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Influence of PEG coating on the biodistribution and tumor accumulation of pH-sensitive liposomes

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Abstract

Liposomes are lipid vesicles widely used as nanocarriers in targeted drug delivery systems for therapeutic and/or diagnostic purposes. A strategy to prolong the blood circulation time of the liposomes includes the addition of a hydrophilic polymer polyethylene glycol (PEG) moiety onto the surface of the vesicle. Several studies claim that liposome PEGylation by a single chain length or a combination of PEG with different chain lengths may alter the liposomes' pharmacokinetic properties. Therefore, the purpose of this study was to evaluate the influence of PEG on the biodistribution of pH-sensitive liposomes in a tumor-bearing animal model. Three liposomal formulations (PEGylated or not) were prepared and validated to have a similar mean diameter, monodisperse distribution, and neutral zeta potential. The pharmacokinetic properties of each liposome were evaluated in healthy animals, while the biodistribution and scintigraphic images were evaluated in tumor-bearing mice. High tumor-to-muscle ratios were not statistically different between the PEGylated and non-PEGylated liposomes. While PEGylation is a well-established strategy for increasing the blood circulation of nanostructures, in our study, the use of polymer coating did not result in a better *in vivo* profile. Further studies must be carried out to confirm the feasibility of the non-PEGylated pH-sensitive liposomes for tumor treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Keywords

Liposomes; Tumor targeting; PEGylation; Drug delivery

Introduction

Over the past decades, drug delivery systems have gained traction as they have the potential to selectively deliver drugs into the desired tissue, resulting in improved therapeutic efficacy and safety [1–3]. Several nanostructured materials have been developed and reported for therapeutic and/or diagnostic purposes. Liposomes, first described by Bangham and coworkers, are spherical vesicles formed spontaneously when phospholipids are exposed to an aqueous environment [4, 5]. Based on a lipid bilayer composition, liposomes could be divided into conventional and long-circulating liposomes (PEGylated liposomes) [3, 6]. Conventional liposomes are usually composed of solid (or fluid) phosphatidylcholines (PCs) and cholesterol (Chol) [6, 7]. After intravenous injection, these molecules, unfortunately, can be recognized by the reticuloendothelial system (RES), leading to an expedited blood clearance that ultimately compromises drug-targeting efficiency [6–8]. To overcome this challenge, PEGylated liposomes emerged in the late 1980s as an alternative strategy to enhance the pharmacokinetic parameters [6]. The surface of this second-generation liposome is coated with polyethylene glycol (PEG), a hydrophilic polymer that can prevent the liposomes from recognition and subsequent clearance by RES [7, 9, 10]. PEGylation increases the hydrophilicity of the liposome and provides a steric barrier against opsonization [9, 11, 12]. The resulting long-circulating liposomes, also known as stealth or sterically stabilized liposomes, are still removed from the blood circulation, however, at a much lower rate [11–13].

Several studies claim that liposome PEGylation may alter the liposomes' pharmacokinetics. According to Sugiyama and coworkers, the combination of PEG with different size chains modifies the conformation of the polymer on the surface of the liposome into a more extended form known as “brush” [14–17]. This conformation leads to a greater fixed aqueous layer thickness (FALT) around the liposome, which is essential for diminishing nanoparticle opsonization. However, the presence of PEG could also show some disadvantages. Large molecules on the surface of liposomes, such as PEG, may reduce the interaction between liposome and cells, hampering the liposomes' internalization into target cells [18, 19]. These findings, therefore, raised the question whether PEGylation is always beneficial to improve the efficiency of a nanocarrier.

A better understanding of the effect of physicochemical characteristics of liposomes on their biodistribution is crucial for the rational development of liposomes for therapeutic applications [20, 21]. Herein, we describe the preparation and characterization of three pH-sensitive liposomes, two of which were coated with PEG (PEG₂₀₀₀ or a mixture of PEG₁₀₀₀/PEG₅₀₀₀) and one liposome with no polymer coating. In order to explore the biodistribution of the nanocarriers utilizing advanced imaging, a radiotracer (^{99m}Tc-HYNIC-βAla-Bombesin_(7–14)) was encapsulated into the liposomes and blood clearance profiles

were determined in healthy mice. Moreover, biodistribution and scintigraphic images were carried out in tumor-bearing mice to compare tumor accumulation.

