Original Article

99mTc-labeling and biological evaluation of conventional liposomes.

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Abstract

Background. Liposomes are self-assembled supramolecular systems, composed by phospholipids and cholesterol, designed for the transportation of drugs or radionuclides. Physical properties of 99mTc (pure gamma emitter with E = 140 KeV, t1/2= 6 hours) makes it useful for scintigraphic imaging.

Purpose. The goal of this study was to evaluate 99mTc labeled conventional liposomes as potential diagnostic agents for malignant lesions.

Methods. The studied liposome composition was hosphatidylcholine: dioleilphosphatidylcholine: cholesterol (1.1:0.4:1molar rate). In order to optimize the labeling, SnF2 was used as reducing agent. Radiochemical purity and labeling efficiency were evaluated using ascending thin layer chromatography. Scintigraphy images were obtained at 1 hour post-injection of labeled liposomes to healthy mice and with spontaneous breast tumors.

Results. Labeled liposomes showed stability during 24 hours, being 138 µg the optimal amount of reducing agent for the technique used.

Conclusions. The described method allows the production of 99mTc labeled liposomes in a simple, efficient and stable way. The radiopharmaceutical showed a physicochemical and biological behavior that allows its potential use as a tumor imaging agent, although further studies are required to confirm this issue.

Key words. liposomes; 99mTc; radiopharmaceutical; scintigraphy.

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1. Introduction

Liposomes are self-assembled supra-molecular systems consisting of an aqueous volume surrounded by one or several phospholipidic layers. In general, conventional liposomes are composed by neutral and/or negative phospholipids and cholesterol [1,3]. Liposomal structure can be rationally designed with high versatility being very flexible systems with an *in vivo* behavior that can be modulated by designing their structure. This fact enables the generation of liposomal formulations with diagnostic or therapeutic purposes [2,4,5].

Size is a critical aspect of these nano-vehicles because it affects their blood elimination and drug incorporation. It has been determined that the size for which there is an optimal ratio between *in vivo* stability and drug incorporation efficiency is around 100 nm ([6].

Liposome labeling with gamma-emitting radionuclides is feasible [7] for use as a tracer for diagnostic imaging [8]. This nano-vehicle has several advantages such as requiring small quantities of radionuclides, in the order of nanograms. Thus, it does not interfere with liposome distribution nor with the physiological processes involved in their biodistribution [8-10].

A radiopharmaceutical is a radioactive compound used for diagnosis or treatment of human diseases. The most widely used radionuclide in nuclear medicine for diagnostic purposes is ^{99m}Tc due to its gamma emission of 140 Kev and a convenient half life of 6 hours. These characteristics allow the acquisition of suitable images with low absorbed doses and fast elimination. Therefore, it is possible to study the *in vivo* biodistribution and pharmacokinetics of radiopharmaceuticals through a non invasive approach [11].

The objective of this study was to develop a liposomal radiopharmaceutical labeled with ^{99m}Tc and to characterize its physicochemical and biological properties.

2. Methods

2.1. Materials

List of materials included: Egg phosphatidylcholine (Epikuron 200, Degussa), cholesterol (Avanti Polar Lipids); dioleylphosphatidylethanolamine (Avanti Polar Lipids); chloroform (Sigma); methanol (Sigma); diethyltriamine penta-acetic acid (DTPA) (Sigma); stannous fluoride, SnF_2 (Sigma); pyridine (Sigma); metylketone (Sigma); sodium bicarbonate (Merck); sodium chloride (NaCl) (Merck); ITLC-SG Ketamine (Kensol); all reagents were analytical quality.

2.2. Preparation of liposomes

Liposomes were prepared by *hand-shaken* method [1] which as follows: a solution of egg phosphatidylcholine: dioleylphosphatidylethanolamine: cholesterol (1.1:0.4:1 molar ratio) in a solvent mixture of chloroform: methanol (9:1 v/v) were added in a balloon. The organic phase was removed by rotary evaporation at 60ºC (Büchi 461 rotary evaporator) and the thin film obtained was vacuum-dried for 12 hours (Lab Line duo vacuum oven).

The phospholipidic film was then hydrated with a 0.1 M sodium bicarbonate solution at 60ºC and a primary suspension of multilamellar liposomes was obtained.

Size reduction was performed by 5 times extrusion through a 400 nm polycarbonate membrane followed by 5 times extrusion through a 100 nm polycarbonate membrane using a miniextruder (Avanti Polar Lipids Inc.). Size distribution of the unilamellar liposomes obtained was determined by laser light diffraction (Coulter LS 230).

