In Vivo ESR Studies on Subcutaneously Injected Multilamellar Liposomes in Living Mice

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Purpose. An innovative, noninvasive, low-frequency electron spin resonance (ESR) spectroscopy method was applied and adapted to investigate the integrity of multilamellar liposomes from hydrogenated phospholipids after subcutaneous injection in living mice. Moreover, the fate of the injected liposomal preparations was examined, as well as the possibility to achieve a depot effect.

Methods. Highly concentrated solutions of the spin probe 2,2,6,6-tetramethyl-4-trimethylammoniumpiperidine-1-oxyl-iodide (CAT-1; 138 mM) were encapsulated in liposomes. They were characterized by laser diffraction, and the liberation of spin probe was investigated by ESR spectroscopy.

Results. Line shape changes allowed the differentiation between encapsulated and released CAT-1 after subcutaneous injection of liposomes. Multilamellar liposomes form a local depot at the site of injection. A sustained release of the spin probe from the depot was monitored by means of ESR. Whereas 40% of the spin probe was released within the first 96 h after administration, 60% remained in intact liposomes under the skin. No depot formation could be observed after injection of CAT-1 solutions, but a fast signal decrease due to systemic distribution and bioreduction of the nitroxide spin probe.

Conclusions. Noninvasive analysis of liposomal integrity in living animals was successfully accomplished using a new L-Band ESR spectroscopy method. The liberation of CAT-1 from liposomes *in vitro* and *in vivo* was monitored by changes in the lineshape of ESR spectra and Heisenberg spin exchange. The significance of liposomal integrity for the formation of a localized drug depot effect was proved.

KEY WORDS: *in vivo* ESR; liposomes; spin exchange, subcutaneous.

INTRODUCTION

Following former in vivo electron spin resonance (ESR) studies on pharmacokinetics and metabolism of parenteral lipid emulsions in living mice (1), low frequency ESR spectroscopy was applied and adapted to investigate the integrity of multilamellar liposomes after subcutaneous injection in mice. Liposomes have received considerable interest as vehicles for drug targeting to the lymphatic system (2). Subcutaneous injection has been the route of administration most extensively investigated for this purpose (3). The targeting of the lymph nodes is of particular interest for the treatment with antitumor, antibacterial, and antiviral drugs (4). Furthermore, the use of subcutaneously administered liposomes in the field of vaccination and rheumatism, with the aim of a prolonged release of antigenes and the formation of a local drug depot, is also in the focus of interest (5,6). However, many drugs have severe tissue damaging effects, (i.e., the vesicant properties of antineoplastics). Therefore, the local administration of the free drugs is not feasible. An advantage of liposomes relates to the observation that the entrapment of highly irritating drugs in liposomes protects surrounding tissue from the direct cytotoxic effect of the drug after s.c., i.d., i.p., and i.m. injection (7). This effect is related to the reduced amount of the free drug at the site of injection. Compared to other formulations, the encapsulation of drugs in multilamellar or multivesicular liposomes leads to a sustained release of the drugs, which can cover days or weeks if the liposomes are administered subcutaneously (8,9,10). The drug release can be modified by the composition of the membrane and is significantly enhanced from liposomes with membranes in the "fluid-state" compared to those in the "gel-state" (11). On the other hand, the size of the liposomes decides on their uptake to the lymphatic system and therefore the distribution in the body (4). Depot formulations on the basis of liposomes proved to be especially suitable for the administration of highly potent drugs such as cytostatics, peptides (i.e., interferone) and analgesics. Corresponding formulations have been already tested in clinical trials or have been permitted for usage (12).

Previous reports have shown that ESR spectroscopy provides information about the physical properties of liposomes; that is, membrane fluidity (13,14) and the pharmacokinetics of drug delivery systems administered to living animals (1,15), respectively. Therefore, in the current report, an ESR spectroscopy method has been described for the noninvasive study of liposomal integrity after their subcutaneous injection in living mice. It will be shown that this method can be used to distinguish between encapsulated spin probes and those released from the liposomes after administration. Furthermore, it was investigated if the multilamellar liposomes used led to the formation of a local depot at the site of injection. The importance of liposomal integrity for the depot effect of liposomes was another subject of interest. Finally, the influence of back-up materials in the hydrogenated egg lecithin Presome C1 on the release of the spin probe CAT-1 from the aqueous interior was investigated.

MATERIALS AND METHODS

Materials

The liposomal lipid systems Presome C1 (hydrogenated egg yolk lecithin/cholesterol; 1 mol:1 mol) and Presome PPG-I (dipalmitoylphosphatidylcholin/cholesterol/dipalmitoylphosphatidylglycerol; 1 mol:0.2 mol) were donated by Nippon Fine Chemical Co., Ltd. (Osaka, Japan). The spin probe 2,2,6,6-tetramethyl-4-trimethylammoniumpiperidin-1-oxyl-iodide (CAT-1) was purchased from Aldrich (Gillingham, UK). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma (Poole, UK), sodium ascorbate from Fluka (Seelze, Germany), halothane Hoechst, used for anesthesia from Hoechst (Frankfurt, Germany), and potassium hydrogenphosphate, disodium hydrogenphosphate, and so-

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dium chloride from Sigma (Seelze, Germany). Female hairless mice (HR-1) were purchased from Kyudo Animal Co. Ltd. (Kumamoto, Japan).

