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Evaluation of liposomes as drug carriers into the skin by one-dimensional EPR imaging

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Summary

One-dimensional electron paramagnetic resonance imaging (1D-EPRI) was used to evaluate quantitatively the influence of the size and composition of liposomes on the translational mobility of liposome-entrapped charged hydrophilic molecules (ASL) into the skin. It was found that only ASL molecules entrapped in small unilamellar vesicles (SUV) or reverse-phase evaporation vesicles (REV) from 'fluid' phospholipids (egg or soya lecithin and phosphatidylserine) can diffuse, while free ASL or ASL entrapped in the multilamellar vesicles (MLV) or REV from 'solid' phospholipids (dipalmitoyl lecithin/dipalmitoylglycerol) do not penetrate. The 1D-EPRI method was found to be useful for the study of diffusion of drugs into the skin.

Introduction

Liposomes, as drug carriers for therapy of skin disorders, can considerably improve the therapeutic efficacy of topically applied drugs and at the same time diminish their side effects (Ganesan et al., 1984; Mezei, 1988). Within the context of drug delivery systems, liposomes possess general characteristics which make them particularly useful. These carrier systems are biocompatible, nontoxic and remarkably flexible. Optimal variations in liposome size, lipid composition and drug-to-lipid ratio can specifically affect the therapeutic benefits arising from liposomal drug delivery.

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Recent research in epidermal cellular and molecular biology recognizes that the principal resistance to drug transport in dermal drug delivery resides in the diffusion processes through the stratum corneum (Barry, 1988). This is a system composed of stacked flat cells, rich in keratin fibrils, that are embedded in the intercellular lipid matrix. The lipid composition is unusual; particularly noteworthy are the exceptionally high contents of ceramides, cholesterol and fatty acids; the first two may be arranged in relatively cohesive lamellar structures, while more nonpolar species may be confined within a separate, relatively loosely bound compartment in the stratum corneum interstices (Curatolo, 1987; Grubauer et al., 1989). The mechanism by which the liposomes affect the penetration of drugs into the skin is not completely understood. The suggestion that lipo-

somes penetrate into the skin or directly transfer the drug between the lipid bilayers has been received skeptically. Also, to date, no direct measurement of how different liposome size and composition influence the penetration of liposome-entrapped drugs into the skin has been made (Gesztes and Mezei, 1988).

With recent development of one-dimensional electron paramagnetic resonance imaging (1D-EPRI), it became possible to follow the rate of penetration of liposome entrapped substances into the skin. The motional processes can be explored directly by entrapping a paramagnetic molecule into the liposomes which serve as the markers for imaging of the diffusion. Hydrophilic or lipophilic nitroxide radicals can be chosen for this purpose. They reflect the behaviour of drugs entrapped in the liposomes. Detailed information about nitroxide mobility is easily evaluated from the 1D-EPRI spectra by comparison with the spectra calculated from the corresponding model (Demsar et al., 1988; Kristl et al., 1989). In this work, we have used 1D-EPRI to evaluate quantitatively the influence of size and composition of liposomes on the translational mobility of liposome-entrapped charged hydrophilic molecules into the skin.

Materials and Methods

Materials

Egg lecithin (EG) was isolated from egg yolk in our laboratory by the method described by Lea et al. (1953). L-α-Dipalmitoylphosphatidylcholine (DPPC) and L-α-dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids (Birmingham, AL). L-α-Phosphatidylserine (PS) from bovine brain was purchased from Sigma (St. Louis, MO), cholesterol (Chol) from Riedel de Haen (Seelze, Hannover, F.R.G.) and *N*-ethylmaleimide (NEM) from Aldrich (Milwaukee, WI). Soya lecithin, Epikuron 200^R (SL) was donated by Lucas Meyer (Hamburg, F.R.G.). All other chemicals were of reagent grade.

Spin probe

ASL (*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-*N*-dimethyl-*N*-hydroxyethylammonium iodide) (Fig. 1) was synthesized in our laboratory. It is readily soluble in water and, due to its charge, does not penetrate the liposome membrane. ASL was incorporated into liposomes during their preparation. Uncaptured ASL was removed by dialysis at 4°C for 24 h. The final concentration of ASL entrapped in liposomes was 0.01 mol/l.

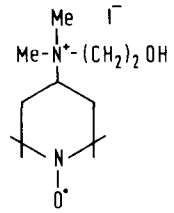

Fig. 1. Chemical structure of ASL spin probe.

Preparation of liposomes

Multilamellar vesicles (MLV) were prepared using a slightly modified method of Bangham et al. (1965). Small unilamellar vesicles (SUV) were prepared by sonication of MLV on a KLN 22/125 ultrasound sonicator at 65 W for 15 min (Šentjurc et al., 1975). Reverse-phase evaporation vesicles (REV) were prepared by the reverse-phase evaporation method (Szoka and Papahadjopoulos, 1978) in water or Tris buffer (pH 7.4, 310 mosmol/l) from different lipid components and at various molar ratios (Table 1). The concentration of lipids in the liposome preparations was 16 mg/ml.