2.3. Liposome labeling technique and quality control

Liposome labeling was performed taking 0.4 ml of liposomal dispersion incubated at room temperature for 20 minutes with 0.9 ml of pertechnetate ($^{99m}TcO_4^-$) solution, 222 MBq

obtained from a $^{99}Mo/^{99m}Tc$ generator (Tecnonuclear, Argentine). Stannous fluoride (SnF_2) was used as a reducing agent. For labeling optimization, different amounts of SnF_2 were used: 69 µg, 138 µg and 207 µg.

Labeling efficiency was evaluated for each case determining the percentage of ^{99m}Tc-liposomes (^{99m}Tc-lip) formed and evaluating radiochemical purity by ascendant instant thin layer chromatography (ITLC-SG) using silica gel coated fiber sheets of 10 cm in length.

For each case, a tiny drop (2-3 µl) of the radiolabeled formulation was applied at a point 1 cm from the end of an ITLC-SG strip. The strip was developed with a suitable solvent mixture or solution to separate the different species that could be present. The solvent front was allowed to reach approximately 10 cm from the origin, then the strip was cut into 10 parts of 1 cm each and the radioactivity in each segment was determined in a scintillation counter (Packard).

The radiochemical species that could have been present were:

- ^{99m}Tc-lip, $^{99m}TcO_4^-$, ^{99m}Tc reduced/hydrolized (^{99m}Tc R/H).

The chromatographic systems used for their discrimination were:

- ITLC-SG, mobile phase NaCl 0.9% solution: R_f ^{99m}Tc-lip = 0; R_f $^{99m}TcO_4^-$ = 1; R_f ^{99m}Tc R/H = 0
- ITLC-SG, mobile phase pyridine:acetic acid:water (3:5:1.5 v/v): R_f ^{99m}Tc-lip = 0.8-1; R_f $^{99m}TcO_4^-$ = 1; R_f ^{99m}Tc R/H = 0.

2.4. In vitro stability studies

In vitro stability of the radiopharmaceutical was evaluated for the different amounts of reducing agent at 2 and 24 hours post labeling in sodium bicarbonate solution 0.1M at room temperature. Radiochemical purity was verified by ITLC-SG using the same mobile phases as described for quality control because the species that could eventually appear as products of radiopharmaceutical decomposition are $^{99m}TcO_4^-$ and ^{99m}Tc R/H.

On the other hand, as a tool for predicting *in vivo* radiolabeling stability, ^{99m}Tc transchelation was challenged against different concentrations of diethyltriamine penta- acetic acid (DTPA). This complexation agent was selected as a competition model molecule for the radionuclide. For that purpose, 0.5 ml of labeled liposomes was passed through a PD10 G25 column (Pharmacie) using normal saline solution as eluting agent to separate $^{99m}TcO_4^-$ and ^{99m}Tc R/H. After that, 0.5 ml of purified labeled liposomes dispersion was incubated at 37ºC with 0.5 ml of 100, 50 and 12.5 mM DTPA solutions.

Radiochemical purity was evaluated at 1 and 2 hours by ITLC-SG using NaCl 0.9% as mobile phase which allowed the separation of DTPA-complex (R_f ^{99m}Tc-DTPA= 1) from the labeled liposomes which remained at the point of application (R_f ^{99m}Tc-lip.= 0).

2.5. Biological evaluation: biodistribution and scintigraphic studies

Biodistribution studies were performed in normal, healthy female CD1 mice, eight weeks old (28-31 g, n=3). They were injected in the tail vein with 0.1 ml of liposomal dispersion composed by 1.25 mg/ml of total lipids labeled with 7.4±2 MBq of ^{99m}Tc pertechnetate.

The mice were sacrificed at 1 and 3 hours after injection. After the removal of the different organs, fluids and tissue they were introduced in pre-weighed tubes, which were weighed again to calculate the weight of organ/tissue and the activity in each one was determined using a solid scintillation counter (Packard). The results were expressed as a percentage of the injected dose.

Scintigraphic studies were performed in a single-head gamma camera, connected to a dedicated computer system with the following conditions: high resolution collimator for low energies, 256x256 matrix and a 10% energy window centered in the photopeak of ^{99m}Tc (140

keV). Studies were performed in CD1 healthy female mice (n=3) and spontaneous breast tumor bearing mice (n=3), all being eight weeks old (27-31 g).