Material and methods

Materials

Phospholipids dioleoylphosphatidylethanolamine (DOPE), distearoylphosphatidylethanolamine (DSPE), DSPE-PEG₁₀₀₀, DSPE-PEG₂₀₀₀, and DSPE-PEG₅₀₀₀ were obtained from Lipoid GmbH. Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma Chemical Company, USA. Glucose, mannitol, and trehalose were purchased from Vetec Química Fina Ltda., Brazil. All other chemicals and reagents used were purchased from Sigma-Aldrich, Brazil. In addition, the peptide HYNIC- β Ala-Bombesin_(7–14) was purchased from GL Biochem, China. Technetium-99m was obtained from an alumina-based ⁹⁹Mo/^{99m}Tc generator from IPEN, Brazil.

Animals

Six-week-old to eight-week-old female BALB/c mice (20–23 g) were used in two separate experiments. In the first, healthy female BALB/c mice were used for blood clearance studies ($n = 6$). In the second, 4T1-tumor-bearing BALB/c mice were used for biodistribution studies and scintigraphic images. Animals were kept under SPF condition with free access to standard food and water. All animal studies were approved by the local Ethics Committee for Animal Experiments of Federal University of Minas Gerais, Brazil.

Liposome preparation

Three types of liposomal formulations were prepared with the same molar concentration of DSPE-PEG₂₀₀₀ (Lip₂₀₀₀), DSPE-PEG_{1000/5000} (Lip_{1000/5000}), or DSPE (Lip). For the preparation, chloroform aliquots of DOPE, CHEMS, and DSPE (PEGylated or not) (lipid concentration of 40 mM; molar ratio of 5.7:3.8:0.5, respectively) were transferred to a flask and the solvent was removed under reduced pressure. The lipid film obtained was solubilized in a 15 mM NaOH solution under vigorous stirring at room temperature. NaOH solution was used to guarantee complete ionization of CHEMS, which is indispensable to form vesicles. Vesicles (liposomes) were formed, and the final pH was adjusted to 7. These liposomes were extruded through 0.4 μ m, 0.2 μ m, and 0.1 μ m polycarbonate membranes (5 cycles for each). The phospholipid concentration was determined using the colorimetric method described by Bartlett [22].

Mean diameter and zeta potential

The mean diameter of the liposomes was determined by dynamic light scattering at 25 °C and an angle of 90°. The zeta potential was evaluated by electrophoretic mobility determination at the angle of 90°. Size and zeta potential measurements were performed in triplicate using the 3000HS Zetasizer equipment (Malvern Instruments, England). The samples were diluted using a 0.9% (w/v) NaCl solution.

Radiolabeling

Twenty milligrams of tricine and 5 mg of EDDA were added to 0.5 mL of 0.9% NaCl (w/v) in an amber-colored sealed vial. Next, 10 µg of HYNIC-βAla-Bombesin₍₇₋₁₄₎ and 10 µL of SnCl₂·2H₂O in an acid solution (4.5 mM) were added and the pH level was adjusted to 7.0. Next, an aliquot of 0.5 mL of Na^{99m}TcO₄ (37 MBq) was added and the solution was heated for 15 min in a water bath at 100 °C and cooled in water.

Radiochemical purity

The radiochemical purity was determined by thin-layer chromatography (TLC) on two solvent systems: methyl ethyl ketone (MEK) to determine ^{99m}TcO₄⁻ and acetonitrile/water (1:1) to determine ^{99m}TcO₂, as previously described [21].

Encapsulation percentage

Aliquots (1.0 mL) of Lip, Lip₂₀₀₀, or Lip_{1000/5000} were placed in 10-mL amber-colored vials with glucose, as a cryoprotectant that was pre-weighed in a sugar/phospholipid (w/w) ratio of 2:1. Afterwards, the solution of ^{99m}Tc-HYNIC-βAla-Bombesin₍₇₋₁₄₎ was added to the vial. The mixture was subjected to three freeze-thaw cycles in liquid nitrogen. By this procedure, liposomes are disrupted during the freezing step and reforming, encapsulating the radiotracer, during the thawing step. Next, non-entrapped ^{99m}Tc-HYNIC-βAla-Bombesin₍₇₋₁₄₎ was removed by ultra-centrifugation (ultra-centrifuge Sorvall Ultra 80, USA) at 150,000g at 4 °C for 90 min. The encapsulation percentage (EP) was calculated according to Eq. (1)

$$\%EP = \frac{\text{Amount of radioactivity(Pellet)}}{\text{Amount of radioactivity(Supernatant + Pellet)}} \times 100 \quad (1)$$

