Labelled Stealth\textsuperscript{R} liposomes in experimental infection: An alternative to leukocyte scintigraphy?


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Summary

Indium-111 (\textsuperscript{111}In) and technetium-99m (\textsuperscript{99mTc}) Stealth\textsuperscript{R} liposomes were compared with \textsuperscript{111}In- and \textsuperscript{99mTc}-labelled white blood cells (WBC) in experimental infection in a rabbit model. Preformed polyethylene glycol-coated liposomes and separated WBC were radiolabelled with either \textsuperscript{111}In-oxine or \textsuperscript{99mTc}-hexamethylene propyleneamine oxime (\textsuperscript{99mTc}-HMPAO). After the intravenous administration of one of the four radiopharmaceuticals to rabbits with focal \textit{Staphylococcus aureus} infection, scintigraphic images were recorded at various time points post-injection and the biodistribution of the radiopharmaceuticals was determined. At 4 h post-injection, uptake of \textsuperscript{111}In-WBC in the abscess was significantly higher than that of the three other products. At later time points, \textsuperscript{111}In-WBC, \textsuperscript{111}In-liposome and \textsuperscript{99mTc}-liposome uptake in the abscess were similar. In contrast, at 20 h post-injection, uptake of \textsuperscript{99mTc}-WBC was significantly lower. The abscess-to-background ratios showed a similar pattern to the absolute abscess uptake: initial high values for \textsuperscript{111}In-WBC, a more gradual increase over time of the liposome preparations to the level of \textsuperscript{111}In-WBC and persistently low values for \textsuperscript{99mTc}-WBC. Clearance from the blood of both labelled WBC preparations was significantly faster and splenic uptake significantly higher compared with those of the labelled liposomes. In conclusion, given the similar \textit{in vivo} characteristics of labelled liposomes and labelled WBC, labelled liposomes may be an attractive replacement for labelled WBC, providing a continuously available, high-quality, \textsuperscript{99mTc}-labelled radiopharmaceutical that can be prepared easily without any need to handle blood.

Introduction

Scintigraphic delineation of focal infection is a clinically important procedure, not only to identify infectious and inflammatory foci, but also to evaluate their activity [1]. Despite the development of new agents, autologous leukocyte (WBC) scintigraphy, using either indium-111 (\textsuperscript{111}In) or technetium-99m (\textsuperscript{99mTc}), is still the imaging procedure of choice in routine clinical practice [2]. Unfortunately, the preparation of radiolabelled autologous leukocytes has several disadvantages which limit its application. In particular, the need to withdraw and handle blood that may be infected constitutes an inherent risk to other patients and staff [3, 4]. Moreover, the isolation and labelling of leukocytes is a relatively complicated and time-consuming procedure that is not possible in every laboratory. One approach to facilitate infection imaging is the application of labelled proteins and peptides [5–7]. These radiopharmaceuticals accumulate in infection by specific receptor or antigen interaction or non-specific deposition [7]. Another method to prevent the handling of blood is the use of Stealth\textsuperscript{R} liposomes. These are phospholipid bilayers with a polyethylene coating that prevent rapid uptake of circulating particles by the mononuclear phagocytic system [8].
These liposomes are radiolabelled analogously to leukocytes either with $^{111}$In or $^{99m}$Tc [9, 10]. In initial animal studies, a number of characteristics of labelled liposomes resembled those of labelled leukocytes in humans in terms of biodistribution and the ability to detect various infections [10]. In this study, the in vivo characteristics of labelled StealthR liposomes are compared with those of labelled leukocytes, both labelled with either $^{111}$In or $^{99m}$Tc in a rabbit model of mild focal Staphylococcus aureus infection.

Materials and methods

Animal model

A calf muscle abscess was induced in female, randomly bred New Zealand white rabbits (body weight 2.5-3.0 kg). Approximately $10^8$ colony forming units (CFU) of S. aureus in 0.5 ml of a 50:50% suspension of autologous blood and normal saline was injected into the left calf muscle [11]. Twenty-four hours after the inoculation, when swelling of the muscle was apparent, the respective radiopharmaceuticals were injected via the ear vein.

