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Interactions of solid lipid nanoparticles with model membranes and leukocytes studied by EPR

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Abstract

Solid lipid nanoparticles (SLN) are colloidal systems which have been proposed for several administration routes. Only limited data are available about the mechanism and rate of interaction of SLN with cells and tissues. The aim of our study was to investigate interactions of SLN with model membranes (liposomes) and cells (leukocytes). SLN dispersions composed of glyceryl tripalmitate, phosphatidylcholine, water, and poloxamer 188 or Tween 20 were prepared by the melt-emulsification process. Spin-labeled phosphatidylcholine (PC(10,3)) and the methylester of doxyl palmitic acid (MeFASL(10,3)) were incorporated into SLN as spin probes (SPs) in order to determine the rate and mechanism of cell interaction by electron paramagnetic resonance (EPR) spectroscopy. Our results indicate that the exchange of SP between SLN and liposomes is much faster for MeFASL(10,3) than for PC(10,3), probably due to the smaller size of the former. In contrast to liposomes, in leukocytes no significant difference in the transfer rates of the two SP was observed after incubation, suggesting that there is an uptake of SLN to leukocytes (endocytosis) although simultaneous SP diffusion is not excluded. The interaction of SLN with leukocytes appears to depend significantly on the stabilizer used. Transfer of PC(10,3) from SLN coated with poloxamer 188 is much faster than from SLN coated with Tween 20.

Keywords: Solid lipid nanoparticles; Interactions; Liposomes; Leukocytes; Electron paramagnetic resonance (EPR)

1. Introduction

Solid lipid nanoparticles (SLN) are colloidal therapeutic systems proposed for several administration routes. They are attractive colloidal drug carriers which combine the advantages of polymeric nanoparticles, liposomes, and fat emulsions but avoid some of their disadvantages (Müller et al., 2000). Drug molecules may be adsorbed on the particle surface, entrapped or dissolved in the solid lipid core or in the outer layer of SLN composed of phospholipid and steric stabilizers (Ahlin et al., 2000a). Distribution depends strongly on the physicochemical characteristics of the drug and components of the SLN. It is most influenced by the partition coefficient of the drug (Ahlin et al., 2000b). Nanoparticles show either almost complete drug release over the first few minutes, or biphasic release with fast drug release during the initial phase, followed by sustained drug release. Rapid release is possibly due to the large surface area and the poor distribution of the drug inside the lipid nanoparticles (zur Mühlen et al., 1998).

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The concept of site-specific drug delivery is an old one, but it is only recently that full consideration has been given to the ways in which this might be achieved in practice, largely due to the advent of the new biosciences. A drug's therapeutic index, as measured by its pharmacological response and safety, relies on the access to and specific interactions of the drug with its candidate receptor, with minimal interaction with non-targeted tissues. Nanoparticulate delivery systems enable the delivery of active compound to the cells of different tissues (Bargoni et al., 1998; Müller and Kreuter, 1999; Yang et al., 1999). In these tissues, the drug can diffuse from carrier system to the site of action after uptake of the particle by a specific cell or by diffusion from the carrier surface into the cell without previous particle internalization. Nevertheless, there are several obstacles to access to the receptor which must be considered, including plasma membranes. The plasma membrane of living cells serves as a dynamic barrier separating the cell interior from the surrounding environment. Transmembrane and intracellular transport plays an essential role in the intracellular activity of drugs. In general there are four main routes by which a substances may cross the membrane barrier: free diffusion, passive transport, active transport, and endocytosis (pinocytosis, phagocytosis) (Alberts et al., 1994).

Limited theoretical data are available about the mechanism and rate of interactions of SLN with cells or tissues, but such data are of vital interest for the design of SLN and their therapeutic function (Fundaro et al., 2000). While most of the challenges relating to targeted drug delivery involve transport processes, little is known how these processes operate in vivo. From the moment that the SLN are administered until the therapeutic effect is observed, specific transport processes are involved. The interactions and processes that determine the ultimate fate of the drug, resulting in a response, occur at membranes or intracellularly.

Electron paramagnetic resonance (EPR) is powerful, sensitive, and versatile technique for probing the structure and organization of supramolecular systems and biomembranes at the molecular level. Detection depends on free radicals, whether formed spontaneously or added to the system. A paramagnetic probe, such as nitroxide, can be used as a model drug, and in such cases EPR can provide important information about the distribution of the spin probe (SP) in SLN and interaction with biological environment. The use of free radicals in biological experiments is limited since nitroxide free radicals are sensitive to reducing or oxidizing agents, pH, and the presence of other radicals, which lead to the formation of non-paramagnetic compounds and consequent decrease or disappearance of the EPR signal (Nordio, 1976).