Liposome Preparation

Multilamellar vesicles (MLV) were prepared from the lipid mixtures Presome C1 or Presome PPG-I. The final lipid concentration was 100 mg/ml. An isoosmolar solution of the spin probe CAT-1 (138 mM), which does not penetrate the liposomal membrane easily due to its charge, was incorporated into the vesicles during hydration (1 h). Before and after hydration, the lipids were dispersed in the aqueous media by using a minishaker. The liposomes were precipitated by centrifugation at 3500 rpm. Non-encapsulated spin probe was removed by washing with isoosmolar phosphate buffer (pH 7.4) until the concentration was below 2%.

Liposome Characterisation

The size of the liposomes was characterised by laser diffraction on a Coulter 230 (Coulter Electronics, Buckinghamshire, UK) with a measuring range from 40 nm to 2000 μm, thus giving the volume distribution, and the frequency distribution of the vesicle diameter was determined.

In Vitro ESR Measurement by X- and L-Band ESR Spectroscopy

In order to determine the concentration-depending lineshape of the ESR-spectra of the spin probe CAT-1, 50 μl of 12 solutions with concentrations in the range between 1 and 250 mM were transferred to disposable capillary tubes and their X-band ESR-spectra were recorded at 32°C with a ESR 300 (ZWG Adlershof, Berlin, Germany) with the following parameters: 9.5 GHz microwave frequency, 2 mW microwave power, 0.1 mT modulation width, 337 mT B_0-field, 8.0 mT sweep, 1 min sweep time.

Encapsulation efficiency was determined by reducing the non entrapped spin probe with sodium ascorbate and comparing the ESR signal intensity after double integration with the one of the original preparation.

The liberation rate of the spin probe was determined by transferring 100 μl of the liposomal preparation to 150 μl of an iso-osmolar sodium ascorbate solution (pH 7.4) and phosphate buffer (pH 7.4), respectively. Both solutions were 139 mM. Fifty microliters of the resulting mixtures were transferred to disposable capillary tubes, and X-band ESR spectra were recorded. Signal intensities were determined after double integration. The share of the spin probe liberated into the buffer has been computed from spectra.

In Vivo ESR Measurement by L-Band ESR Spectroscopy

Female hairless mice (HR-1) weighing about 20 g were anesthetized with halothane, and 60 μl of the liposomal preparations and a CAT-1 solution were injected in the back, respectively. The still anesthetized mice were placed in a loop-gap resonator of an L-band ESR spectrometer, and spectra were recorded either over the injection site or the chest of the animals with a JES-RE-1L (JEOL, Tokyo, Japan) using the following parameters: 1.1 GHz microwave frequency, 3 mW microwave power, 0.2 mT modulation, 44.0 mT B_0-field, 12.0 mT B_0-scan, 120 s sweep time. DPPH served as standard and was fixed under the body of the animals at the site of injection and the chest, respectively.

Analysis of *in Vitro* and *in Vivo* ESR Measurements

The obtained spectra were assessed concerning their dynamic parameters. In addition to any of the diffusional models, as represented by the rotational correlation time τ_R, it is possible to account approximately for the effects of isotropic Heisenberg spin exchange between probe molecules with a rate specified by ω_{SS} (16–18). The integral share and the linewidth (ΔH) of ESR spectra were determined with a computer program for the calculation of ESR spectra (19). Quantitative analysis was performed by double integration of ESR spectra. The rate constants of reduction and spin probe liberation were determined by fitting procedures.

RESULTS

Lineshape of ESR Spectra

If high concentrations of the spin probe CAT-1 are encapsulated in liposomes and the nonencapsulated parts are removed from the preparation, one can distinguish between encapsulated spin probe and that released from the liposomes. This is possible because ESR spectra of solutions with high concentrations of nitroxyl spin probes are broad single line signals, whereas free nitroxyl spin probes released from liposomes are detected as sharp triplet lines due to their low concentration in the surrounding media. Figure 1A shows the ESR spectra of six different CAT-1 concentrations. The transition of the spectrum from a sharp triplet line to a broad single line signal with increasing concentrations of CAT-1 is due to dipol-dipol interactions and increasing spin exchange between the molecules. The spin exchange has been determined ranging from 2.00×10^{-7} s at a concentration of 1 mM to 1.35×10^{-9} s at a concentration of 250 mM (Fig. 1B). Simultaneously, the rotational correlation times increase from 4.21×10^{-10} s to 1.10×10^{-8} s due to the increasing viscosity of the solutions.

At low concentrations of CAT-1, the typical three-line signal of a spin probe can be observed. Increasing the concentration up to 50 mM still gives a three-line signal but with increasing linewidths. Above that concentration the signal shape changes gradually to a one-line signal due to spin exchange. Further increase in concentration exceeding 138 mM now shows a decrease in linewidth of the one-line signal. Therefore not only a dilution of the spin probe solution can be monitored by analyzing the linewidth but also the loss of water from the inside of the liposomes. General considerations of spin exchange are described in (20).

Liposome Characteristics

The commercially available liposomal systems Presome C1 and Presome PPG-I allow a fast production of multilamellar vesicles by hydrating the phospholipids under stirring with the solute that is supposed to be entrapped. Laser diffraction data of liposomes that were obtained out of the production process are presented in Table I. The distribution of liposome sizes made from Presome PPG-I shows two maxima at 0.432 μm and 1.919 μm, respectively. The majority of the particles (99%) were smaller than 2.540 μm. The size of liposomes from Presome C1 also showed a bimodal distribution but with their maxima at 0.755 μm and 1.919 μm, respectively. Ninety-nine percent of the particles had diameters below 2.933 μm.