Preparation of liposomes

Partially hydrogenated egg-phosphatidylcholine with an iodine value of 40 (PHEPC) was obtained from Asahi Chemical Industry Co., Ltd (Ibarakiken, Japan) [12]. The polyethylene glycol (PEG) 1900 derivative of distearoyl-phosphatidyl-ethanolamine (PEG-DSPE) was a kind gift from Liposome Technology, Inc. (Menlo Park, CA) and prepared as described previously [13]. The cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO) and the glutathione from E. Merck (Darmstadt, Germany). A chloroform/methanol mixture (10/1, v/v) containing PEG-DSPE, PHEPC and cholesterol was prepared in a molar ratio of 0.15:1.85:1. A lipid film was formed by rotary evaporation followed by high vacuum to remove residual organic solvent [14].

For encapsulation of glutathione (for subsequent $^{99m}$Tc labelling), the film was hydrated at room temperature with 50 mM glutathione in HEPES buffer (10 mM HEPES, 135 mM NaCl, pH 7.4) at an initial phospholipid concentration of 120 mM. For encapsulation of Desferal (for subsequent 111In labelling), the lipids were dispersed at room temperature in 6 mM Desferal in 0.9% HEPES buffer (10 mM HEPES, 135 mM NaCl, pH 7.5) at an initial phospholipid concentration of 120 mM. The liposomes were sequentially extruded through polycarbonate filters of 0.6, 0.2, 0.1 and 0.08 μm pore size (Poretics, Livermore, CA, USA). Unentrapped glutathione or Desferal was removed by gel filtration on an EconoPac 10DG column (Bio-Rad, Richmond, CA, USA). The particle size distribution was determined by dynamic light scattering with a Malvern 4700 system using a 25 mW He-Ne laser and the automeasure 3.2 software (Malvern, UK). As a measure of particle size distribution of the dispersion, a polydispersity index was determined, ranging from 0.0 (entirely monodisperse) to 1.0 (completely polydisperse). The mean size of the lipidosome dispersions was 120 nm, with a polydispersity index of 0.2.

Radiolabelling of liposomes

Preformed glutathione-containing liposomes were labelled by transporting $^{99m}$Tc as a lipophilic $^{99m}$Tc-hexamethypropyleneamine oxime ($^{99m}$Tc-HMPAO) complex through the bilayer. The $^{99m}$Tc-HMPAO is subsequently irreversibly trapped in the internal aqueous phase due to reduction by the encapsulated glutathione [15]. One milligram of $^{99m}$Tc-HMPAO was incubated with 2.5 GBq $^{99m}$Tc-pertechnetate. The liposomes were incubated for 15 min at room temperature with 1 MBq $^{99m}$Tc-HMPAO per μmol phospholipid. Removal of unencapsulated $^{99m}$Tc-HMPAO was achieved by gel filtration on a EconoPac 10DG column (Bio-Rad, Richmond, CA) with 5% glucose as the eluent. Thus, a radiochemical purity of more than 98% was achieved. Altogether, 200 MBq $^{99m}$Tc-liposomes were injected intravenously.

Preformed Desferal-containing liposomes were labelled with $^{111}$In, essentially as described previously [16, 17]. $^{111}$In was transported in the form of $^{111}$In-oxine through the bilayer and trapped irreversibly in the internal aqueous phase by the encapsulated Desferal. Briefly, the liposomes (75 μmol phospholipid per ml) were incubated for 1 h at room temperature with 75–100 kBq $^{111}$In-oxine (Mallinckrodt Medical, Petten, The Netherlands) per μmol phospholipid. Removal of unencapsulated $^{111}$In-oxine was achieved by gel filtration on an EconoPac 10DG column (Bio-Rad, Richmond, CA). A radiochemical purity of more than 98% was achieved. Twenty MBq $^{111}$In-liposomes were injected intravenously.