Although there are several publications on SLN there are still only a few on the interactions of SLN, for example with human granulocytes, murine peritoneal macrophages or cell lines, determined by chemiluminescence or fluorescence (Müller et al., 1997a,b; Bocca et al., 1998). Attention has been focused mainly on the viability of cells after being in contact with SLN or on the effects of entrapped drugs (Migletta et al., 2000). Despite the increasing attention paid to SLN as carrier systems for drug targeting to specific tissue sites in the body, their interactions with different membranes have not been studied in detail.

The aim of this study was to investigate the transport of two model compounds from SLN to model membranes (liposomes) and to living cells. Leukocytes were chosen as cells on the basis of their particle depletion role in tissues and blood. The influence of membrane type and the molecular structure of the SP have been characterized.

2. Materials and methods

2.1. Chemicals

Glyceryl tripalmitate was obtained from Dynasan® 116, Hülls AG, Germany; non-hydrogenated soya bean lecithin (Phospholipon® 80) from Nattermann, Germany; poloxamer 188 (Pluronic® F68) from BASF AG, Germany; and Tween® 20 (KgaA) from Merck, Darmstadt, Germany. Glycerol was from Pharmachem, Ljubljana, Slovenia and Trypan Blue Stain dye from GIBCO, Paisley, Great Britain.

The spin-labeled derivative of fatty acid, methylester of 5′-doxyl palmitic acid (MeFASL(10,3)), and spin-labeled phosphatidylcholine (PC(10,3), 1-palmitoyl-2-palmitoyl(5′-doxyl)-*sn*-glycero-3-phosphocholine) were synthesized at the Faculty of Pharmacy, Ljubljana (Fig. 1).

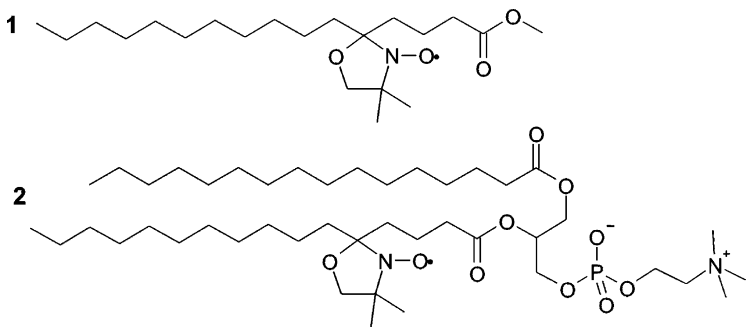

Fig. 1. Structural formula of: (1) spin-labeled derivative of fatty acid, MeFASL(10,3) and (2) spin-labeled phosphatidylcholine, PC(10,3).

2.2. Preparation of nanoparticles

SLN were prepared by the melt-emulsification process (Ahlin et al., 1998). Total of 2 ml of heated poloxamer 188 or Tween® 20 water solution (0.5%) was added to the melted triglyceride (50 mg), phospholipid (20 mg), and SP, which was deposited as a thin film on the wall of glass tubes. The concentration of SP in SLN dispersions was 4×10^{-4} M for experiments with living cells and 5×10^{-3} M with liposomes. The mixture was stirred 8 min at 20,000 rpm at 70–80 °C using a Lab Tek rotor-stator homogenizer (Omni International, Gainesville, USA). After cooling to room temperature (10 min of stirring at 5000 rpm) the lipids were solidified and nanoparticles were formed with a solid lipid core and an outer layer composed of phospholipid and steric stabilizer. For evaluating the interactions of SLN with leukocytes SLN dispersions were made isotonic with glycerol.

2.3. Preparation of liposomes

A dispersion of 1% (w/w) phospholipid forming liposomes was prepared as for SLN. The phospholipid was heated in a glass tube to 70 °C. Purified water, heated to the same temperature, was added and stirred at high shear rate (20,000 rpm) for 5 min at the same temperature with a rotor-stator Lab Tek homogenizer (Omni International). The liposome dispersion was cooled to room temperature for 10 min at 5000 rpm and extruded successively through polycarbonate membranes with pore diameters of 800, 400, and 200 nm (LiposoFast extruder, Avestin, Canada). The mean diameter of the liposomes was 250 ± 15 nm.

