free amikacin was detected. The result indicates that for LAI liposome integrity is maintained during LPLC analysis. Of note, the amikacin drug peak was only observed at 200 nm, not at 254nm (Figure 2c). As a result, amikacin leakage was measured at 200 nm. The liposome contribution to 200 nm was calibrated by different concentrations of blank DPPC/CH liposomes and free drug leakage was recalculated by subtracting the contribution from the liposomal peak at 200 nm (i.e., free drug %= free drug peak intensity at 45 min/ ((free drug peak intensity at 45 min) +(encapsulated drug peak intensity at 17 min)-(blank DPPC/CH liposome contribution at 17min)).

Radiolabeling chemistry and analysis of LAI

Analysis and purification of control samples—The ^{99m}Tc-radiolabeled LAI and the control LAI (a mixture of LAI with $^{99m}TcO_4^-$in the absence of the reducing agent, $SnCl_2$) were evaluated by LPLC. The LPLC for the control LAI (i.e., LAI+^{99m}Tc pertechnetate) demonstrated that a single radioactivity peak eluted at a retention time of 45 min, identical to the retention time of ^{99m}Tc pertechnetate. No radioactivity (<1.0%) was observed at the liposome peak position (between 10 and 20 min) (Figure 2). This result indicates that the phosphate moiety of LAI could not make a coordination complex with pertechnetate in the absence of a reducing agent and that ^{99m}Tc pertechnetate did not undergo encapsulation inside LAI during the labeling process. The mixture of amikacin drug and ^{99m}Tc pertechnetate also demonstrates no interaction in the absence of $SnCl_2$.

The control samples were also analyzed by the ITLC method developed with acetone as a solvent. Free $^{99m}TcO_4^-$eluted along with the solvent at the solvent front. Unbound ^{99m}Tc was separated from LAI in the absence of the reducing agent and moved quickly to the solvent front. In contrast, the ^{99m}Tc labeled LAI in the presence of the reducing agent remained at the origin of the sample application when the ITLC was developed with acetone.

For the purification of ^{99m}Tc-labeled LAI, we tested several cartridges and columns including Bio-Gel P-6 cartridges (BioRad), 10 DG desalting column (BioRad), PD-10

column (GE Healthcare) and Oasis Max anion exchange cartridge (Waters). We found that Oasis®Max syringe cartridge was satisfactory, quickly eluting LAI and efficiently removing ^{99m}Tc pertechnetate whereas other columns and cartridges we tested were easily clogged or the flow was very slow when LAI was eluted.

Radiolabeling efficiency and stability of ^{99m}Tc- labeled LAI

The ^{99m}Tc labeling of LAI was very efficient in the presence of stannous chloride (100 μM) in 0.91mM ascorbic acid, producing ^{99m}Tc-LAI with a high labeling yield: 100.0± 0.0% (n=3) and 89.3±3.2% (n=3), when determined by ITLC and LPLC, respectively. The radiochemical purity after the purification with Oasis Max cartridge was >99% determined by ITLC and 92.9±3.2% (n=3) by LPLC. The ^{99m}Tc labeled LAI exhibited a good radiochemical stability and a good integrity of liposome size (see below for details). In contrast, labeling in 1 mM HCl solution appeared to increase the viscosity of the liposome, thereby making it difficult to purify the labeled liposome using the anion exchange cartridge. ^{99m}Tc labeling of LAI in HCl solution can cause liposome aggregation at a HCl concentration around 1 mM.

The stability of ^{99m}Tc-LAI stored at 4°C was analyzed by LPLC using a UV detector set at both 254nm and UV 200nm and an on-line radioactivity detector (Figure 3 and Table 1). The LPLC profiles of ^{99m}Tc-LAI demonstrate that a major peak detected by a UV monitor at 200 nm and 254 nm overlapped with the major radioactivity signal peak. The peak shape and retention time of these peaks was identical to those of the original LAI, indicating that the ^{99m}Tc labeling condition did not change the size of LAI. The stability data suggest that the LAI kept at 4°C provides a stable storage condition. The particle size distributions shown by dynamic light scattering demonstrate that the distribution of ^{99m}Tc-LAI sample three months after the radiolabeling overlapped with the particle size distribution of the original LAI (see Figure 4). This finding also strongly supports the LPLC data, demonstrating that the liposome integrity of LAI remains unchanged after the radiolabeling.