Radiotracer leakage

Ultra-filters (MW 10 kDa; Amicon Ultra, Millipore, USA) were used to estimate the amount of ^{99m}Tc-HYNIC-βAla-Bombesin₍₇₋₁₄₎ leaked from Lip, Lip₂₀₀₀, or Lip_{1000/5000}. Aliquots of 90 µL of each sample were incubated, under agitation, at 37 °C with 1.0 mL of fresh mice plasma. Radiotracer leakage was determined from samples taken at 10 min, 30 min, 60 min, 90 min, 120 min, 240 min, 360 min, and 1440 min after incubation. The samples were placed into ultra-filters and centrifuged at 14,000×g for 30 min. The percentage of leaked ^{99m}Tc-HYNIC-βAla-Bombesin₍₇₋₁₄₎ was calculated according to Eq. (2)

$$\text{Leakage percentage(\%)} = \frac{\text{Amount of radioactivity(Filtrate)}}{\text{Amount of radioactivity(Filter + Filtrate)}} \times 100 \quad (2)$$

Blood clearance

The ^{99m}Tc-HYNIC-βAla-Bombesin₍₇₋₁₄₎-loaded Lip, Lip₂₀₀₀, or Lip_{1000/5000} was administered (*n* = 6) through a tail vein, and the blood samples were collected at 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, and 240 min. A small incision was made in the distal tail to facilitate rapid and reliable blood drawing. Each sample was

weighed, and the radioactivity was determined in an automatic scintillation apparatus. The percentage of injected dose (%ID) was determined, and the data were plotted as a function of time. The terminal elimination rate constant (K_{el}) was estimated from the slope of the terminal exponential phase of blood curve, by means of linear regression, applied to the semi-log plot of the concentration-time data. The elimination half-life ($T_{1/2}$) was then calculated as $\text{Ln}(2) / K_{el}$.

Tumor cell inoculation

Cell culture—Human breast cancer 4T1 cells were grown in DMEM medium (D-MEM, Gibco, USA), supplemented by 10% (v/v) fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL). Cells were kept in humidified air (95%) containing 5% CO₂ at 37 °C. After 3–5 days of culture, the cells were trypsinized and cell viability was assessed after staining with trypan blue. After centrifugation (5 min at 330×*g*), the cells were re-suspended in DMEM inoculated in the BALB/c mice.

Inoculation—Aliquots (100 µL) with 2.5×10^6 4T1 cells were subcutaneously injected into the right flank of female BALB/c mice. Tumor cells were allowed to grow in vivo for 10 days. 4T1 tumor-bearing mice were used for biodistribution studies and scintigraphic imaging.

Biodistribution studies

Aliquots (100 µL) of Lip, Lip₂₀₀₀, or Lip_{1000/5000} (3.7 MBq) were injected intravenously into 4T1 tumor-bearing mice ($n = 6$). At 1 h and 4 h after administration, mice were anesthetized with a mixture of xylazine (15 mg/kg) and ketamine (80 mg/kg). Liver, spleen, kidneys, stomach, heart, lungs, blood, muscle, thyroid, and tumor were removed, washed with distilled water, dried on filter paper, and placed in pre-weighed plastic test tubes. Radioactivity was measured using an automatic scintillation apparatus. A standard dose containing the same injected amount was counted simultaneously in a separate tube, which was defined as 100% radioactivity. The results were expressed as the percentage of injected dose/gram of tissue (%ID/g).

Scintigraphic images

Anesthetized mice were horizontally placed under the gamma camera (Mediso, Hungary) with a low-energy high-resolution collimator. Images were acquired at 1 h and 4 h post injection using a $256 \times 256 \times 16$ matrix size with a 20% energy window set at 140 keV for a period of 300 s.

Statistical analysis

All data are expressed as mean $\pm$ SD. Means between the various groups were compared by analysis of variance (ANOVA), followed by Tukey's test. A *P* value of < 0.05 was considered to indicate a statistically significant difference. All data were analyzed using GraphPad Prism software, version 5.00.