Liposome gel

Liposomes loaded with ASL were dispersed in hydrogel composed of 0.6 g xanthan gum (Keltrol^R, Kelco, San Diego, CA) and 24.4 g

TABLE 1

Composition and shape of the liposomes used

Lipid composition	Molar ratio	Liposomes
EL : Chol	7 : 3	REV
EL : Chol	1 : 1	REV
EL : PS	7 : 3	REV
DPPC : DPPG	4 : 1	REV
EL : Chol	7 : 3	SUV
EL : Chol	7 : 3	MLV
SL	1	MLV
EL : PS	7 : 3	MLV

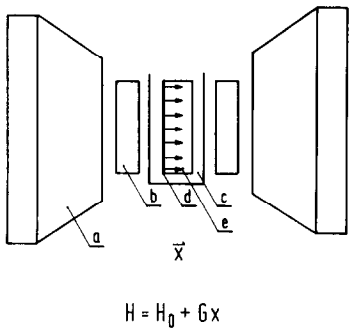

Fig. 2. The geometry of the sample, magnetic field and magnetic field gradient for 1D-EPRI. The liposome gel is applied at the left edge and then diffuses in the direction indicated by the arrows. a, electromagnet; b, Helmholtz coils; c, microwave cavity; d, thread with liposome gel; e, skin.

purified water. The final concentration of lipids was 1% (w/w).

Stability of liposomes

Leakage and chemical transformation of ASL entrapped in the liposome gel during storage were monitored. For this purpose ASL entrapped liposome gel was stored in a dialysis tube and dialyzed against water for more than 1 week in order to remove released ASL. The EPR spectral intensity of ASL remaining in the liposome gel was measured at different time points during dialysis. It was found to remain constant over a period of 1 week, indicating that no appreciable leakage of ASL from the liposome gel occurred during the measurement period.

Skin for EPR spectroscopy

Pig ear skin soaked in a 20 mmol/l solution of NEM for 24 h, in order to prevent nitroxide reduction, was used. It was stored in a desiccator at 75% relative humidity (over a saturated NaCl solution) at 4°C for no longer than 10 days.

1D-EPRI

This was based on the use of a conventional EPR spectrometer with superimposed Helmholtz field gradient coils parallel with the modulation coils on a Varian E-9 X-band spectrometer. A gradient of 0.25 T/m was used. The laboratory magnetic field was parallel with the gradient direction.

A slice (approx. 1 mm thick and 1 cm long) of pig ear skin was placed in the tissue cell. A thread (1 cm long) soaked with the liposome gel was placed in contact with the narrow surface of the skin and both were put together into the microwave cavity with the large plane parallel with the magnetic field gradient. The geometry of the sample in the magnetic field is shown in Fig. 2. The EPR spectra in the magnetic field gradient were measured at various intervals after being in contact with the liposome gel for different periods. The change in shape of the spectra reflects the concentration profile of the spin probe diffusing into the skin.

Results and Discussion

EPR spectra of ASL in pig ear skin, after contact with ASL-loaded liposome gel for different times, are presented in Fig. 3. The observed asymmetry in the EPR spectral lineshape reflects the concentration profiles of the spin probe diffusing into the skin. From the 1D-EPRI spectra, the parameter $l = I_2/I_1$ (Fig. 3), which reflects the asymmetry of the first absorption line of the spectrum, can be calculated. It depends strongly on the diffusion of the spin probe into the sample.

The experimental value of l can be compared with l obtained from the lineshapes of the simu-

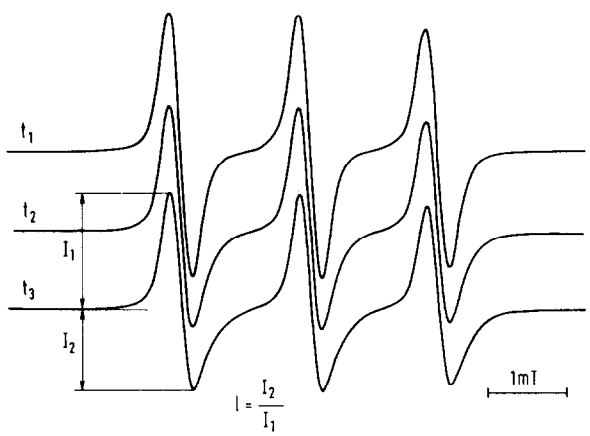

Fig. 3. 1D-EPRI spectra of ASL diffused into pig ear skin from ASL entrapped liposome gel at $t_1 = 5$ min, $t_2 = 30$ min, and $t_3 = 50$ min. The peak size parameter is defined as $l = I_2/I_1$, and is related to the rate of diffusion of ASL into the skin.

lated spectra which were calculated according to a model described elsewhere (Demsar et al., 1986). The concentration distribution of ASL diffusing into the skin can be calculated by solving the differential equation for one-dimensional diffusion:

$$\frac{\partial C(x,t)}{\partial t} = \frac{\partial}{\partial x} D \frac{\partial C(x,t)}{\partial x}$$

taking into account the approximation of a semi-finite sample with a constant surface concentration of the spin probe C_0, with the boundary conditions: $C(0,t) = C_0$, $C(x,0) = C_0$, at $x = 0$ and $C(x,0) = 0$ at $x > 0$.

