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The measurement of liposome entrapped molecules' penetration into the skin: A 1D-EPR and EPR kinetic imaging study

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Summary

One-dimensional electron paramagnetic resonance imaging (1D-EPRI) and EPR reduction kinetics were used to follow continuously the transport of liposome entrapped substances into the skin. Through 1D-EPRI the concentration distribution of the paramagnetic probe, which was applied to the skin entrapped in liposomes, could be followed, while through EPR reduction kinetics the chemical transformation of the paramagnetic probe, after it had been released from the liposomes, to an EPR-invisible form could be measured. Through the combination of both methods, and with the application of a model, in which the heterogeneity of different skin layers and the metabolism of the released substance was taken into account, liposome decay in the skin, as well as the time evolution of concentration distribution profiles for ASL in skin, was followed separately for both the entrapped substance and that released from liposomes. MLV (multilamellar vesicles) and REV (reverse-phase evaporation vesicles) obtained from egg lecithin and cholesterol (7:3 mol/mol) with the entrapped spin probe ASL (*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-*N*-dimethyl-*N*-hydroxyethylammonium iodide), which does not penetrate the liposome membrane easily, were applied to pig ear skin and the results were compared with those obtained for ASL dissolved in water and applied to the skin. The rapid decay of liposomes in the stratum corneum was measured, being much faster for MLV than for REV. In addition, a rate of transport 100-times faster was observed for ASL applied to the skin in REV than that observed for ASL applied in MLV or in solution. Our observations show that the rapid decay of liposomes takes place in the stratum corneum, however, some of the ASL molecules remain protected from the reducing agents in the skin, which indicates that some REV liposomes can penetrate deeper into the skin, or at least their lipids protect the entrapped substance from metabolic transformation.

Introduction

It is generally accepted that drugs applied to the skin entrapped in specially designed liposomes can be efficiently delivered into and through the skin. There are several well written reviews (Mezei, 1988; Schäfer-Korting et al., 1989; Egbaria and Weiner, 1990) on drug delivery into the skin via liposomes. The results show a greater concentration of the delivered drug in different skin layers (Wholrab et al., 1989), or increased pharmacological effects (Geszetes and Mezei, 1988), when the drug is applied to the skin entrapped in liposomes compared to the application of the free drug. In spite of the well documented enhanced delivery of liposome entrapped molecules, there is no detailed explanation about the mechanism by which such large objects as

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liposomes can cross the skin layers more efficiently than smaller molecules. Some authors argue that neither liposomes nor the phospholipids which make up these liposomes penetrate through the skin (Ganesan et al., 1984; Lasch et al., 1991) or that the efficiency of drug acceptance is increased by the presence of liposomes (Westerhof, 1985). Others believe that the penetration of liposomes is possible during the second stage when some liposomes, which have already been degraded, trace the conditions for the transport of the remaining liposomes (Lautenschläger and Röding, 1989). This idea is supported by some electron micrographs (Foldvari et al., 1990), where intact liposomes, loaded with an electron-dense marker, are observed in different locations within the tissue, which indicates that the entrapped molecules are transported into the skin via the penetrating liposomes. Cevc and Blume (1991) have observed the rapid transport of specially designed intact liposomes (transfersomes) into the deeper layers of the skin. They have suggested that it is the high water concentration gradient across the epidermis which forces the liposomes to penetrate into the deeper skin layers. It is obvious that due to the heterogeneity of the skin and the multiple physiological tasks of the skin (Wiechers, 1989) no simple approach concerning the penetration of liposome entrapped substances into the skin is possible.

In our previous work (Gabrijelčič et al., 1990) we measured the penetration of spin-labeled molecules into the skin in free and liposome entrapped form by 1D-EPRI. Through this method it was possible to follow continuously the evolution of concentration profiles in skin for liposome entrapped substances, monitoring the variations in the lineshape of the EPR spectra, and to calculate the corresponding penetration rates. No detectable transport into the inner skin layers of free, charged spin probe molecules or of the same molecules entrapped in MLV was observed during the time period of 1 h. However, for spin probe molecules entrapped in liposomes of an appropriate size and composition, significant penetration into the inner skin layers was detected and the diffusion coefficients were determined. Using this method it is not possible to distinguish the penetration of spin-labeled molecules entrapped in liposomes from those which are released after disintegration of the liposomes. Another disadvantage of this method is that the reducing agents in the skin transform the paramagnetic spin probe, released from disintegrated liposomes, to the diamagnetic EPR-invisible form. As a consequence, the EPR spectral intensity decreases with time, which complicates the calculation of concentration profiles appreciably. In our next experiment, the reducing capability of skin was used to introduce the EPR kinetic imaging method. Namely, from the rate of reduction of the spin probe molecules which had been released after destruction of liposomes, it was possible to follow continuously the evolution of concentration profiles into the skin, separately for liposome entrapped substance and for the released, free molecules, as well as to measure the decay rate of the liposomes during their penetration into the skin (Schara et al., 1990; Gabrijelčič et al., 1991). This method indentified the relationship between penetration of molecules released from and/or entrapped in liposomes and the reduction rates.