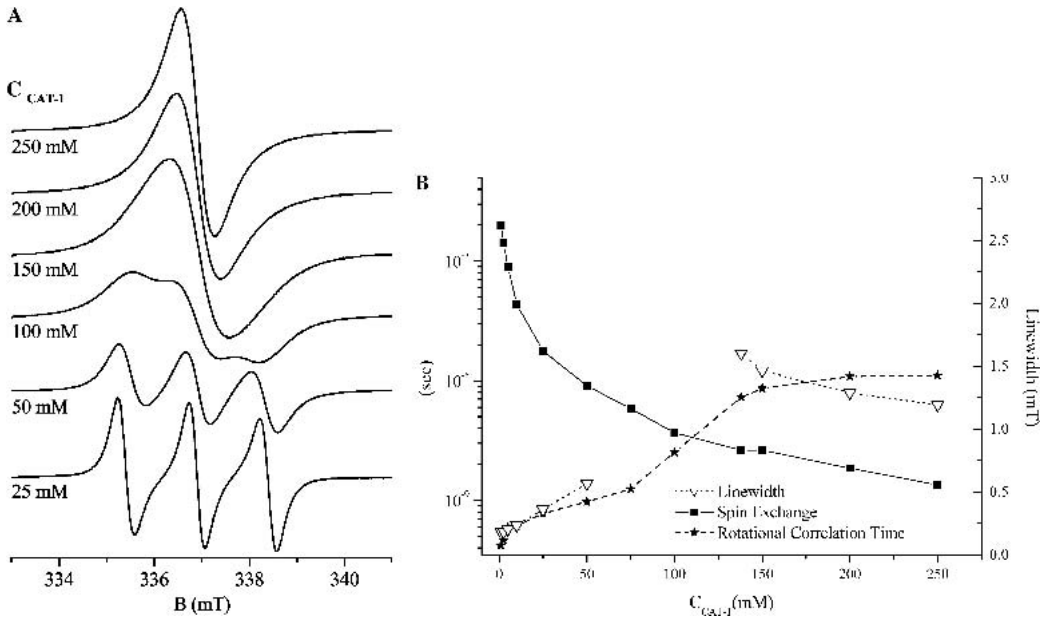

Fig. 1. X-Band ESR spectra of the spin probe CAT-1 (A), Heisenberg spin exchange and rotational correlation times (B), depending on the concentration of the spin probe CAT-1.

According to the laser diffraction data 40% of the liposomes made from Presome PPG-I have diameters below 0.452 μm. According to Oussoren *et al.* (4), liposomes with diameters between 0.04 μm and 0.40 μm are gradually taken up into the lymphatic system and therefore distributed in the body. The uptake rate to the lymphatic system depends on the particle size. Only 10% of the liposomes made from Presome C1 have diameters below 0.40 μm, thus only a small amount of those particles will be distributed by the lymphatic system.

Even though that there are differences in the particle sizes between liposomes made from Presome C1 and Presome PPG-I, the encapsulation efficiency of the spin probe CAT-1 in the multilamellar liposomes (i.e., the fraction of the spin probe that is localized inside the liposomes, related to the overall amount of spin probe used for the preparation; that is 53% in case of Presome C1 and 52% in case of Presome PPG-I), which was determined after reduction of the non-entrapped spin probe with sodium ascorbate, is almost the same.

Table I. Selected Properties of Liposomes Made from Presome C1 and Presome PPG-I

	Presome C1	Presome PPG-I
Diameter of liposomes (μm) and frequency distribution		
5%	0.343 ± 0.230	0.111 ± 0.011
10%	0.452 ± 0.141	0.154 ± 0.019
50%	1.051 ± 0.023	0.602 ± 0.050
90%	2.331 ± 0.384	2.053 ± 0.012
95%	2.576 ± 0.438	2.225 ± 0.010
99%	2.933 ± 0.523	2.540 ± 0.007
Encapsulation efficiency (%)	51.8 ± 0.9	52.8 ± 0.1
First-order rate constant of liberation		
In sodium ascorbate buffer (h^{-1})	0.010	0.012
In phosphate buffer (h^{-1})	4.46×10^{-5}	6.61×10^{-5}

Liberation of CAT-1 from Liposomes

The liberation process of CAT-1 from the liposomes to the outer medium has been investigated at 32°C, in order to obtain information on the changes that the lineshape of ESR spectra undergoes while the spin probe is released. Because nitroxides are reduced to the corresponding diamagnetic hydroxylamines inside the body by reducing agents such as ascorbic acid and thioredoxin reductase (21,22), the liberation process was investigated both in sodium ascorbate solution and phosphate buffer, respectively.

The addition of sodium ascorbate to the liposomal formulations leads to a reduction of the released CAT-1. Due to the decreasing radical concentrations in the inner phase of the liposomes, the spin-spin interactions between the nitroxide radicals are also decreasing. As an effect a transition of the corresponding ESR spectra from a broad single line signal (ΔH 1.60 mT) to a triplet line signal (ΔH 0.18 mT) occurs (Fig. 2A). The intensity of the latter one decreases until only 1% of the initial concentration remains in the formulation after 168 h.