2.4. Characterization of SLN

The mean diameter of SLN and polydispersity index (PI) as a measure of dispersion homogeneity were estimated using photon correlation spectroscopy (PCS, Zetasizer 3000, Malvern, UK) at a fixed angle of 90°. Zeta potential was measured on the principle of electrophoretic mobility of charged particles in electric field and was performed on the same instrument.

2.5. Isolation of leukocytes

Fresh bovine blood, with citrate as an anticoagulant, was centrifuged and distilled water was added to lyse the sedimented blood cells. NaCl solution was added to stop the haemolysis. After centrifugation, leukocytes were isolated in the pellet and washed. The hypotonic shock treatment was repeated to obtain only leukocytes in the pellet. Before incubation with SLN, leukocytes were counted with a microscope using Bürker Turk comora. No differential count was performed, but neutrophils were assumed to be in the majority. The number of leukocytes was set to 1×10^9 ml^{-1}.

2.6. Leukocyte viability assay

Viability of leukocytes was assessed by the Trypan blue exclusion assay. After incubation with nanoparticles and washing with PBS, cells were diluted (50 times) and colored (1:1) with Trypan blue solution (0.4%) on glass of Bürker Turk comora. In this experiment, the cells with damaged cytoplasm membranes were colored and determined in sight field of

microscope. Viability was expressed as a percentage of the uncolored cells. In control experiments leukocytes were incubated in PBS.

2.7. Incubation of spin-labeled SLN with liposomes

One part of SLN dispersion was incubated with 50 parts of liposome dispersion for a defined time (5 min to 5 h) at room temperature. Samples were drawn into the capillary and EPR measurements performed. SLN dispersion diluted with purified water in the same ratio was the control.

2.8. Incubation of spin-labeled SLN with leukocytes

Leukocytes were incubated with SLN at 37 °C. One part of leukocyte suspension (1×10^9 cells/ml) was mixed with one part of SLN dispersion (40 mg SLN/ml cell) and incubated for a defined time (from 5 min to 3 h). The sample was then centrifuged to separate cells from SLN, which didn't interact with the cells. The supernatant was drawn into the capillary and EPR intensity measured. The cells were washed with PBS, drawn into the capillary and EPR intensity measured. Cell viability was assessed in parallel with EPR measurements.

2.9. EPR measurements

EPR spectra were recorded on a Brukker ESP 3000 X-band spectrometer. The measurement parameters were: center field (340 mT), modulation amplitude (0.15 mT), modulation frequency (100 kHz), microwave power (30 mW), and temperature (37 °C).

3. Results and discussion

Interaction of SLN with membranes was determined from changes in EPR spectral width or intensity with time of incubation of spin-labeled SLN with liposomes or leukocytes. In leukocytes, SP molecules are reduced by metabolic processes, leading to a decrease in intensity, in contrast to liposomes. This is consistent with the observed viability of the cells.

3.1. Characterization of SLN dispersions

The SLN prepared as described exhibited a mean diameter of 130 ± 10 nm, PI 0.3 and zeta potential −30 mV. SLN dispersions were characterized just to minimize variability of the results connected with these parameters. Mean diameter, PI, and zeta potential did not change over the period in which measurements were performed.

3.2. Interactions of SLN with model membranes

After interaction of spin-labeled SLN with liposome membrane a passive transfer of SP molecules to the membrane occurs. Since there can be no endocytosis or other protein-dependant processes in liposomes, only surface components can exchange their location in contact with nanoparticles. Both the SPs used, MeFASL(10,3) and PC(10,3), are due to their physicochemical characteristics preferentially located in the surface layer of the nanoparticles. The determination of transport of SP from the nanoparticle surface to the liposome membrane provides an estimate of the passive transport of substances into cells or parts of the cell membrane with poor endocytic activity.

Since liposomes could not be separated from SLN (both have approximately the same size and density), interaction between them was evaluated by measuring the change in width of the middle peak of the EPR spectra. For this purpose, high concentrations of SP (5 mM) were incorporated in SLN dispersions causing the EPR spectra to broaden as a result of spin–spin exchange interactions. Transfer of SP from SLN to liposomes led to the spectral lines becoming narrower (Fig. 2a and b). This phenomenon confirmed that SLN interacted with liposomes and that the SP distributed between them. The quantitative determination of interaction of SLN with liposomes is shown in Fig. 3. It shows the relative EPR line broadening as a function of incubation time. The spectra of MeFASL(10,3) narrowed faster than those of PC(10,3) leading to the conclusion that exchange between SLN and liposomes is much faster for MeFASL(10,3) than for PC(10,3). The initial slopes of both curves are very steep, indicating fast interaction of SLN with the liposome bilayer. Half the MeFASL(10,3) diffused into liposome bilayers in 5 min and after 30 min it had reached equilibrium. PC(10,3), which is a larger