Preparation and radiolabelling of leukocytes

For each labelling, 100 ml of blood was withdrawn carefully from an anaesthetized donor rabbit by carotid artery cannulation into 60 ml syringes, each containing 10 ml 0.33% methylcellulose in acid citrate dextrose (ACD). The total white blood cell count of the donor rabbits was $8.5 \times 10^9$ l$^{-1}$ ($^{99m}$Tc labelling) and $12 \times 10^9$ l$^{-1}$ ($^{111}$In labelling) with approximately 50% granulocytes. The erythrocytes were allowed to sediment for 1 h. The supernatant was removed and centrifuged for 10 min at 450 g. The cell pellet was washed with 5 ml phosphate buffered saline (PBS) (pH = 7.4). 1% HSA and centrifuged once more for 10 min at 450 g. The cell pellet was resuspended in 1.5 ml in PBS, 1% HSA. Then, 2 GBq
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"Tcm-HMPAO or 100 MBq In-oxine solution (Mal- linckrodt Medical, Petten, The Netherlands) in 0.2 M Tris-hydroxymethyl-aminomethane (pH = 8.0) was added to the cell suspension. The cells were incubated at room temperature for 30 min and centrifuged for 10 min at 450 g. The cell pellet was resuspended in 5 ml PBS, 1% HSA (\textsuperscript{111}In-WBC) or 5% glucose (\textsuperscript{99}Tcm-WBC). Morphological integrity of the WBC was checked by light microscopy examination. The labelling efficiency (cell associated activity/total activity) was higher than 95% for \textsuperscript{111}In-WBC and 75% for \textsuperscript{99}Tcm-WBC. Twenty MBq \textsuperscript{111}In-WBC or 200 MBq \textsuperscript{99}Tcm-WBC were administered intravenously.

Study design

Twenty-four hours after S. aureus infection, groups of four rabbits each were injected via the ear vein with either \textsuperscript{99}Tcm-liposomes, \textsuperscript{99}Tcm-WBC, \textsuperscript{111}In-liposomes or \textsuperscript{111}In-WBC. The rabbits were immobilized and placed prone on a single-head gamma camera equipped with a parallel-hole, low-energy (\textsuperscript{99}Tcm studies) or medium-energy (\textsuperscript{111}In studies) collimator (Siemens Orbiter, Siemens Inc, Hoffmann Estate, IL). Each rabbit was imaged at 5 min, 1 h, 4 h, 8 h, 20 h and 48 h (\textsuperscript{111}In only) after injection. Images (100,000 counts per rabbit) were obtained and stored in a 256 x 256 matrix.

The scintigrams were analysed by drawing regions of interest over the abscess, over the non-infected contralateral calf muscle (used as a background region), over the heart (representing blood pool activity), the lung (labelled leukocytes) and over the whole animal. Whole-body retention, blood clearance, lung clearance, abscess-to-background ratios and percentage activity remaining in the abscess (abscess-to-whole body ratio) were calculated.

After acquiring the final images, the rabbits were sacrificed with an overdose of sodium phenobarbital and biodistribution was determined. Blood was obtained by cardiac puncture. Tissues (infected left calf muscle, right calf muscle, liver, spleen, kidney, duodenum, right femur and bone marrow from the right femur) were dissected, weighed and their activity was measured in a shielded well-type gamma counter. To correct for physical decay and to permit calculation of the uptake of the radiopharmaceuticals in each organ as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results were expressed as

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{(A) \textsuperscript{111}In-WBC and (B) \textsuperscript{111}In-liposomes 48 h post-injection; (C) \textsuperscript{99}Tcm-WBC and (D) \textsuperscript{99}Tcm-liposomes 20 h post-injection. In all images, the S. aureus abscess can be clearly seen (arrow). For all four radiopharmaceuticals, spleen (WBC > liposomes), liver and bone marrow uptake can be visualized. The \textsuperscript{99}Tcm-labelled preparations show renal uptake and both liposome products residual blood pool activity (heart). Gut activity is only apparent after injection of \textsuperscript{99}Tcm-WBC.}
\end{figure}
percent injected dose per gram (%ID g⁻¹). Abscess-to-muscle, abscess-to-blood and abscess-to-bone marrow ratios were calculated.