Static images were obtained 1 hour post-injection of 0.1 ml of ^{99m}Tc-liposomes (1.25 mg/ml total lipids, 10±3 MBq) in the tail vein. Mice were anesthetized with intraperitoneal Ketamine injection, using a dose of 100 mg/kg. Animal studies were conducted in accordance with the local Ethics Committee of the University Hospital.

3. Results

Liposomal size distribution was obtained by laser light diffraction (figure 1). An unimodal size distribution was observed: 81.7% of total liposomes were between 50 and 250 nm (p=0.05) with a mean vesicle size of 136 nm.

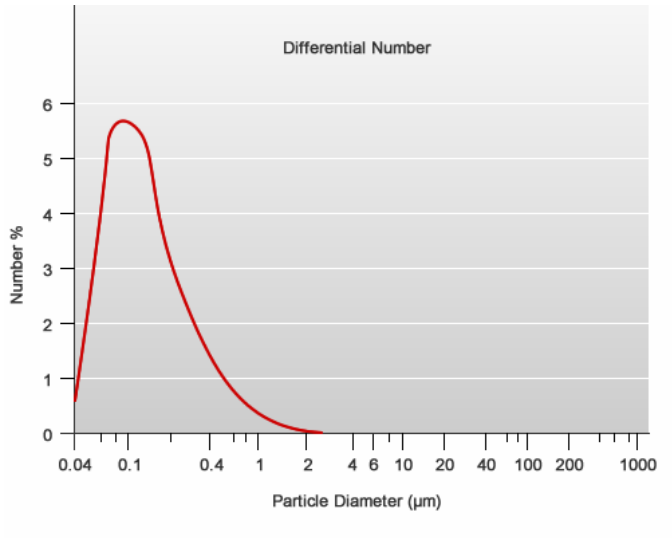

Figure 1. Percentage number versus particle diameter.

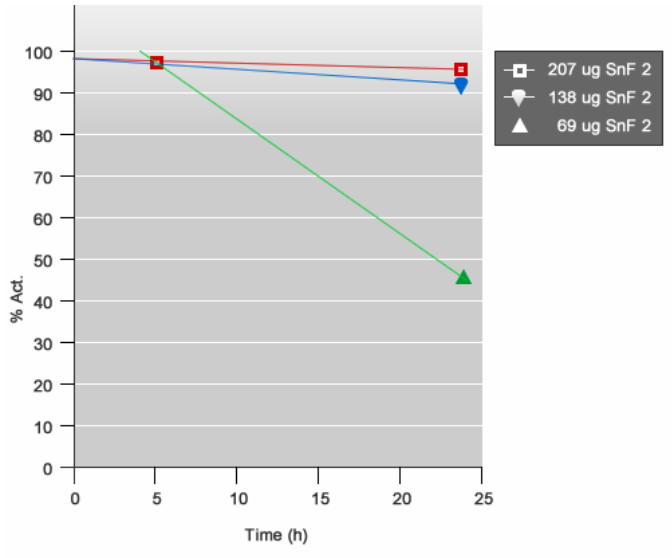

Figure 2. Percentage of labeled liposomes versus time.

Labeling efficiency was over 95 % for the 3 quantities of reducing agent at 1 hour (figure 2). Labeling stability was different according with the amount of SnF_2used; it was >90% for 138 and 207 µg of SnF_2 and <50% for 69 µg; of SnF_2 during the studied period. It was also observed that only at high DTPA concentrations relevant transchelation appeared (figures 3 and 4).

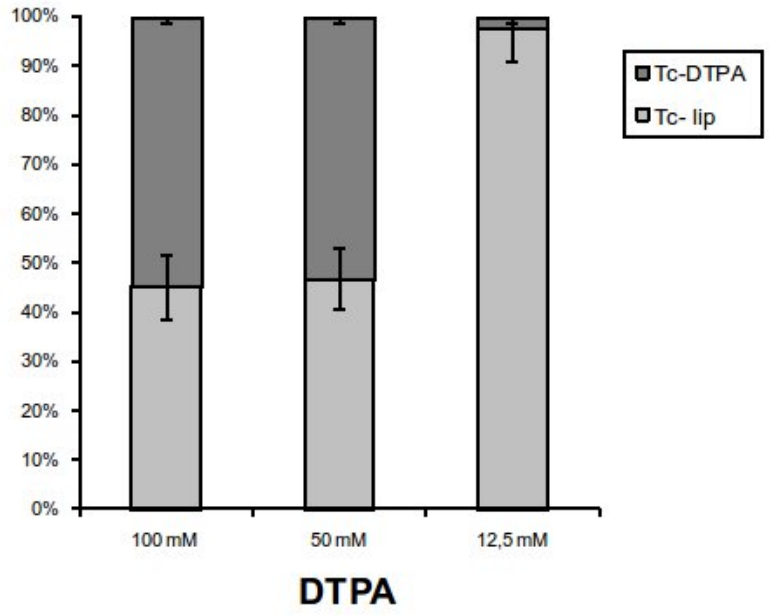

Figure 3. Transchelation study at 1 hour post injection.