Here $C(x,t)$ is the spin probe concentration, x the distance from the surface which is held at a constant concentration, and t the time elapsed from the start of the diffusion.

The distribution function ($F(x,t)$), which is the solution of the above equation, is:

$$F(x,t) = C(x,t)/C_0 = 1 - \mathrm{erf}\left(x/2\sqrt{Dt}\right)$$

where erf is the error function.

The EPR spectral lineshape $S(\mathrm{H})$ is:

$$S(\mathrm{H}) = \sum_i F(x_i) \sum_{m=1}^{-1} L'_m(H(x_i))$$

where $H(x_i) = H_0 + Gx_i$ (H_0, laboratory magnetic field; G, magnetic field gradient) and L'_m are the first derivatives of the Lorentzian lineshapes. The parameters necessary for determination of the Lorentzian lineshapes (linewidth and isotropic hyperfine splitting) are obtained from the EPR spectra of the same samples as for 1D-EPRI, but without a gradient.

From comparison of the calculated I and the value measured from the experiment, the diffusion constant D can be obtained.

The diffusion constants of ASL into the skin for liposomes of different size and composition are listed in Table 2. The values were calculated from measurements taken 60 min after the liposome gel was put into contact with skin. From Table 2 one can see that the diffusion of ASL dissolved in water or hydrogel into the skin is negligible within the time of measurements. Also, we could not detect any diffusion of ASL molecules in the case of spin label incorporated into MLV or the REV from 'solid' phospholipids (DPPC/DPPG, with a phase transition temperature at 41° C). However, for REV composed of 'fluid' phospholipids, appreciable diffusion of ASL into the skin was observed. In order to exclude a possible effect of the liposome ingredients on the barrier of the horny layer, in a control experiment the hydrogel with free ASL and empty penetrant REV was used. Also, for this system no penetration of ASL into the skin was observed, proving that the charged molecule ASL can be transmitted into the skin only when incorporated into liposomes. We cannot say as yet whether the liposomes remain intact and diffuse together with ASL through the skin layers, or are destroyed on contact with the epidermis and ASL alone diffuses through the skin. The extent to which the liposomes penetrate into the skin without being destroyed will be the subject of further investigations.

TABLE 2

Diffusion constant of ASL (D, cm^2/s) into pig ear skin, from ASL entrapped liposomes of various lipid composition and size, as well as free ASL in water and in hydrogel (HG)

Lipid composition of the liposomes	Diffusion constant ($\times 10^{-6}$) (cm^2/s) [a]
REV (EL : Chol = 7 : 3) in HG	0.6 ± 0.1 (2)
REV (EL : Chol = 1 : 1) in HG	1.8 ± 0.5 (4)
REV (EL : PS = 7 : 3) in HG	1.5 ± 0.1 (2)
REV (EL : PS = 7 : 3) in Tris	2.5 ± 0.9 (5)
REV (DPPC : DPPG = 4 : 1) in HG	0.0 ± 0.0 (6)
SUV (EL : Chol = 7 : 3) in water	1.2 ± 0.5 (5)
MLV (EL : Chol = 6 : 4) in HG	0.0 ± 0.0 (3)
MLV (EL : PS = 7 : 3) in Tris	0.0 ± 0.0 (2)
MLV (SL) in HG	0.0 ± 0.0 (3)
Free ASL in water	0.0 ± 0.0 (3)
Free ASL in HG	0.0 ± 0.0 (2)
REV (EL : Chol = 7 : 3) + free ASL in HG	0.0 ± 0.0 (2)

[a] Mean ± S.D.

The number of measurements on different skin samples is shown in parentheses. The measurements were taken using various samples of pig ear skin and at different time intervals after killing of the animal. There were no significant differences in D when measurements were performed on fresh skin or skin which had been stored at 4° C for 10 days.

From Table 2, one can also observe that the differences in D for liposomes of various compositions which penetrate into the skin are not significant, indicating that the major factors influencing the penetration of liposomes are liposome shape and fluidity of phospholipid components, other characteristics appearing to be of minor importance only.

The results obtained provide direct evidence of the diffusion of substances entrapped in liposomes through the skin layers, and prove that liposomes can be considered as a promising new drug delivery system for topical application. Its main ingredients, phospholipids and water, are native components of the skin, and are thus highly compatible, biodegradable and nontoxic. Another advantage of this system is that it promotes the penetration of charged molecules into the skin, which would otherwise be unable to do so because of their charge. However, our results also show that the size and phospholipid composition of liposomes are important for efficient delivery through the skin.

These investigations also demonstrate the usefulness of the 1D-EPRI method for the study of liposome diffusion through the skin from the point of view of its application as a topical drug delivery system. This procedure will facilitate further investigation of the mechanisms by which liposomes enhance the penetration of drugs into the skin.

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