Due to the liberation of the spin probe a concentration gradient inside of the liposomes occurs. This can be proved by the simulation of the experimental spectra recorded after 10 and 24 h, respectively. The simulation indicates that the recorded spectra consist of a superimposition of three single spectra namely a singlet (ΔH 1.62 mT), a triplet with broad (ΔH 0.81 mT), and a triplet with narrow single lines (ΔH 0.34 mT), respectively. These spectra are assigned to three different concentrations inside the liposomes. While the originally encapsulated solution leads to a single broad line, the triplet with sharp narrow lines proves the existence of areas with low concentrations of the spin probe within. The third spectrum contributing to the experimental spectrum can be assigned to an intermediate concentration. The result makes clear that CAT-1 is released from the liposomes by permeation through the liposomal membrane and not by the disintegration of the

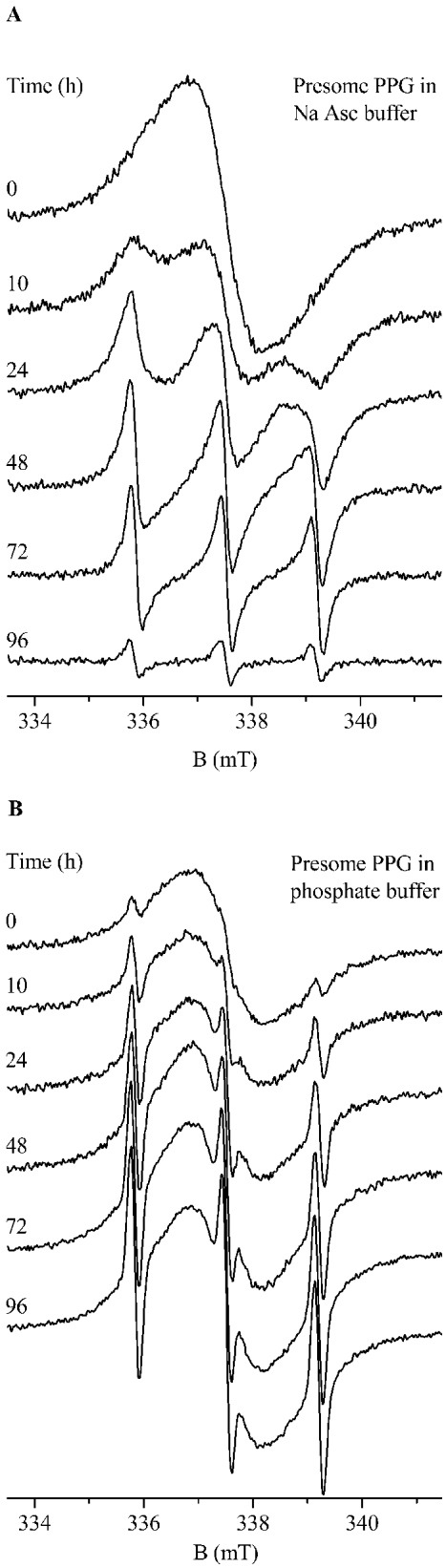

Fig. 2. L-band ESR spectra of CAT-1 encapsulated in Presome PPG-I liposomes depending on the time after the addition of sodium ascorbate (A) and phosphate buffer (B) to the liposome preparations, respectively.

particles. Otherwise the signal intensity would decrease without a transition of the lineshape.

Spectra of CAT-1 encapsulated in liposomes that were incubated in phosphate buffer are presented in Fig. 2B. The spectra prove the possibility to distinguish between encapsulated CAT-1 (singlet, ΔH 1.62 mT) and the spin probe released to the dispersion media of the liposomes (triplet, ΔH 0.17 mT). In the course of the liberation, the amplitude of the triplet signal is increasing. In contrast to the liposomes, that were incubated in sodium ascorbate solution, the lineshape of the originally recorded ESR spectra largely remains unchanged throughout the experiment. The analysis of the liberation constant shows, that the liberation of the spin probe from liposomes that were incubated in sodium ascorbate solution is accelerated, in comparison to those that were incubated in phosphate buffer (Table I). This can be explained by an elevated concentration gradient which is due to the continuous reduction of the spin probe outside of the liposomes. Contrary the spin probe molecules, which were released from liposomes in phosphate buffer, they remain at the surface of the liposomes due to their opposite charge. Therefore the concentration gradient as well as the liberation constant decrease.

The results show that encapsulated and non encapsulated CAT-1 can be distinguished due to the lineshape of the ESR spectra. Furthermore, the method gives evidence whether the spin probe is liberated by diffusion or by disintegration of the liposomes. The enhanced liberation of CAT-1 in sodium ascorbate solution indicates that its liberation will also be accelerated if reducing factors (i.e., enzymes) interact with subcutaneously administered liposomes.

In Vivo ESR Measurements

After the subcutaneous injection of the liposomally encapsulated spin probe in hairless mice, the ESR spectra recorded over the site of injection were a superimposition of three single components, namely a triplet with a low signal intensity (ΔH 0.24 mT), a signal with a narrow single line (ΔH 0.21 mT), and a broad singlet (ΔH 1.54 mT) with a small amplitude (Fig. 3). While the narrow single line was assigned to DPPH, which was used as a standard in order to calibrate the accuracy of the signal intensity, the encapsulated parts of the spin probe CAT-1 were detected as a broad single line. In the course of 96 h, ESR spectra could be recorded over the site of injection showing these two components and therefore prove the integrity of the liposomes under the skin. The third component of the ESR spectra recorded at the beginning of the investigation, namely the three-line signal, was assigned to the nonencapsulated parts of the spin probe and to the parts released during the subcutaneous injection, respectively. The transition to a three-line signal is caused by the dilution of the spin probe and therefore by the reduction of spin-spin and dipole-dipole interactions when it is released from the liposomes. Within the first hours after injection, the small shares of the nonencapsulated spin probe were reduced to such an extent that a differentiation between the signal and the noise of the ESR spectra was difficult. In one of the investigated formulations, no triplet compound could be observed already after 2 h (Fig. 3A). The fast reduction due to bioreduction of the nonencapsulated parts of the spin probe gives evidence for the importance of the vesicle structure for the depot effect