Results

Size distribution and zeta potential

Blank liposomes (Lip, Lip₂₀₀₀, and Lip_{1000/5000}) showed similar mean diameter (~ 130 nm), low polydispersity index (PDI < 0.2), and zeta potential close to neutrality (data not shown). After the freeze-thaw process, for radiotracer encapsulation, the same parameters were evaluated and the results are expressed in Table 1.

The vesicles showed a good homogeneity, indicated by the low polydispersity index. Furthermore, the mean diameter was smaller than 500 nm for all three formulations which is a criterion to be compatible with intravenous administration [20]. The zeta potential was close to neutrality for all formulations. Lip showed a more negative value of zeta potential compared to the other formulations, likely due to the absence of the PEG chain. Additionally, no significant loss of phospholipids was observed during the preparation of the liposomes.

Radiochemical purity

The radiopurity of ^{99m}Tc-HYNIC-βAla-Bombesin_(7–14) was shown to be 96.2% ± 2.5% (*n* > 6). These results were highly reproducible, and the complex remains stable up to 24 h, as previously described by de Barros et al. [21]. High radiolabeling purity and good in vitro stability are essential for a useful and reliable radiotracer since the technetium-99m must be attached to the molecule of interest throughout the images' time frames. These results indicated that ^{99m}Tc-HYNIC-βAla-Bombesin_(7–14) can be used as a probe to track the liposomal formulations used in this study.

Encapsulation percentage and radiotracer leakage

The encapsulation rates of Lip, Lip₂₀₀₀, and Lip_{1000/5000} for ^{99m}Tc-HYNIC-βAla-Bombesin_(7–14) showed a similar rate (26.3% ± 7.1%, 31.3% ± 8.4%, and 27.4% ± 6.4%, respectively). These values are consistent with the literature utilizing a parallel methodology [23, 24]. The amounts of radiotracer trapped into the liposomes are suitable for further in vivo imaging and biodistribution studies; however, ^{99m}Tc-HYNIC-βAla-Bombesin_(7–14) leakage must be determined in order to certify high payloads in long-term assessments. Otherwise, free peptide distribution will compromise the nanoparticle in vivo profile impairing the outcome interpretations.

Figure 1 shows the leakage profile of the radiotracer from Lip, Lip₂₀₀₀, and Lip_{1000/5000}. An initial release was observed for all formulations at 10 min, and no additional release was observed within 6 h. After 24 h, 32.7% of ^{99m}Tc-HYNIC-βAla-Bombesin_(7–14) leaked from Lip, and lower amounts were leaked from PEGylated liposomes, Lip₂₀₀₀, and Lip_{1000/5000} (17.1% and 18.6%, respectively). These results provide the platform to allow the use of all formulations in further in vivo studies.

Blood clearance

The blood clearance for the three formulations followed a bi-exponential curve, with a rapid decline of injected dose up to 45 min and a second phase representing terminal elimination

(Fig. 2). Lip, Lip₂₀₀₀, and Lip_{1000/500} showed terminal half-lives ($T_{1/2}$) of 118.3 min, 160.0 min, and 125.3 min, respectively. The similar blood clearance for all formulations encouraged us to evaluate the in vivo tumor uptake of liposomes with and without PEG coating.

Biodistribution and scintigraphic images

The biodistribution profiles for Lip, Lip₂₀₀₀, and Lip_{1000/5000} are shown in Fig. 3. The three formulations showed significant up-take by the liver and spleen and moderate uptake in the kidneys, at 1 h and 4 h. Other organs evaluated presented no significant uptake, especially the stomach and thyroid. The low uptakes in these organs indicate the stability between the chelating agent and technetium-99m [21].

Lip, Lip₂₀₀₀, and Lip_{1000/5000} showed higher tumor uptake compared with the contralateral muscle at 1 h and 4 h. These results demonstrate the ability of each formulation to reach the tumor site probably due to the enhanced permeability and retention (EPR) effect [20, 25, 26]. The tumor-to-muscle ratio shown in Fig. 4 was obtained from biodistribution studies and was also similar among the formulations.

Scintigraphic images of 4T1 tumor-bearing mice are presented in Fig. 5. The findings were consistent with the biodistribution data. In addition, a visible tumor signal was achieved by scintigraphic images, mainly at 4 h post injection (arrows). The quantitative analyses of the scintigraphic images showed no statistical difference in tumor-to-muscle ratio, at 1 h and 4 h, between biodistribution and scintigraphic studies (Table 2).