**Statistical analysis**

All mean values are given as %ID g⁻¹ or ratios ± one standard error of the mean (s.e.m.). Statistical analysis was performed using one-way analysis of variance (ANOVA). The level of significance was set at P < 0.05.

**Results**

Figure 1 shows images recorded at 48 h (¹¹¹In label) and 20 h (⁹⁹Tc⁹⁹m label) post-injection. The abscess can be clearly seen with all four radiopharmaceuticals. Quantitative analysis of the scintigraphic data revealed very similar whole-body retention over time of ¹¹¹In-WBC and ¹¹¹In-liposomes (Fig. 2A). The ⁹⁹Tc⁹⁹m preparations cleared significantly faster from the body than the ¹¹¹In-labelled preparations (after 1 h post-injection, P < 0.05).

From 8 h post-injection onwards, the whole-body retention of ⁹⁹Tc⁹⁹m-WBC was significantly lower than that of ⁹⁹Tc⁹⁹m-liposomes. In contrast, blood clearance of both liposome formulations was significantly slower than the blood clearance of labelled WBC (P < 0.001 at 1 h post-injection and all later time points; see Fig. 2B). There was no difference between ¹¹¹In- and ⁹⁹Tc⁹⁹m-liposome blood clearance, nor between ¹¹¹In- and ⁹⁹Tc⁹⁹m-WBC blood clearance. While both liposome formulations showed low pulmonary activity, both ¹¹¹In- and ⁹⁹Tc⁹⁹m-labelled WBC showed initial margination in the lungs (image not shown). As shown in Fig. 2C, after the initial entrapment in the lungs, fast clearance of the labelled cells from the lungs into the circulation was observed with a first half-life of less than 1 h. From 4 h post-injection onwards, the relative pulmonary uptake of ⁹⁹Tc⁹⁹m-WBC was significantly lower than that of ¹¹¹In-WBC (P < 0.05).

Figure 3A shows abscess uptake over time as determined scintigraphically. At 4 h post-injection, uptake of ¹¹¹In-WBC was significantly higher than that of the other three radiopharmaceuticals (P < 0.05). At later time...
points, uptake of $^{111}$In-WBC, $^{111}$In-liposome and $^{99m}$Tc-liposome in the abscess was similar. In contrast, at 20 h post-injection, uptake of $^{99m}$Tc-WBC was significantly lower than the uptake of the other three radiopharmaceuticals ($P < 0.05$). The abscess-to-background ratios (Fig. 3B) and the remaining activity in the abscess (Fig. 3C) showed a similar pattern to the absolute abscess uptake: initial high values for $^{111}$In-WBC, a more gradual increase over time of the liposome preparations to the level of $^{111}$In-WBC and persistently low values for $^{99m}$Tc-WBC ($P < 0.05$).

The biodistribution data (see Table 1) confirmed the scintigraphic data obtained at 48 h ($^{111}$In-labelled preparations) and 20 h ($^{99m}$Tc-labelled preparations) post-injection. Abscess uptake and abscess-to-muscle ratios were high for both $^{111}$In-WBC and $^{111}$In-liposomes. Abscess uptake of $^{99m}$Tc-liposomes was higher than that of $^{99m}$Tc-WBC ($0.11 \pm 0.01$ vs $0.03 \pm 0.003$ %ID g$^{-1}$; $P < 0.01$), as were the abscess-to-muscle ($P < 0.05$) and abscess-to-bone marrow ratios ($P < 0.05$). The low blood levels of $^{99m}$Tc-WBC resulted (despite lower abscess uptake) in relatively high abscess-to-blood ratios compared with $^{99m}$Tc-liposomes ($P < 0.01$). Both the $^{111}$In- and the $^{99m}$Tc-labelled liposomes showed higher blood levels than the $^{111}$In- and $^{99m}$Tc-WBC ($P < 0.01$ and $P < 0.001$, respectively). $^{111}$In-WBC and $^{111}$In-liposome uptake was very similar in all organs except the spleen, where it was markedly higher for $^{111}$In-WBC ($3.25 \pm 0.12$ vs $0.70 \pm 0.07$ %ID g$^{-1}$; $P < 0.001$). Although less striking than for the $^{111}$In-labelled products, splenic uptake of $^{99m}$Tc-WBC was higher than that of $^{99m}$Tc-liposomes ($0.66 \pm 0.14$ vs $0.35 \pm 0.07$ %ID g$^{-1}$; $P < 0.05$). Lung and duodenal uptake of $^{99m}$Tc-liposomes were higher than those of $^{99m}$Tc-WBC ($P < 0.01$).