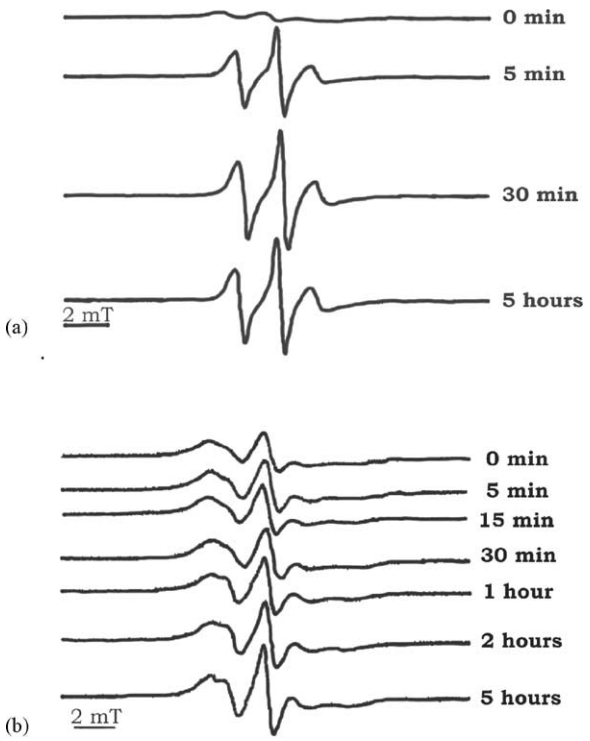

Fig. 2. Changes of EPR spectral linewidth due to the transfer of SP from poloxamer 188 coated SLN to liposomes for: (a) MeFASL(10,3) and (b) PC(10,3) after different times of incubation of SLN with liposomes.

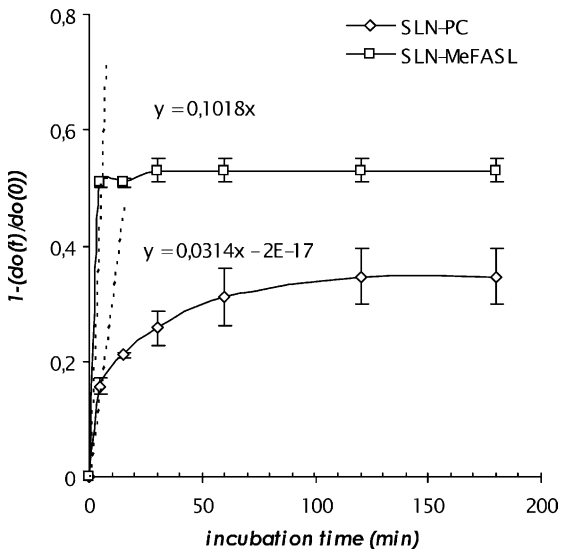

Fig. 3. Transfer of PC(10,3) and MeFASL(10,3) from poloxamer 188 coated SLN to liposomes. The ordinate shows the narrowing of the EPR spectral linewidth as a percentage of the initial linewidth. ($d_o(t)$ = width of the middle line of EPR spectrum after time t, $d_o(0)$ = width of the middle line of EPR spectrum before addition of liposomes ($n = 3$; mean ± S.D.)). The slopes of dashed lines indicate the rate of interaction of SLN with liposomes.

and less mobile molecule, diffused into liposomes more slowly so that equilibrium was attained after 2 h. The difference in passive diffusion between these molecules is evident also from the calculated slopes of the initial intensity curve (Fig. 3). This difference is mainly a consequence of molecular size and shape (Fig. 1). Despite the same structure of the chain carrying the nitroxide ring, the additional lipophilic chain of PC(10,3) contributes to a stronger inclusion in the nanoparticle phospholipid layer, and it therefore transfers more slowly into liposome membranes. On the other hand, MeFASL(10,3) is a more lipophilic molecule, and would be expected to change its location in the membrane-like environment more rapidly.