Impaction study to evaluate aerosol particle size distribution

The liposome deposition in the lung that depends on aerosol droplet size can be assessed by inertial impaction tests using an Andersen cascade impactor and nebulizer. Heavier aerosol particles travel shorter distance and deposit on earlier stages, and lighter particles travel longer distance and deposit on later stages (Patton, 1996) (Figure 5a). The aerodynamic particle size distribution (PSD) was determined by both gravimetric mass and ^{99m}Tc radioactivity measurement. A great correlation was demonstrated between the two measurements (Figure 5b). No deposition on the filter was observed. About 65% of the ^{99m}Tc-LAI formulation was deposited on the middle stages: stages 3 (cut-off diameter 3.3 μm) and 4 (cut-off diameter 2.1 μm) of ACI. More than 88% was deposited between stages 2 and 5. No deposition on the filter and the increased deposition amount on the middle stage suggest that a stable aerosol of ^{99m}Tc-LAI was generated. The impinger study was also performed to determine the stability of ^{99m}Tc-LAI (Figure 6). The LPLC shows that ^{99m}Tc-LAI aerosolized through eFlow nebulizer and recaptured in theimpinge exhibited the original liposomal LPLC peak position. The LPLC analysis of impaction and impinger

samples indicates that 86% and 66%, respectively, of the total amikacin remained as encapsulated drug even after storage for 48 hrs (Table 1).

Discussion

The size exclusion high-performance liquid chromatography (HPLC) has been a very useful technique for liposome analysis since 1980s (Ollivon et al., 1986, Lesieur et al., 1991, Lesieur et al., 1993, Grabielle-Madelmont et al., 2003). This method permits liposomes to be separated from small solutes (i.e., free drugs) and to be subdivided into different sized-liposomes. In addition, this analysis provides information on liposome stability and drug encapsulation, as well as average size and size distribution. However, this method was found to be destructive for cholesterol rich liposomes (especially, size >300nm), which has limited its use (see also (Lesieur et al., 1993, Grabielle-Madelmont et al., 2003). The liposome associated DPPC was recovered well from the size exclusion HPLC whereas the liposome associated DPPC/cholesterol showed a poor recovery (Lesieur et al., 1991). For these reasons, LPLC method was instead chosen as the analytical method.

Comparing the stability data obtained from both ITLC and LPLC, the ^{99m}Tc-LAI was very stable for 24 h without ^{99m}Tc pertechnetate detected when the ITLC was used whereas the LPLC analysis showed about 10% ^{99m}Tc pertechnetate detected at 0, 3 and 24 h. This appears to indicate that about 10% of ^{99m}Tc is bound unstably to LAI which dissociates when eluted through the LPLC column. However, it is still associated to LAI when a gentle analysis method, ITLC is used. Although the LPLC might destabilize ^{99m}Tc binding, the LPLC profiles traced by the UV monitor indicate that the LPLC did not disrupt the integrity of LAI with respect to the leakage of amikacin, showing a minor peak (< 2% of the total) representing amikacin leaked out of LAI. This finding indicates that encapsulated amikacin remained stably inside the liposome and the liposome structure was not disrupted by the ^{99m}Tc labeling condition or the LPLC elution process. The advantage of LPLC equipped with a UV monitor and an on-line radioactivity detector in sequence was that it enabled us to simultaneously provide information on the structural integrity of liposome, the radiochemical stability of ^{99m}Tc-LAI and the liposome stability in regards to leakage of encapsulated amikacin. Of note, the LPLC stability data for ^{99m}Tc-LAI are similar to those determined by a dialysis method.(Weers et al., 2009)