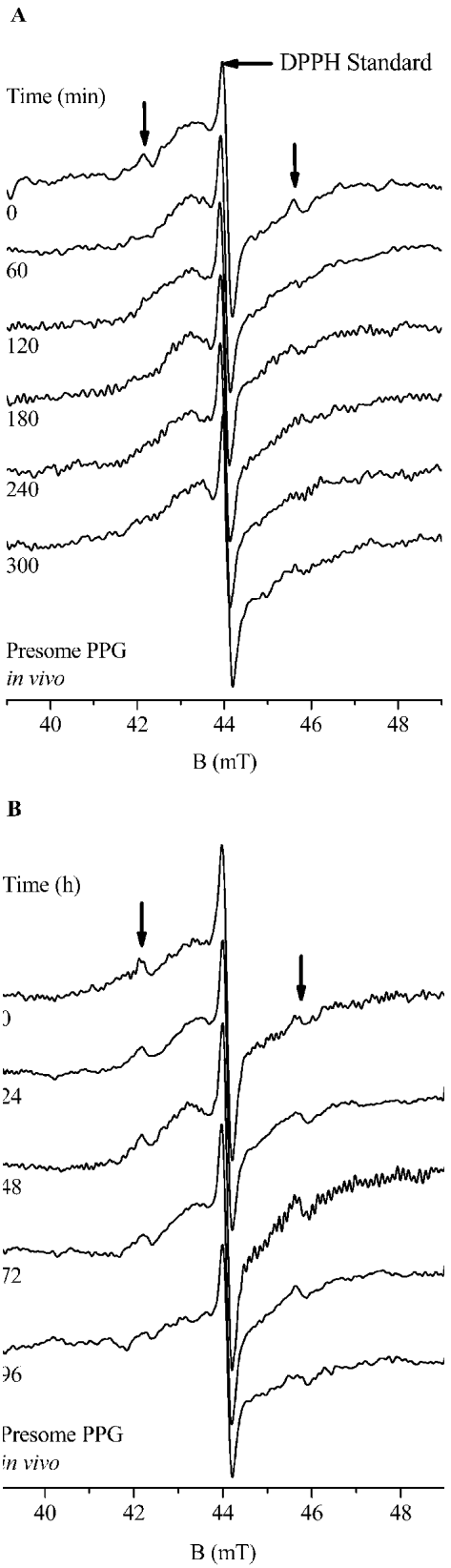

Fig. 3. Two L-band *in vivo* ESR experiments (A and B) involving CAT-1 (138 mM) encapsulated in Presome PPG-I liposomes after subcutaneous injection in hairless mice with DPPH as external standard, in dependency on the time. The low and high field peaks of the three-line signal from released CAT-1 are indicated by arrows.

of liposomes. On the other hand, the triplet signal was clearly detectable in the ESR spectra which were recorded in the time between 24 and 96 h after the subcutaneous injection of the liposomal formulations (Fig. 3B). This proves that there is still a continuous release of the spin probe from the liposomes after 24 h.

After the subcutaneous injection, there were no ESR spectra of CAT-1 recordable over the chest of the animals, that is, we could not observe an uptake of liposomes into the lymphatic system and the systemic distribution of the spin probe.

The determined linewidths and the integral shares of the single components of the recorded ESR spectra remain nearly unchanged over 96 h (Fig. 4).

After the subcutaneous injection of CAT-1 solutions, a fast decrease of the spin probe concentration at the site of injection was registered by the changes of the ESR lineshape (Fig. 5). The ESR spectra recorded directly after the injection were a superimposition of the DPPH signal (ΔH 0.20 mT), a broad single line of the CAT-1 solution (ΔH 1.69 mT), and a three-line signal (ΔH 0.37 mT). The three-line signal could be assigned to the spin probe, which was distributed in the surrounding tissue and therefore underlying a dilution. Within 30 min, the triplet signal became the most dominant component of the recorded spectra (Fig. 5A). The existence of the two singlet components could only be proved by fitting of a calculated spectrum to the recorded spectrum. The contribution of the three-line signal to the experimental spectra increases from 9% directly after the injection to 88% after 60 min (Fig. 6). Simultaneously, the amplitude of the three-line signal increased due to the decreasing spin-spin interactions and the ongoing decrease of the linewidth of the signal. Further on the contribution of the three-line signal decreases until after 180 min, the three-line signal disappears at the injection site. The increase of the relative share of the DPPH signal in the recorded spectra to 100% after 180 min proved the reduction and the systemic distribution of the spin probe, respectively. The systemic distribution could also be observed by means of the ESR spectra, which were recorded over the chest of the animals (Fig. 5B). A three-line signal was detected 15 min after the injection of the spin probe solution next to the single line signal of the DPPH standard. Up to 45 min the amplitude

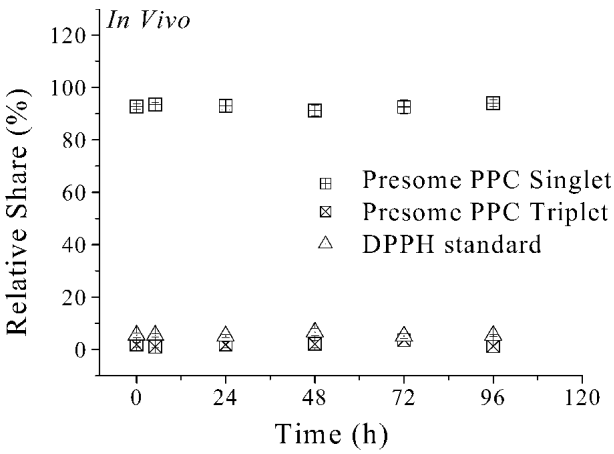

Fig. 4. Relative integral share of the singlet and triplet component of ESR spectra depending on the time after subcutaneous injection of encapsulated CAT-1 in hairless mice ($\bar{x}$ ± range, n = 3).