3.3. Interactions of SLN with leukocytes

In order to determine whether the whole SLN or only the SP molecules cross the leukocyte membrane, the integrity of the membrane was determined by Trypan blue exclusion assay. Whereas only intact cellular membranes perform active processes like endocytosis, the cell viability during the experiments was evaluated. After a 3-h incubation period the cell viability decreased by approximately the same percentage for nanoparticles with PC(10,3) and MeFASL(10,3), using poloxamer 188 or Tween 20 as steric stabilizers (Table 1). Different SPs or steric stabilizers did not exhibit significantly different effects on cell viability, although it was reduced by approximately 10% compared with the control. Similar effects of SLN on cell viability have been reported (Müller et al., 1997a; Schöler et al., 2002).

SLN interaction with leukocytes was determined by a slightly different procedure from that used for liposomes. EPR spectral intensity was calculated as a product of the amplitude of the middle absorption line and the square potency of the corresponding peak-to-peak line width. After incubation of leukocytes with nanoparticles, leukocytes were separated by centrifugation and washed twice to remove

Table 1
The viability of leukocytes after a 3-h incubation with various SLN dispersions

	Sample			
	Control	Polox-SLN-PC	Polox-SLN-MeFASL	Tween-SLN-PC
Viability (%)	97 ± 1	88 ± 4	83 ± 5	84 ± 4

Polox-SLN-PC, poloxamer 188 coated SLN with PC(10,3); Polox-SLN-MeFASL, poloxamer 188 coated SLN with MeFASL(10,3); Tween-SLN-PC(10,3), Tween 20 coated SLN with PC(10,3) ($n = 3$; mean ± S.D.).

non-adsorbed and non-internalized nanoparticles. To investigate whether incubation results in adsorption of SLN to cells and uptake into the cells, supernatant and cells were analyzed separately. The EPR spectral intensities of SP in the pellet were determined after a defined time of cell incubation with two different types of nanoparticles and presented as a part of the initial intensity in the SLN dispersion (Fig. 4). From the EPR spectra, it is not possible to conclude whether the whole nanoparticles with incorporated SP or only

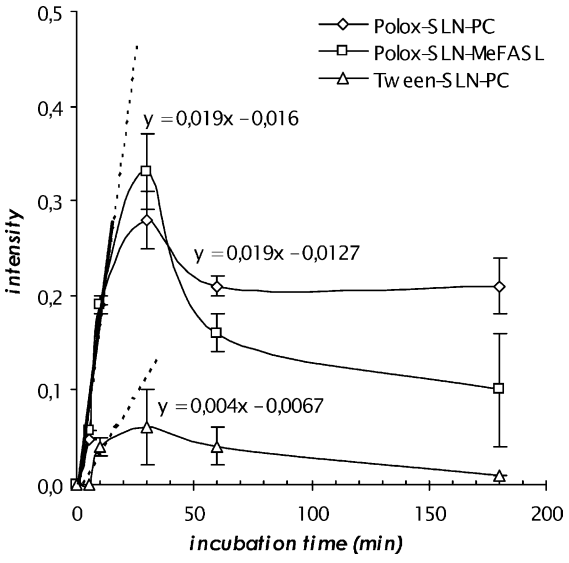

Fig. 4. Interaction of SLN with leukocytes. EPR spectra intensities in leukocytes after different times of incubation with SLN: Polox-SLN-PC, poloxamer 188 coated SLN with PC(10,3); Polox-SLN-MeFASL, poloxamer 188 coated SLN with MeFASL(10,3); Tween-SLN-PC(10,3), Tween 20 coated SLN with PC(10,3). Intensity is represented as a ratio of the spectral intensity in leukocytes after incubation with SLN dispersion to the intensity measured after the SLN dispersion was diluted with PBS at the same volume ratio as with leukocytes ($n = 3$; mean ± S.D.).

SP molecules transfer into the cells. But comparison of the kinetics of uptake of MeFASL(10,3) and PC(10,3) in the first minutes of incubation of poloxamer coated SLN with leukocytes, shows (see the equations for the intensity–time slopes), that there is no significant difference in uptake kinetics, whereas passive diffusion into liposomes is much faster for MeFASL(10,3) (Fig. 3). We may, therefore, conclude, that poloxamer coated nanoparticles were rapidly internalized in living cells by endocytosis.