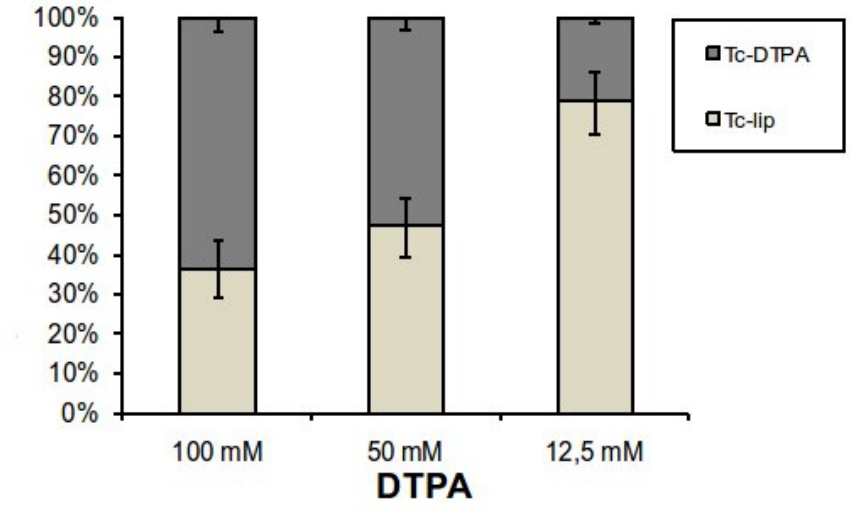

Figure 4. Transchelation study at 2 hours post injection.

Biodistribution of ^{99m}Tc-liposomes at one hour post-injection in healthy mice showed high blood clearance. Blood activity was less than 5% with the liver showing the highest uptake (figure 5).

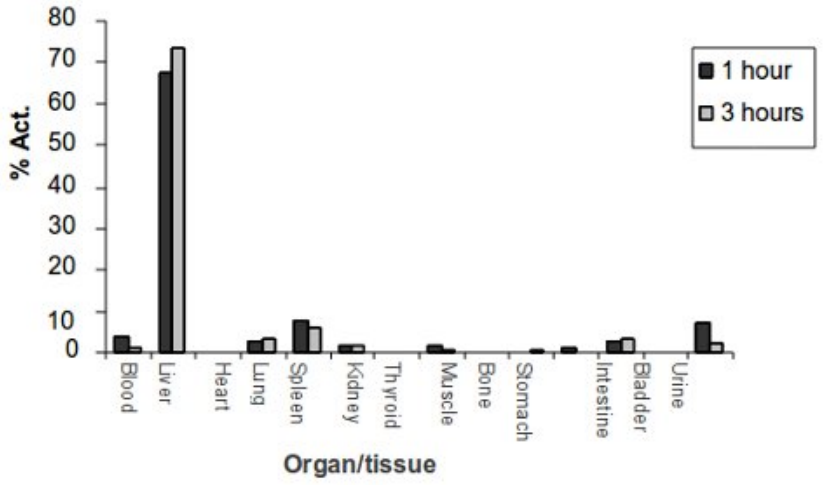

Figure 5. Biodistribution study for 99mTc- liposomes in healthy mice at 1 and 3 hours post injection.

Images of a CD1 healthy mouse acquired at one hour post-injection (figure 6) demonstrated, as mentioned, high liver uptake without relevant accumulation in other organs. Besides, in mice with spontaneous breast lesions images showed tracer accumulation in tumors with high tumor-to-muscle ratios (figure 7).