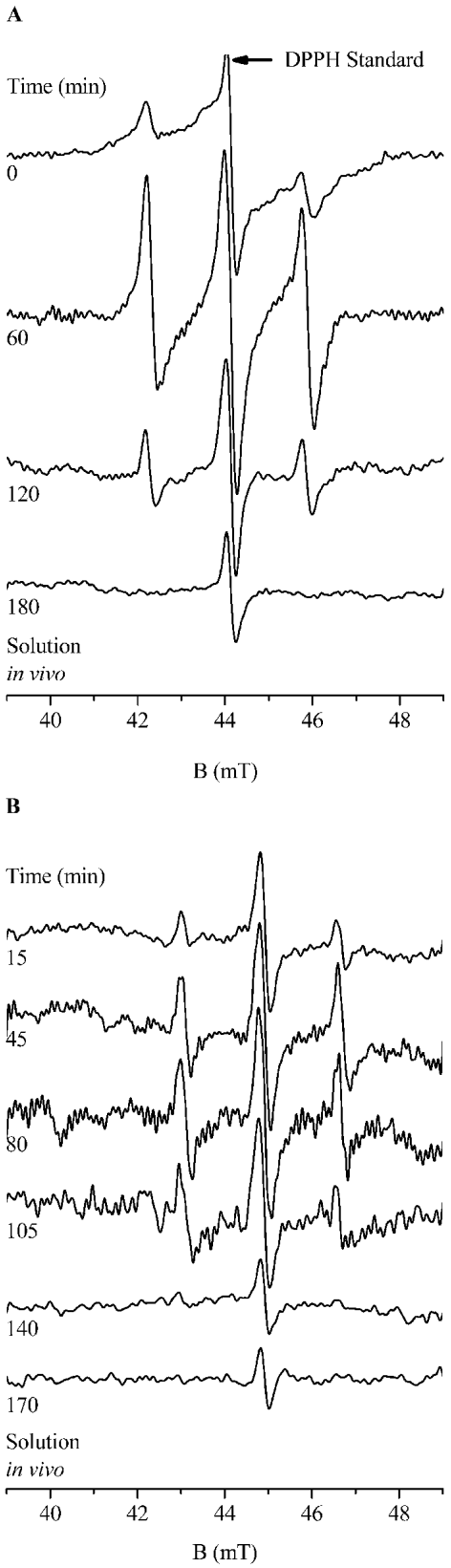

Fig. 5. L-band *in vivo* ESR spectra of CAT-1 (138 mM) in phosphate buffer after subcutaneous injection in hairless mice with DPPH as standard, recorded over the back (A) and the chest (B) in dependency on the time.

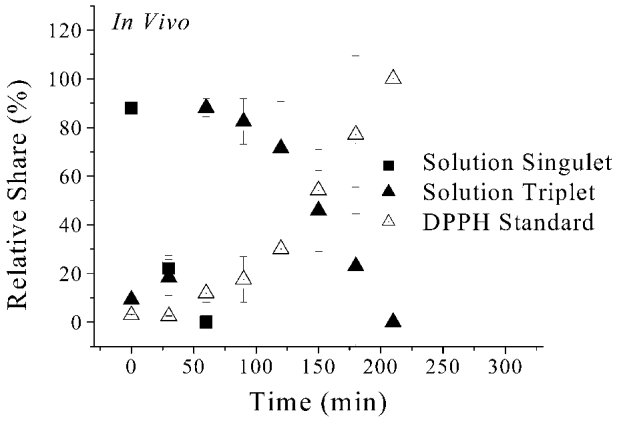

Fig. 6. Relative integral share of the singlet and triplet compound of ESR spectra depending on the time after subcutaneous injection of CAT-1 in hairless mice, with DPPH as standard ($\bar{x}$ ± range, n = 3).

of the three-line signal increased, then remained nearly unchanged in the spectra recorded after 45 and 80 min. After that, the amplitude decreased until after 170 min, the three-line signal diminished. These results prove a fast distribution and reduction of the injected spin probe CAT-1 inside of the animals.

Quantitative Analysis of *in Vivo* ESR Spectra

By means of the quantitative analysis of the *in vivo* ESR spectra, which were recorded over the site of injection, differences between liposomes and solutions can be shown as far as the depot effect and the systemic distribution of the liposomes are concerned. After the injection of the CAT-1 solutions the signal intensity decreased by a first order kinetic (Fig. 7). The rate constant was $^1k = 0.011\ h^{-1}$. The formation of an edema at the site of injection resulted in a decrease of the rate constant of the spin probe reduction ($^1k = 0.004\ h^{-1}$). Therefore, CAT-1 ESR signals were detectable over 300 min. On the other hand the period, while ESR signals were detectable over the chest of the animals, was not influenced.