**Discussion**

StealthR liposomes labelled with either $^{111}$In or $^{99m}$Tc depicted focal abscess as well as $^{111}$In-labelled leukocytes. Abscess accumulation of $^{111}$In-WBC was initially fast and high compared with both liposome formulations. However, by 4 h post-injection this difference had
Comparison of labelled liposomes and leukocytes

Table 1. Mean (± s.d.) biodistribution data (%ID g⁻¹) and ratios for ¹¹¹In-labelled leukocytes and Stealth® liposomes (48 h post-injection) and for ⁹⁹Tcm-labelled leukocytes and Stealth® liposomes (20 h post-injection).

<table>
<thead>
<tr>
<th></th>
<th>¹¹¹In-WBC</th>
<th>¹¹¹In-liposomes</th>
<th>⁹⁹Tcm-WBC</th>
<th>⁹⁹Tcm-liposomes</th>
</tr>
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<tbody>
<tr>
<td>Abscess</td>
<td>0.14 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>0.03 ± 0.003</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.005 ± 0.001</td>
<td>0.008 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>Blood</td>
<td>0.18 ± 0.01</td>
<td>** 0.31 ± 0.03</td>
<td>** 0.04 ± 0.005</td>
<td>** 0.27 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.10 ± 0.02</td>
<td>— 0.11 ± 0.03</td>
<td>** 0.04 ± 0.001</td>
<td>** 0.08 ± 0.005</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.25 ± 0.12</td>
<td>** 0.70 ± 0.07</td>
<td>* 0.66 ± 0.14</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>0.24 ± 0.02</td>
<td>— 0.20 ± 0.03</td>
<td>— 0.10 ± 0.02</td>
<td>— 0.13 ± 0.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.15 ± 0.01</td>
<td>— 0.11 ± 0.01</td>
<td>— 0.13 ± 0.02</td>
<td>— 0.15 ± 0.01</td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.02 ± 0.005</td>
<td>— 0.04 ± 0.01</td>
<td>** 0.01 ± 0.001</td>
<td>** 0.04 ± 0.005</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.34 ± 0.02</td>
<td>— 0.36 ± 0.07</td>
<td>— 0.18 ± 0.03</td>
<td>— 0.19 ± 0.02</td>
</tr>
<tr>
<td>Femur</td>
<td>0.02 ± 0.005</td>
<td>— 0.02 ± 0.005</td>
<td>— 0.007 ± 0.001</td>
<td>— 0.01 ± 0.002</td>
</tr>
<tr>
<td>Abscess-to-muscle ratio</td>
<td>29.9 ± 9.8</td>
<td>20.9 ± 3.3</td>
<td>24.2 ± 2.7</td>
<td>* 38.6 ± 6.7</td>
</tr>
<tr>
<td>Abscess-to-blood ratio</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>** 0.4 ± 0.1</td>
</tr>
<tr>
<td>Abscess-to-bone marrow ratio</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>* 0.6 ± 0.1</td>
</tr>
</tbody>
</table>

—, Not significant; ** P < 0.01; *** P < 0.001.