The structure of the ^{99m}Tc-LAI complex is still not well understood at the present time. It is possible that the oxidation state of $^{99m}Tc^{+7}$ was reduced to $^{99m}Tc^{+4}$ and then complexed with the phosphate moieties on the surface of the liposome (Phillips, 1999). The schematics of the radiolabeled ^{99m}Tc-DPPC structure is shown in Figure 7 by adapting a structure of ^{99m}Tc-methyl disphosphonate (Qiu, 2011). Previous studies on ^{99m}Tc labeling on preformed liposomes using $SnCl_2$ have yielded different results with variable and inconsistent labeling efficiency (i.e., the labeling efficiency <50% in some cases)(Caride, 1990, Phillips, 1999, Baljosevic et al., 2002). We think that the high labeling efficiency and improved stability analyzed by LPLC may be the result of the following factors: 1) the presence of ascorbic acid; 2) the improvement in the removal of ^{99m}Tc pertechnetate and tin-colloid by the anion exchange cartridge; 3) a stable coordination between $^{99m}Tc^{4+}$ and liposomal surface functional groups at a high concentration, and 4) an optimum liposome

size (~200 nm). The liposome size used in the previous published studies was <100 nm for SUV and about >1μm for multilamellar vesicles (MLV), and in many studies the liposome size was often not well characterized. Further structural investigation needs to quantify each factor.

The results from the impaction study with a condition similar to that used in a patient study suggest that this nebulization process does not appreciably deteriorate the radiochemical stability of ^{99m}Tc-LAI or the liposome integrity with respect to the leakage of amikacin. These findings suggest that ^{99m}Tc-LAI can provide reliable monitoring of the deposition and clearance of LAI in the lung .These results together with the test results of sterility and LAL showing “no bacteria growth” and acceptable endotoxin unit of < 4.25 EU/8.5 ml (n=3) for the patient doses formulated in this study support that these methods of ^{99m}Tc labeling, purification and dose formulation under aseptic conditions are suitable for clinical trials.

Conclusions

The ^{99m}Tc labeling of LAI with stannous chloride dissolved in ascorbic acid produced ^{99m}Tc- LAI with a quantitative labeling yield without a significant impact on the liposome size and stability. The development of LPLC analysis for the LAI cholesterol rich liposome system allowed a quick and robust assessment of ^{99m}Tc-labeled LAI for radiochemical stability and liposome integrity, a key step toward successful clinical trials. The ^{99m}Tc labeling, purification and dose formulation methods under aseptic conditions provided a patient dose (7 mCi/560 mg amikacin in 8.5 ml) that is suitable for human studies. We believe that our labeling approach and evaluation methods are significantly improved methodologies important in the radiolabeling of liposomes. Establishment of these methodologies is an important key step that is required before initiating studies on the uptake of radiolabeled liposomal drugs in disease sites and studies to evaluate treatment efficacy in the clinic.