After 30 min incubation, about 30% of both SPs from poloxamer coated SLN and only 6% of SP from Tween 20 coated SLN were detected in leukocytes. Nanoparticles with Tween 20 as steric stabilizer were taken up by leukocytes five-fold less than by poloxamer coated SLN. Thus, Tween 20 has a better surface masking effect and, in vivo, a longer circulating time can be expected, compared with poloxamer coated nanoparticles. This phenomenon can be partially explained by differences in the structures of the stabilizers. Decreased uptake of colloidal carriers may be caused by increasingly hydrophilic nature of the surface of nanoparticles and by increased packing density of the coating layer (Bocca et al., 1998). Tween 20 has a larger lipophilic part, which enables the molecule to anchor in the nanoparticles with its hydrophilic chains at the particle surface. Poloxamer 188 is preferentially adsorbed on the particle surface and can be desorbed, leaving the surface “unprotected.” This can explain the faster and more extensive uptake of poloxamer stabilized SLN than of Tween 20 coated nanoparticles.

After a time, the EPR spectral intensities measured in leukocytes started to decrease (Fig. 4). EPR spectra intensity of PC(10,3) in supernatant shows a different time course from that for leukocytes (Fig. 5). It decreases over the first 30 min—further changes are within experimental error. Thus, interaction was very

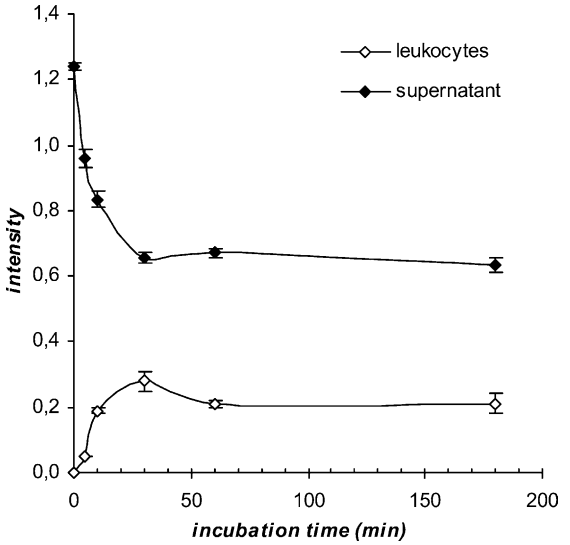

Fig. 5. Comparison of EPR spectral intensities of PC(10,3) in leukocytes and in supernatant after different incubation times of leukocytes with poloxamer coated SLN. Intensity is represented as the ratio of the spectral intensity in leukocytes or in supernatant after incubation with SLN dispersion to the intensity obtained after SLN dispersion was diluted with PBS at the same ratio as with leukocytes ($n = 3$; mean ± S.D.). At time zero the intensity in the supernatant is greater than 1 because the distribution of SLN into the leukocytes does not occur instantaneously after addition of SLN.

fast and the uptake of SLN was stopped after a short period of contact with leukocytes. Approximately 60% of SP remained in supernatant even after 3-h incubation. We suppose that a saturable endocytic process prevents further uptake of the particles. In addition to SP uptake, the intensity changes are also caused in leukocytes by the reduction of SP. The spectral intensity in leukocytes first increases and then starts to diminish when the reduction of nitroxide groups by cellular redox systems becomes faster than the transport of SP into the cells.

MeFASL(10,3) is reduced faster than PC(10,3) in leukocytes. Similarly, in liposomes, the passive diffusion process is faster for MeFASL(10,3), indicating that, in leukocytes, it is the faster mobility of the smaller MeFASL(10,3) that explains the result. The spectral intensity decrease of PC(10,3) was not observed in the supernatant because extracellular reduction could occur only if cells were damaged. The kinetics of reduction were monitored after different times of incubation of spin-labeled SLN with leukocytes. After 5 min incubation the reduction was much faster than after 15 min incubation and both were faster than after 1 h incubation (results not shown). The slow down of reduction explains why after 3-h of incubation the EPR signal was still detected in leukocytes. This could be due to different locations of SP in SLN and the presence of steric stabilizers on the particle surface, which protect SP from reduction. SP molecules located on the surface of SLN and those SP which diffused from the surface of SLN to the leukocyte membranes are more accessible to cell redox systems than probes located deeper in SLN.

4. Conclusion

Liposomes have been used as model membranes to study the passive transport of two different spin-labeled compounds, PC(10,3) and MeFASL(10,3), from SLN to liposomes. It was observed that the passive transport across the membrane was faster for the smaller MeFASL(10,3). In contrast, no significant difference in the transfer rate was observed for the same compounds after incubation of SLN with leukocytes. It is, therefore, presumed that there is an uptake of SLN to leukocytes, probably by endocytosis, although simultaneous SP diffusion is also not excluded. The rate of this interaction depends on the stabilizer used.

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