disappeared. The Stealth® liposomes labelled with either ⁹⁹Tcm or ¹¹¹In showed similar abscess uptake on scintigraphy. In previous studies using a rat model of infection, absolute abscess uptake of liposomes appeared to be higher than in the present study [9, 10]. However, the present study was not only performed in a different species, but also the inflammatory response was much lower in the rabbits employed. We were forced to use relatively low S. aureus doses (approximately 4 x 10⁸ CFU per kg body weight in rabbits vs 5 x 10⁸ CFU per kg body weight in rats), since a higher bacterial load causes sepsis and subsequent mortality. In contrast to the other three formulations, ⁹⁹Tcm-WBC showed relatively low abscess uptake. This could not be explained by the poor quality of the radiopharmaceutical, since (for both ¹¹¹In-WBC and ⁹⁹Tcm-WBC) the leukocytes were morphologically intact and the two in vivo quality requirements were met: fast pulmonary transit and low liver uptake [18].

With regard to the kinetics of the radiopharmaceuticals, both the label and the pharmaceutical formulation (cell or liposome) played an important role. Whole-body retention was mainly determined by the radiolabel and the labelling method. In contrast to the ¹¹¹In formulations, which were totally retained in the body, the ⁹⁹Tcm-labelled agents showed considerable excretion. There was significantly less ⁹⁹Tcm excretion when labelled liposomes (10% ID over 20 h) were used compared with ⁹⁹Tcm-WBC (30% ID over 20 h). This could be explained by the purification procedure of the ⁹⁹Tcm-liposomes, which removes the majority of unbound ⁹⁹Tcm-HMPAO (resulting in more than 95% radiochemical purity of the ⁹⁹Tcm-liposomes). ⁹⁹Tcm-WBC can only be purified by repeated washing of the cell suspension with 5% glucose solution, while gel filtration is impossible. In contrast to whole-body retention, blood clearance of the radiopharmaceuticals was determined by the pharmaceutical formulation: both leukocyte preparations cleared significantly faster than the labelled liposomes, resulting in relatively lower background activity of the labelled leukocytes, especially at the earlier time points.

The most striking difference in biodistribution between leukocytes and liposomes was the relatively low splenic uptake of the latter. In humans, this would not only improve image quality in the upper abdomen, but would also reduce the radiation burden to the spleen and long-living T-lymphocytes, especially when using ¹¹¹In [19]. In liver, kidney and bone marrow, uptake of the labelled WBC and liposomes was similar. For the ⁹⁹Tcm preparations, lung uptake of liposomes was higher than that of leukocytes. This was most probably related to the higher blood level of ⁹⁹Tcm-liposomes. This may also have been the case for duodenal wall activity, especially since the ⁹⁹Tcm-liposome images revealed a much clearer picture of the abdomen than the ⁹⁹Tcm-WBC images. Similar to the lower renal excretion rate of ⁹⁹Tcm-liposomes, compared with ⁹⁹Tcm-WBC, the lower intestinal activity could again be explained by the extra purification required to remove non-liposomal ⁹⁹Tcm-HMPAO.

When comparing the labelled Stealth® liposomes with non-PEGylated liposomes, liver and spleen uptake was much lower than that of non-PEGylated larger liposomes [20, 21]. Also, bone marrow uptake is lower for Stealth® liposomes. Blood clearance and uptake of Stealth® and non-PEGylated ⁹⁹Tcm-labelled liposomes in

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other organs are similar [20]. Whole-body excretion of 
radioactivity is also similar for both formulations. Over-
all, targeting of the infectious focus with $^{99m}$Tc-$\text{Stealth}^R$
liposomes is at least as good as, or even better than, that
of $^{99m}$Tc-$\text{non-PEGylated liposomes}$ [10, 20].

Given the similar in vivo characteristics of labelled
liposomes and labelled leukocytes, the advantage of the
liposome formulation would be the continuous avail-
ability of a high-quality, $^{99m}$Tc-labelled radiopharma-
ceutical that can be prepared easily from a lyophilized
kit without the need to handle blood. In patient care,
$^{99m}$Tc-labelled liposomes would provide an attractive
alternative to the laborious method of labelled leuko-
cytes, especially in acute infectious disease.

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mark of Liposome Technology, Inc., Menlo Park, CA,
USA.

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