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Declaration of interest

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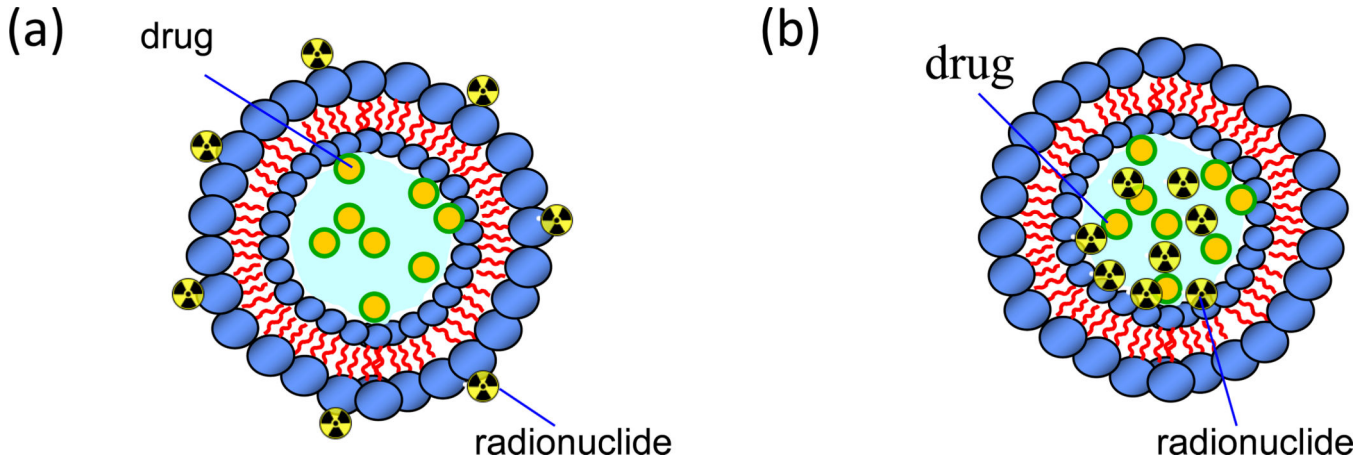

Figure 1.

Strategies for the radiolabeling of therapeutic drug encapsulated liposomes. a) Radionuclides such as ^{99m}Tc are bound on the surface of preformed drug encapsulated liposome through a chelating reaction; b) Encapsulation of radionuclides into an enclosed compartment by a pH gradient loading method with preformed liposomes or from co-encapsulation during liposome formation using dissolved lipids and radionuclide building blocks.

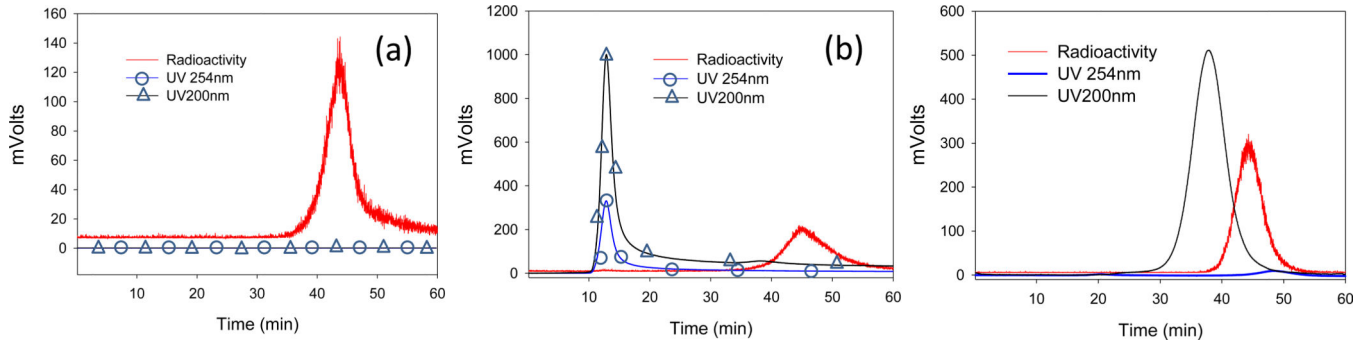

Figure 2.

Analysis of control samples by LPLC: (a) ^{99m}Tc pertechnetate; (b) the mixture of LAI and ^{99m}Tc pertechnetate in the absence of $SnCl_2$; and (c) the mixture of amikacin and ^{99m}Tc pertechnetate in the absence of $SnCl_2$.