Discussion

It has been demonstrated that the liposomes are drug delivery systems that are able to modify tissue distribution, metabolism, and in vivo elimination of drugs [3, 27]. PEG hydrophilic polymer has been widely used to modify the pharmacokinetics of nanoparticles after intravenous administration. This polymer increases the circulation of the particle by reducing the recognition by the mononuclear phagocytic system. Recent studies [14–17] also demonstrated that the combination of PEG with different size chains modifies the conformation of the polymer on the surface of the liposome, which may contribute to reducing nanoparticle opsonization [14, 15]. However, the addition of PEG to the liposomal surface may also impair the uptake by target cell [11]. In this work, we investigated the influence of PEG chain size on the blood circulation time, tissue distribution, and tumor uptake of pH-sensitive liposomes in order to identify the best composition for achieving tumor accumulation. It is worth mentioning that pH-sensitive liposomes have been reported by our group as a promising strategy to selectively release their content in acidic environments, such as tumor [28].

Regarding the mean diameter, Lip has shown a slight increase in size likely due to the fusion of the vesicular membranes during the encapsulation. After liposome preparation, no difference in vesicle diameter was found for all three liposomal formulations. However, the freeze-thaw process led to an increase in liposome size only for Lip formulation. These data suggest that the absence of PEG may have favored vesicular aggregation; once, it is already

reported that the presence of PEG creates a hydrophilic layer around the liposomes that can reduce the interaction between the lipids of the bilayer, hindering the aggregation process [29].

Zeta potential values close to neutrality were already anticipated to these formulations. Noteworthy was the more negative zeta potential for Lip, which might be explained by the absence of PEG. PEG-coated liposomes show low electrophoretic mobility due to the hydrodynamic resistance given by this polymer [30]. Besides that, without the hydrophilic polymer on the surface, the predominant negative charge presents in the structural lipids are exposed, leading to the reduction of the zeta potential for Lip [31].

^{99m}Tc -HYNIC- β Ala-Bombesin₍₇₋₁₄₎ has been used by our group, entrapped into liposomes, as a stable radiotracer [32]. Herein, we proposed to encapsulate this radiometal complex into pH-sensitive liposomes to track their biodistribution. In order to attain this aim, we must guarantee that most of the radiotracer remains encapsulated into the liposomes; otherwise, the study will no longer reflect the nanoparticles fate. In this concern, leakage profiles in the presence of plasma were determined. An initial ^{99m}Tc -HYNIC- β Ala-Bombesin₍₇₋₁₄₎ leakage for all formulations due to the presence of radiotracer adsorbed on the surface of the vesicles was observed [21]. An additional release from the formulations was observed after 24 h of study; however, PEGylated liposomes showed higher retention of the radiotracer. In fact, the presence of PEG could lead to a slight increase in liposome stability, resulting in a higher retention of the encapsulated drug [33, 34]. Nevertheless, even for liposomes with no PEG coating, a low ^{99m}Tc -HYNIC- β Ala-Bombesin₍₇₋₁₄₎ leakage was observed (~ 70% of the radioactive probe remains stable into the liposome for up to 24 h). Therefore, the leakage profile ensures that the whole system will be monitored during the experiments, not only the free peptide, and that all formulations could be used for further in vivo studies [35, 36].

In the present study, blood clearance was very similar among the formulations. Although Lip₂₀₀₀ presented the longest $T_{1/2}$, it is interesting to mention that, unlike it was reported in previous studies [14, 16, 17], the presence of PEG did not cause a considerable increase in blood circulation time. It may suggest that, for this liposome composition, the presence of PEG was not able to prolong significantly the blood circulation time.

In regard to the biodistribution studies, significant uptake by the liver and spleen was expected, since nanostructures are normally recognized by macrophages present in these organs [36, 37]. Additionally, the kidney uptake might be explained by the release of ^{99m}Tc -HYNIC- β Ala-Bombesin₍₇₋₁₄₎ from the liposomes. These results corroborates with the in vitro release profile, indicating that a certain amount of the radio-tracer is leaked out from the liposomes. However, the substantial payload is kept into the liposomes within the first 4 h, which allowed biodistribution studies and scintigraphic images of the formulations. Low uptake in the stomach and thyroid is extremely important since it confirms the radiolabel stability over time avoids data interpretation errors since it is well established that free technetium-99m is taken up by these organs [21].