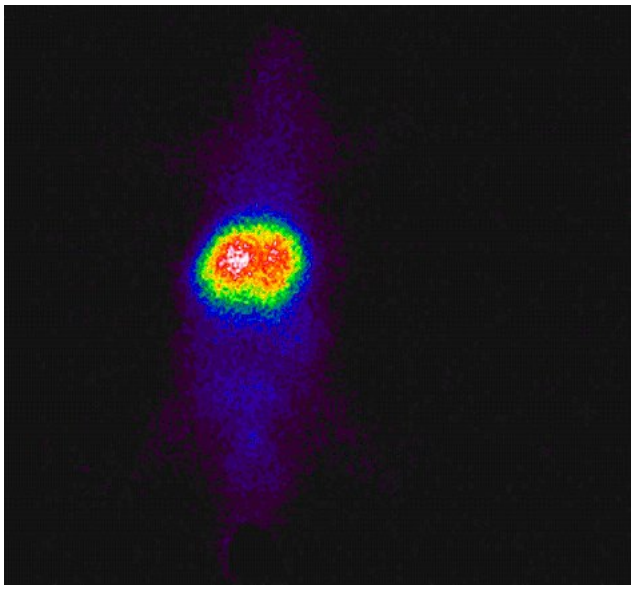

Figure 6. Scintigraphic image of healthy CD1mouse at 1 hour post injection of 99mTc- liposomes with 10 ± 3 MBq.

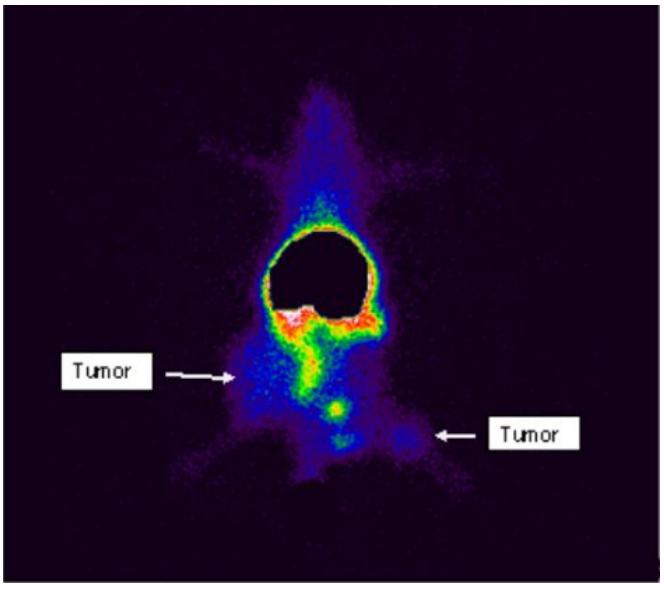

Figure 7. Scintigraphic image of CD1 mouse with tumor at 1 hour post injection of 99mTc- liposomes with 10 ± 3 MBq.

4. Discussion

In this study, the composition of liposomes was designed with dioleoyl phosphatidylethanolamine to provide amine groups in the phospholipid layer. Thus, liposomes chelate with the radionuclide and constitute a stable radiopharmaceutical capable to minimize radionuclide interchange with plasmatic proteins. This was proved by in vivo and in vitro studies. In vitro studies demonstrated high stability of radiolabeled liposomes against a model molecule that has high affinity for ^{99m}Tc. In vivo studies showed high rate of blood clearance without significant blood pool uptake demonstrating radiolabel stability.

The optimal labeling method was the one that generated a stable radiopharmaceutical with the less amount of reducing agent [13-17].

The stable labeled liposome obtained with the chosen technique and lipid composition allowed suitable tracing of the nano-vehicle. The technique was optimized so that the desired radiopharmaceutical can be formulated in a quick, easy and efficient way.

Scintigraphic and biodistribution studies performed in healthy mice demonstrated that liposome elimination takes place mainly through the liver with no significant uptake by other organs [18]. In cases where the radiocompound is not stable in vivo, thyroid and stomach activity should become evident; in this study, no such activity was noted. Besides, there was no bladder uptake as expected, because the liposome size does not allow renal excretion. In vivo stability is critical and it cannot be easily achieved by radiopharmaceutical design. There are studies where bladder uptake was evident after the injection of radiolabeled conventional liposomes [19]; for this reason, galactosylated liposomes were designed to achieve in vivo stability.

In order to evaluate the in vivo performance of these nanosystems, a spontaneous breast cancer mice model was evaluated; these are slightly irrigated solid tumors. Scintigraphy studies showed a high tumor-to-muscle ratio; it can be postulated that this accumulation could be related to angiogenic processes at the tumor periphery associated with inflammatory lesions that produce blood vessel disruption and extravasation of the nanovehicle.

5. Conclusions

The designed liposomes showed suitable physicochemical and biological properties for their potential use as radiotracers in oncology imaging. Further studies using different tumor models will allow the confirmation of these preliminary observations.

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