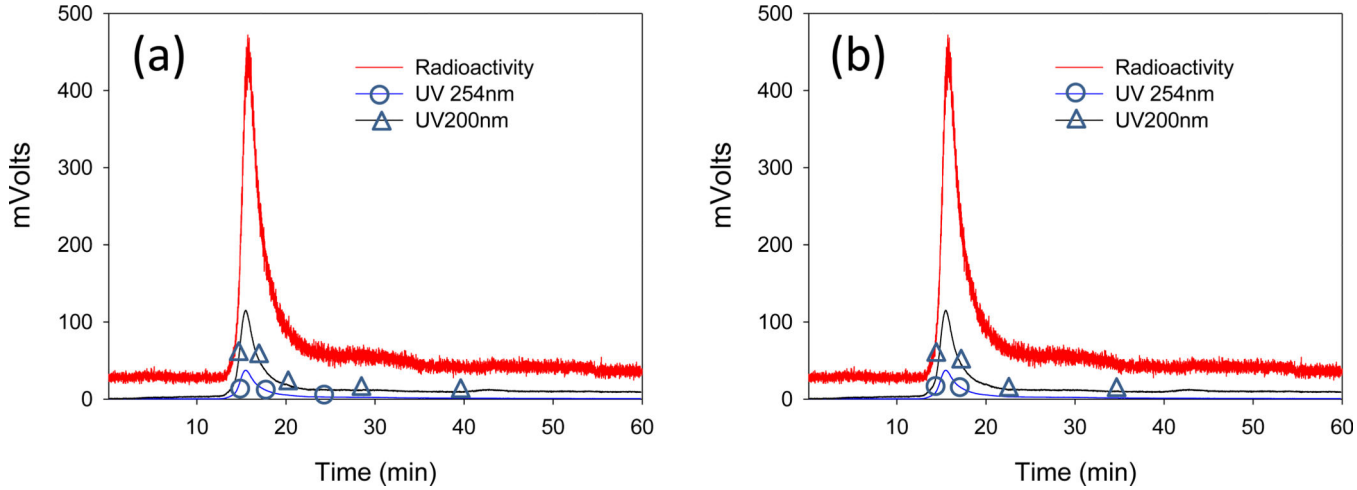

Figure 3.

LPLC analysis to determine the stability of ^{99m}Tc-LAI. Samples from the patient dose were stored at 4°C and analyzed at the designated time points by both a UV detector set at 254nm and 200nm and an on-line radioactivity detector connected in sequence: (a) 0 hr; (b) 3 hr. The UV peaks overlap with the radioactivity peak, indicating that the radiolabel did not change the integrity of LAI.