In contrast to solutions the reduction of the signal intensity in liposomal preparations did not decrease exponentially

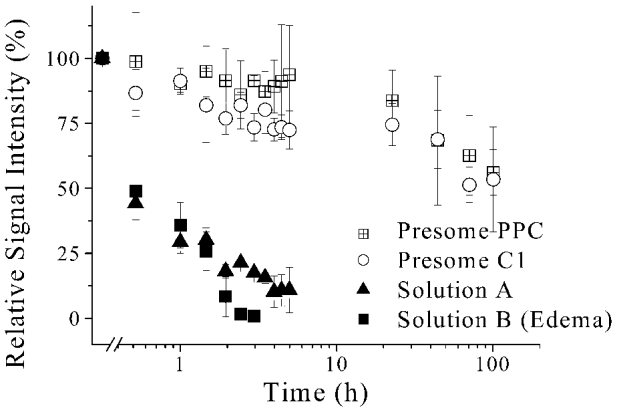

Fig. 7. Signal intensity of CAT-1 depending on the time after subcutaneous injection of Presome C1 liposomes, Presome PPG liposomes, and phosphate buffer in hairless mice, with (solution A) and without (solution B) formation of an edema at the site of injection, respectively ($\bar{x}$ ± range, n = 3).

but linear. The rate constant of the zero order kinetic was ${}^0k = 0.478\%\ h^{-1}$ for Presome C1 liposomes and ${}^0k = 0.441\%\ h^{-1}$ for Presome PPG-I liposomes, respectively. According to the rate constants, the spin probe is not released faster from hydrogenated egg lipids than from hydrogenated semisynthetic lipids. Within 96 h, that were monitored during this study, the signal intensity of the spin probe decreased by 40%. This means that the main part of the spin probe was available over a longer period of time.

DISCUSSION

In this study, the integrity of subcutaneously administered liposomes in living mice was analyzed noninvasively by an innovative method using *in vivo* low-frequency ESR spectroscopy (1,23). The method is based on the possibility to differentiate CAT-1 solutions with high and low concentrations by means of the shape of their ESR signals. Whereas the ESR signals of solutions with high concentrations of CAT-1 are broad single lines due to spin exchange, the diluted solutions of the spin probe show a three-line ESR signal. Therefore, it was possible to observe the release of the spin probe by the change of the lineshape *in vitro* and *in vivo*, respectively. The change of the spectrum from a broad single line into a three-line signal, that occurs in the moment the spin probe is liberated, is caused by a decrease of the Heisenberg spin exchange (Fig. 1). The current method is quite unique and completely different from radioactive tracer methods. The currently applied radioactive tracer methods, which are commonly used for such examinations, are characterized by the enclosure of radioactively marked drug in liposomal preparations and/or marking constituents of the membrane, followed by tracking the distribution process in the body (23). But contrary to the newly developed ESR method, the radioactive signal of the tracers is not inevitably bound to the integrity of the liposomes. The method differs from previously used ESR methods, where solutions with low concentrations of the spin probe are encapsulated, and the existence of an ESR signal in the skin is supposed to indicate the integrity of the particles. By means of the current method, we were able to show that not only an ESR signal remains until 96 h after the subcutaneous injection of the liposomes, but also by the lineshape of the signal that its existence is bound to the integrity of the injected liposomes. These results underline the importance of the liposome integrity for the depot effect. It is also substantiated by the fact that nonencapsulated parts of the spin probe, which were liberated during the injection, are reduced within a few hours before a continuous release of CAT-1 from the spin probe depot starts. The extent, by which liposomes can prolong the presence of the spin probe and therefore of potential drugs at the site of injection, was shown by comparison of the signal intensities after the injection of liposomes and solution, respectively. According to that about 60% of the spin probe remains at the site of injection up to 96 h after the injection when applied in form of liposomes. Whereas a complete distribution in the body and reduction takes place when a spin probe solution was injected.

When liposomes are introduced into any body compartment, essentially two things can happen: 1) liposomal contents may be released, with or without disintegration of the vesicles under the influence of factors in the biologic environment and/or 2) vesicles can be transported through the tissues in circulating lymph fluid or blood where some or all of the dose may be taken up by cells (24). The linewidths and line shapes of the singlet components of the ESR spectra of the liposomal preparations does not alter after injection. This is a hint that the concentration of the CAT-1 solution inside the liposomes under the skin does not significantly change throughout the study. Moreover, a loss of water from the liposomes can be excluded as well as a partial release of the spin probe from the liposomes to the surrounding. The permeation of the spin probe through the liposomal membrane can be also excluded because the analysis of the experimental ESR spectra gives rise to only two species, namely the spectrum of the encapsulated and the spectrum of the released spin probe. A concentration gradient, as it was discussed above for the liberation experiment with ascorbic acid, could not be observed. Thus from the analysis of the experimental spectra, the conclusion can be drawn that the decrease of the spin probe concentration at the site of injection is not related to a release under maintenance of the liposomal integrity. It could be rather related to an uptake and removal of the liposomes by the lymphatic system or a disintegration of the particles due to phagocytosis, adsorption, or fusion of the liposomes with the cells of the surrounding tissue. The signal decrease for formulations made from Presome C1 and Presome PPG-I is nearly identical, which does not speak for a removal of the liposomes from the place of injection. In case of a massive removal of the liposomes by the lymphatic system the rate constants should have been significantly different since in formulations made from Presome PPG-I 40%, and in formulations from Presome C1 only 10% are available for a potential uptake into the lymphatic system, due to particle size as it was discussed by Oussoren *et al.* (3). The typical spectrum of the spin probe CAT-1 cannot be observed in the spectra that were recorded over the chest of the animals, which confirms that the injected liposomes remain at the site of injection. Thus, the decrease of the signal intensity is most probably related to a disintegration of the liposomes in the local depot. Under the given conditions, no differences were detected between the liposomes made from semisynthetic lipids and those from egg lipids concerning the disintegration of the vesicles and spin probe reduction, respectively.

As a conclusion, it can be stated that we successfully accomplished the noninvasive analysis of liposomal integrity in living animals by using a new L-Band ESR spectroscopy method. The liberation of CAT-1 from liposomes *in vitro* and *in vivo* was monitored by changes in the lineshape of ESR spectra and Heisenberg spin exchange. The significance of liposomal integrity for the formation of a localized drug depot effect was proved.