It is important to underscore the higher tumor uptake compared to contralateral muscle for all tested formulations. It is well known that tumors require the formation of new blood

vessels to maintain the energy supply for their accelerated growth. Such vessels, however, have sufficiently large pores, from 100 to 800 nm, allowing nanovesicle leakage [24, 38, 39]. It was expected that PEG coating would promote an increase in blood concentration, and this would lead to a higher bioavailability and, consequently, a greater tumor uptake [14]. Notwithstanding, no differences in tumor uptake were found for the formulations developed herein. As the blood circulation time of a system is crucial for its tumor accumulation by EPR effect [40], this might be a reason for our results since no major differences in the blood clearance for the three formulations were observed. The low rigid liposome membrane conferred by the lipid composition might explain this fact [40]. Therefore, despite the structural difference among formulations, their ability to reach the tumor area was similar, suggesting that PEGylation, in this case, did not improve in vivo properties of the liposomes. Collectively, the results showed in this study raised a question about the use of PEG indiscriminately for all formulations. Although PEGylation is a well-established strategy for increasing blood circulation of nanostructures, the use of polymer coating did not result in a prolonged half-life. The same would be true for other formulations, which might be unfavorable in terms of cell penetration. Therefore, an evaluation of the advantages of PEGylation for each system individually is crucial for determining the best formulation for tumor delivery.

Conclusion

Three radiolabeled pH-sensitive liposomes were successfully prepared with adequate mean diameter and stability for intravenous administration. In vivo assays showed similar blood clearance and high tumor accumulation for all liposomes, indicating that PEG did not improve in vivo properties of the pH-sensitive liposomes used in this study. Further studies must be carried out to confirm the feasibility of the non-PEGylated pH-sensitive liposomes for tumor treatment.

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Fig. 1. Release profile of the ^{99m}Tc -HYNIC- β Ala-Bombesin $_{(7-14)}$ from Lip, Lip2000, and Lip1000/5000 in the presence of plasma at 37 °C ($n = 3$). The asterisk means statistical difference at 1440 min (24 h)

Fig. 2.
Blood clearance curve of Lip, Lip₂₀₀₀, and Lip_{1000/5000} after intravenous administration in healthy mice ($n = 6$)

Fig. 3.

Biodistribution of Lip, Lip₂₀₀₀, and Lip_{1000/5000}, at 1 h (**a**) and 4 h (**b**) after intravenous administration in 4T1 tumor-bearing mice ($n = 6$). Results were expressed as the average injected dose of ^{99m}Tc -HYNIC- β Ala-Bombesin₍₇₋₁₄₎, per gram tissue, $\pm$ standard deviation

Fig. 4. Tumor-to-muscle ratio for Lip, Lip₂₀₀₀, and Lip_{1000/5000} at 1 h and 4 h post injection ($n = 6$)

Fig. 5. Scintigraphic images of Lip, Lip₂₀₀₀, and Lip_{1000/5000} at 1 h and 4 h after intravenous administration in 4T1 tumor-bearing mice ($n = 6$)

Table 1

Mean diameter, PDI, zeta potential, and the diameter distribution of the vesicles after the freeze-thaw process

Formulation	Mean diameter (nm)	PDI	Zeta potential (mV)	Diameter distribution of vesicles (%)	
				< 200	200–500
Lip	212.3 ± 17.9 ^a	0.20 ± 0.09	− 9.7 ± 1.2 ^a	88.0 ± 1.9	11.9 ± 1.9
Lip ₂₀₀₀	137.0 ± 2.1 ^b	0.13 ± 0.07	− 4.1 ± 0.6 ^b	99.6 ± 0.1	0.4 ± 0.1
Lip _{1000/5000}	146.6 ± 7.6 ^b	0.11 ± 0.03	− 3.0 ± 0.1 ^b	99.7 ± 0.2	0.3 ± 0.2

The results were expressed as the mean ± standard deviation ($n = 3$). Different letters show statistically significant differences ($P < 0.05$)

Table 2

Target/non-target ratios obtained from scintigraphic images

Formulation	Target/non-target ratio	
	1 h	4 h
Lip	2.58 ± 0.81	4.11 ± 0.85
Lip ₂₀₀₀	1.61 ± 0.79	2.89 ± 1.01
Lip _{1000/5000}	1.79 ± 1.01	2.95 ± 0.59

The results were expressed as the mean $\pm$ standard deviation ($n = 6$)