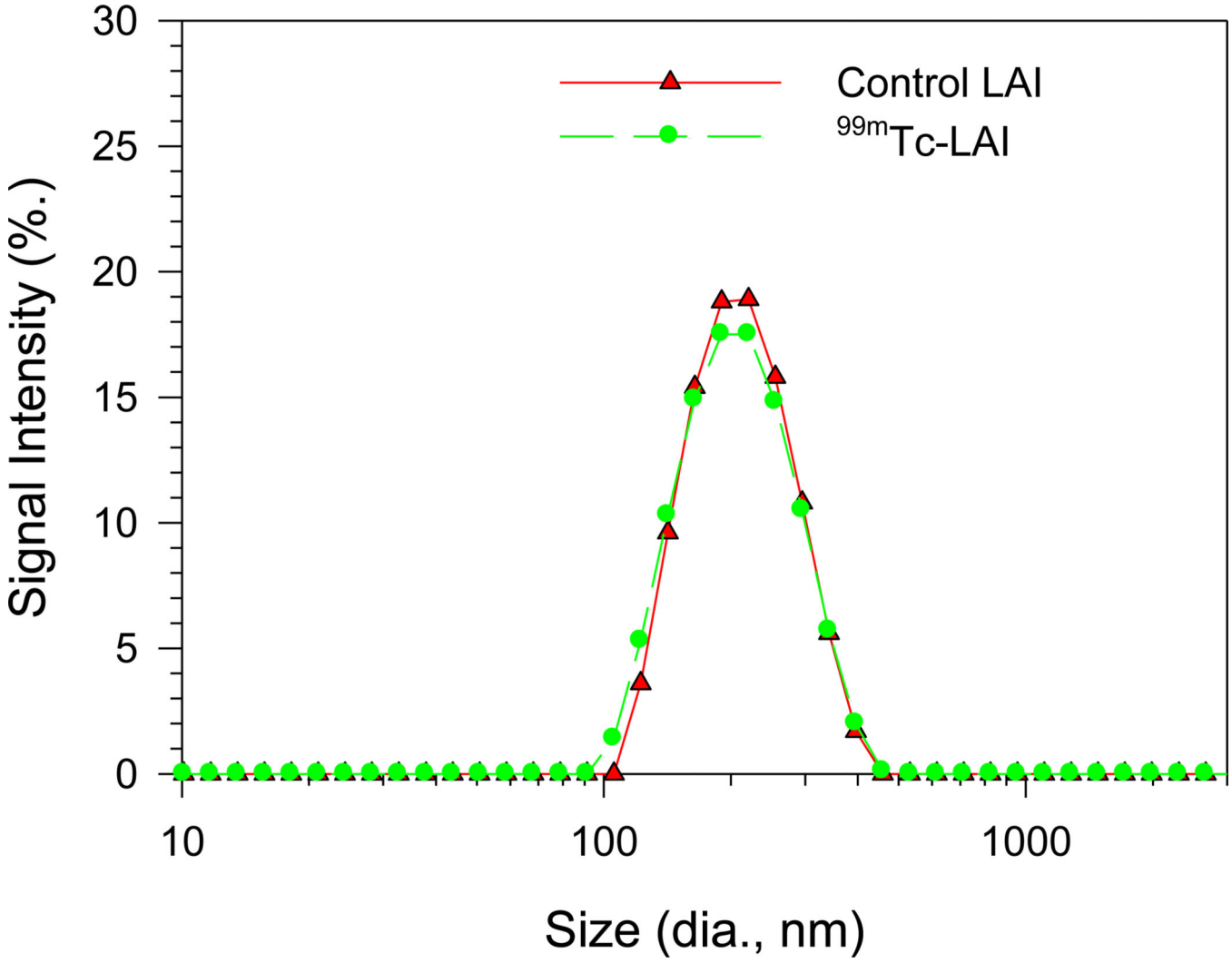

Figure 4.

Particle size distribution determined by dynamic light scattering. The control LAI and ^{99m}Tc-LAI were stored for 3 months at 4°C. The particle size distribution measured with a dynamic light scattering instrument showed the same liposome size distribution between the control LAI and ^{99m}Tc-LAI.

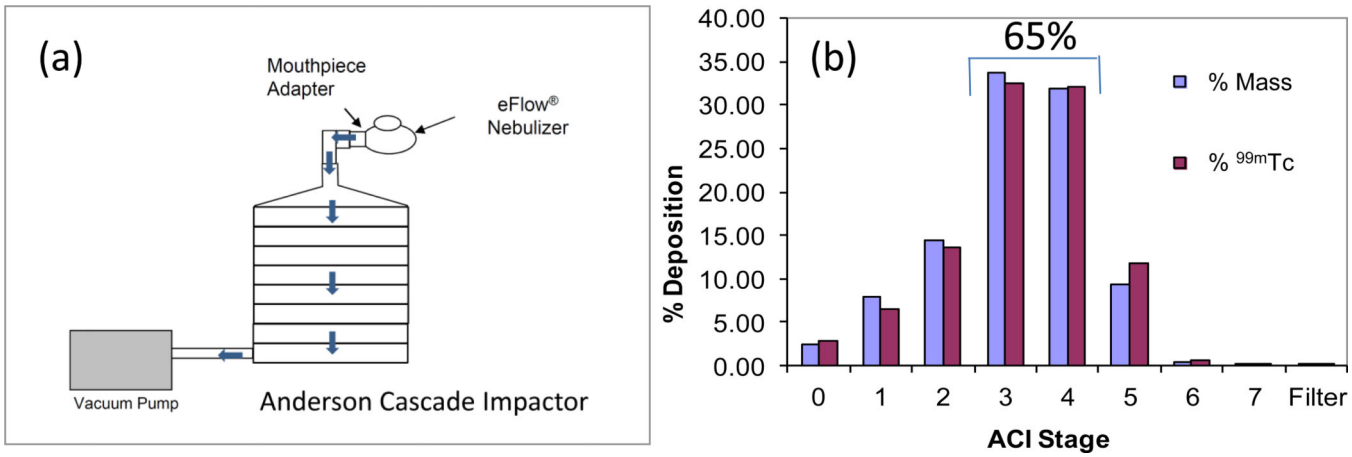

Figure 5.