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REFERENCES

1. T. Yamaguchi, S. Itai, H. Hayashi, S. Soda, A. Hamada, and H. Utsumi. In vivo ESR studies on pharmacokinetics and metabolism of parenteral lipid emulsions in living mice. *Pharm. Res.* **13**:729–733 (1996).

2. G. Storm, C. Oussoren, P. A. M. Peeters, and Y. Barenholz. Tolerability of liposomes in vivo. In G. Gregoriadis (ed.), *Liposomes as Drug Carriers*, J. Wiley & Sons, New York, 1988, pp. 345–383
3. C. Oussoren, M. Velinova, G. Scherphof, J. J. van der Want, N. van Rooijen, and G. Storm. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection: IV. Fate of liposomes in regional lymph nodes. *Biochim. Biophys. Acta* **1370**: 259–272 (1998).
4. C. Oussoren, J. Zuidema, D. J. A. Crommelin, and G. Storm. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. *Biochim. Biophys. Acta* **1328**:261–272 (1997).
5. I. Babai, S. Samira, Y. Barenholz, Z. Zakay-Rones, and E. Kedar. A novel influenza subunit vaccine composed of liposome-encapsulted haemagglutinin/neuramidase and IL-2 or GM-CSF. I. Vaccine characterisation and efficacy studies in mice. *Vaccine* **17**:1223–1238 (1999).
6. M. L. Corvo, O. C. Boermann, W. J. G. Oyen, J. C. S. Jorge, M. E. M. Cruz, D. J. A. Crommelin, and G. Storm. Subcutaneous administration of superoxide dismutase entrapped in long circulating liposomes: In vivo fate and therapeutic activity in an inflamation model. *Pharm. Res.* **17**:600–606 (2000).
7. C. Oussoren, W. M. C. Elling, D. J. A. Crommelin, G. Storm, and J. Zuidema. The influence of the route of administration and liposome composition on the potential of liposomes to protect tissue against local toxicity of two antitumor drugs. *Biochim. Biophys. Acta* **1369**:159–172 (1998).
8. S. Mantripragada. A lipid based depot (DepoFoam® technology) for sustained release drug delivery. *Prog. Lipid Res.* **41**:392–406 (2002).
9. M. P. Ramprasad, G. M. Anatharamaiah, D. W. Garber, and N. V. Katre. Sustained-delivery of an apolipoprotein E-peptidomimetic using multivesicular liposomes lowers serum cholesterol levels. *J. Control. Rel.* **79**:207–218 (2002).
10. N. V. Katre, J. Asherman, H. Schaefer, and M. Hora. Multivesicular liposome (DepoFoam) technology for the sustained delivery of insulin-like growth factor-I (IGF-I). *J. Pharm. Sci.* **87**: 1341–1345 (1998).
11. M. L. van Slooten, O. Boerman, K. Romoren, E. Kedar, D. J. A. Crommelin, and G. Storm. Liposomes as sustained release system for human interferon-γ: Biopharmaceutical aspects. *Biochim. Biophys. Acta* **1530**:134–145 (2001).
12. Homepage SkyePharma. http://www. Skyepharma.com, 2002.
13. K. Vrhovnik, J. Kristl, M. Sentjurc, and J. Smid-Korbar. Influence of liposome bilayer fluidity on the transport of encapsulated substance into the skin as evaluated by EPR. *Pharm. Res.* **15**:525–530 (1998).
14. L. Coderch, J. Fonollosa, M. De Pera, J. Estelrich, A. De La Maza, and J. L. Parra. Influence of cholesterol on liposome fluidity by EPR: Relationship with percutaneous absorption. *J. Control. Rel.* **68**:85–95 (2000).
15. L. Honzak, M. Sentjurc, and H. M. Swartz. In vivo EPR of topical delivery of a hydrophilic substance encapsulated in multilamellar liposomes applied to the skin of hairless and normal mice. *J. Control. Rel.* **66**:221–228 (2000).
16. D. J. Schneider and J. H. Freed. Calculating Slow Motional Magnetic Resonance Spectra. In L. J. Berliner and J. Reuben (eds.), *Biological Magnetic Resonance*, Vol. 8., Plenum Press, New York, 1989, pp. 1–76.
17. D. E. Budil, S. Lee, S. Saxena, and J. H. Freed. Nonlinear-least squares analysis of slow-motion EPR spectra in one and two dimensions using a modified Levenberg-Marquardt algorithm. *J. Magn. Reson.* **120**:155–189 (1996).
18. J. H. Freed. Theory of Slow Tumbling ESR Spectra for Nitroxides. In L. J. Berliner (ed.), *Spin Labeling, Theory and Applications*, Academic Press, New York, 1976, pp. 53–132.
19. D. Pfeiffer. Computer program ISOTROP, Federal Institute for Materials Research and Testing, Berlin, Germany.
20. Yu. M. Molin, K. M. Salikhov, and K. I. Zamaraev. *Spin Exchange*, Springer, Berlin Heidelberg, 1980.
21. J. Fuchs. Free radical reduction by thioredoxin reductase. *J. Invest. Dermatol.* **91**:92–93 (1988).
22. J. Fuchs, H. J. Freisleben, M. Podda, G. Zimmer, R. Milbradt, and L. Packer. Nitroxide radical biostability in skin. *Free Rad. Biol. Med.* **15**:415–423 (1993).
23. K.-P. Moll. PhD Thesis, Humboldt-University of Berlin, Berlin, 2004, pp. 161–171.
24. J. Senior. Fate and behaviour of liposomes in vivo: a review of controlling factors. *Crit. Rev. Therapeut. Drug Car. Syst.* **3**:123–193 (1987).