Schematic experimental set up for impaction study (a) and aerodynamic particle size distributions of ^{99m}Tc-LAI (b). Andersen cascade impactor (ACI) was used for this experiment in which the levels of LAI droplets on individual stages were measured gravimetrically (mass) and via quantification of radioactivity (counts per minute (CPM) of ^{99m}Tc). The aerosol particle distribution determined gravimetrically overlapped with that determined by the radioactivity measurement. About 65% of the total deposition was found in the middle stages.

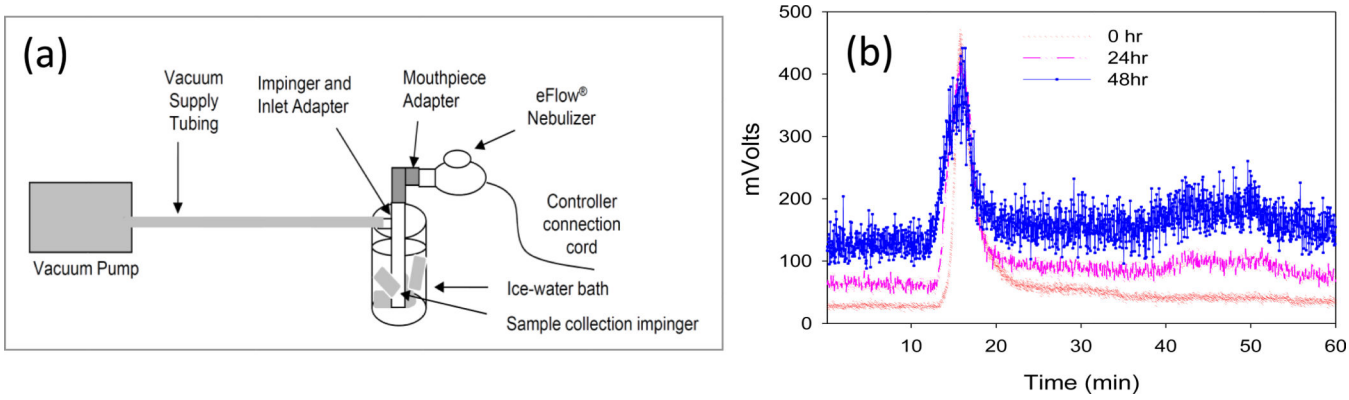

Figure 6.

Impinger Study and its stability analysis: (a) Schematic diagram for impinger study to determine the stability of ^{99m}Tc-LAI after aerosolization with the eFlow electronic nebulizer; and (b) LPLC analysis for the stability of ^{99m}Tc-LAI after impinger study at different time points, showing that aerosolized ^{99m}Tc-LAI maintains its original liposomal peak position.

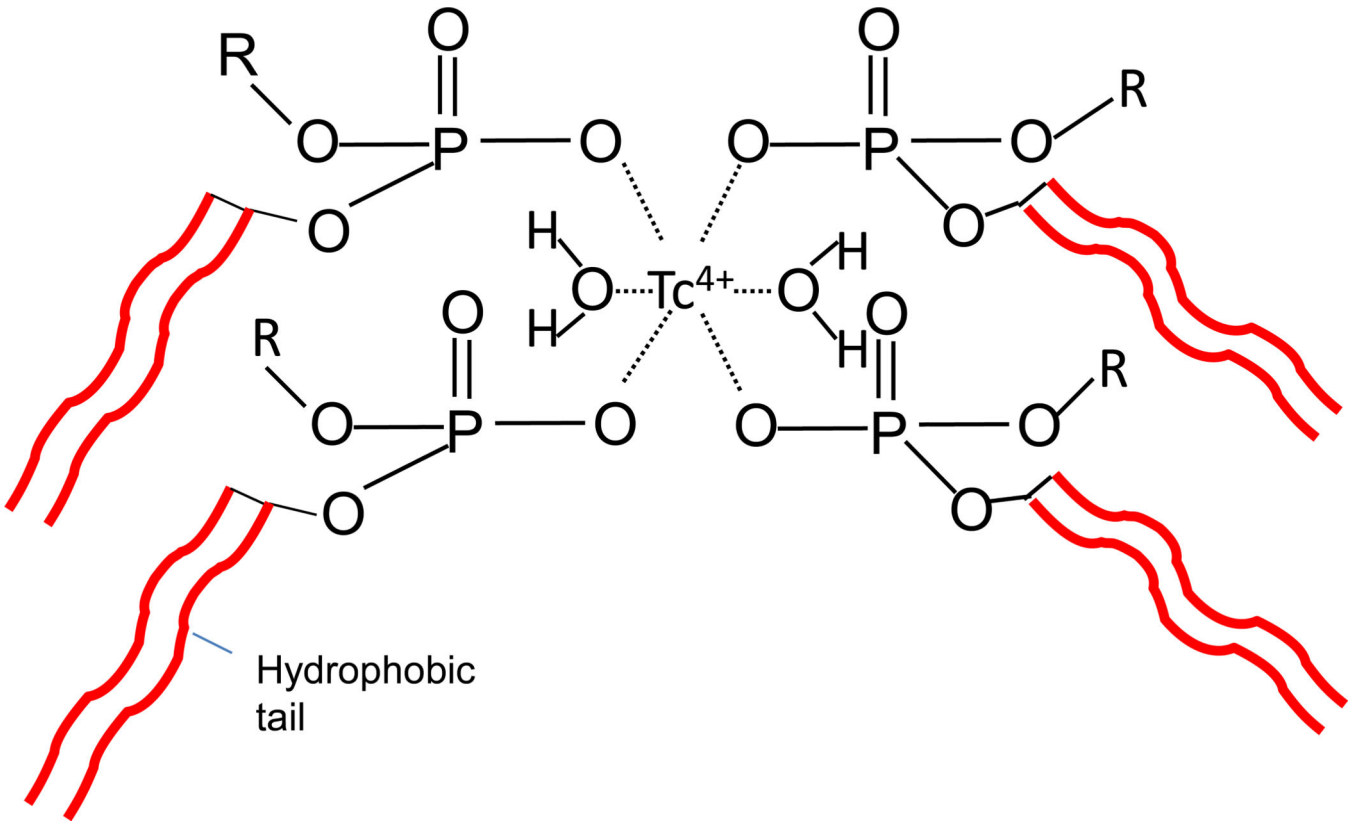

Figure 7.

Schematics of the radiolabeled ^{99m}Tc-DPPC structure adapted from a structure of ^{99m}Tc-methyl disphosphonate. The stannous chloride ($SnCl_2$) as a reducing agent changes ^{99m}Tc oxidation state from its native +7 state to a +4 state. Thus, the ^{99m}Tc binds with phosphate group of DPPC.

Table 1

LPLC Analysis Result of Radiolabeled LAI

	% free 99mTc	% free drug
Before purification	10.7±3.2	- *
After purification	7.1±3.2	- *
Mixed dose, 3hr	10.4±0.8	1.4±0.0
Mixed dose, 24hr	10.1±1.5	1.6±0.6
Mixed dose, 48hr	31.2±9.1	1.9±0.6
After impaction, 48hr**	14.6	14
After impinger, 0hr	17.3	21.3
After impinger, 24hr	35.0	31.9
After impinger, 48hr	10.7±3.2	34.0

All the data represent mean±S.D. (n=4) except impaction and impinger studies.

* Free drug was not detectable.

** Data was obtained from analysis of stage 3 sample