In the present work, a synthesis of both kinetic and 1D-EPR imaging methods for the evaluation of transport is presented. Concentration profiles calculated from the reduction kinetic imaging experiment were used to fit the lineshapes of EPR spectra obtained by 1D-EPRI. This combination of two independent experiments requires a refined model for the calculation of concentration profiles, taking into account the heterogeneous layer structure of the skin, thus providing a detailed insight into the transport characteristics of liposomes and liposome entrapped substances.

Materials and Methods

Liposomes

Multilamellar vesicles (MLV) were prepared using a slight modification of the method reported by Bangham et al. (1965). Reverse-phase evaporation vesicles (REV) were prepared by the reverse-phase evaporation method (Szoka and Papahadjopoulos, 1987).

The lipids used for the preparation of liposomes were egg lecithin (EL), isolated from egg yolk in our laboratory according to the method described by Lea et al. (1953), and cholesterol (Chol), obtained from Riedel de Haen (Seelze, Hanover, Germany), in a molar ratio of 7:3. During preparation, a spin probe (ASL; *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-*N*-dimethyl-*N*-hydroxyethylammonium iodide) was incorporated into the liposomes. This spin probe is readily soluble in water and, due to its charge, does not penetrate the liposome membrane easily. Uncaptured ASL was removed by dialysis at 4°C for 24 h. The EPR spectra were measured before and after dialysis and from the intensities of the EPR spectra of ASL, before and after dialysis, the entrapped volume for liposome dispersion was determined. For REV it was 0.08 ml/ml and for MLV 0.02 ml/ml. The final concentration of ASL entrapped in the liposomes was 0.01 mol/l. The lipid concentration in the liposome dispersion was 16 mg/ml.

Skin

Skin samples were cut from fresh pig ear delivered from the slaughterhouse. The ear sample was rinsed in water, the apex was cut through and for EPR measurements a slice approx. 1 mm thick, 5 mm wide and 10 mm long was placed in the tissue cell, with the narrow surface mounted in contact with a 0.3 mm thick cotton thread soaked with the liposome dispersion. The measurements were taken within 1–10 h of killing the animal. Before measurement the skin was stored at 4°C in a desiccator at 75% relative humidity.

EPR method

One-dimensional EPR imaging (1D-EPRI) For 1D-EPRI a magnetic field gradient of 0.25 T/m was used in the direction perpendicular to the narrow surface of the sample and parallel with the direction of the magnetic field. Due to the diffusion of the spin probe into the skin, the lineshape of 1D-EPRI spectra became asymmetric. The experimental spectra were fitted with the spectra evaluated from the corresponding concentration profiles of the spin probe in the tissue sample, taking into account the relation:

$$H_i = H_0 + Gx_i$$

$$S(H) = \sum_m \sum_i C(x_i,t) F'_m(H - H_i) \qquad (1)$$

where H_0 is the resonance magnetic field in the absence of magnetic field gradient G in the direction of the laboratory magnetic field, $F'_m(H - H_i)$ denotes the first derivative of the EPR spectral lineshape for the hyperfine component m and $C(x_i,t)$ is the spatial distribution function of the spin probe molecules (concentration profile) at time t.

For a quick reference, asymmetry was measured by the parameter l ($l = I_2/I_1$) (Fig. 1) at various intervals after being in contact with the liposome dispersion.

EPR kinetic imaging The kinetics of reduction of the spin probe ASL was determined by measuring the intensity decrease of the EPR spectra with time for the whole sample (thread and skin). The amplitude of the first hyperfine component of the EPR spectra was measured, as the lineshape did not change with time significantly.

The reduction kinetics and evolution of the concentration profiles of the spatially spread reacting molecules were calculated using a model which included the transport of intact liposomes and of released spin-labeled molecules into the skin, and the decay rate of the liposomes, as well as the differences between stratum corneum and viable skin layers. A system of coupled differential equations describing the diffusion-reaction coupled system was used:

$$\frac{\delta C(x,t)}{\delta t} = D^{(j)} \frac{\delta^2 C(x,t)}{\delta x^2} - k_1^{(j)} C(x,t)^{(R)} C(x,t) + k_2^{(j)} C(x,t)^{(L)} \qquad (2)$$

$$\frac{\delta C(x,t)^{(R)}}{\delta t} = -k_1^{(j)} C(x,t)^{(R)} C(x,t) \qquad (3)$$

$$\frac{\delta C(x,t)^{(L)}}{\delta t} = D^{(j)(L)} \frac{\delta^2 C(x,t)^{(L)}}{\delta x^2} - k_2^{(j)} C(x,t)^{(L)} \qquad (4)$$

where $C(x,t)$ and $C(x,t)^{(L)}$ are the concentration distribution functions of ASL released after liposome disintegration (free ASL) and of ASL entrapped in liposomes (or another form which protects ASL from reduction – entrapped ASL), while $C(x,t)^{(R)}$ denotes the concentration distribution of endogenous reducing agent. Index j denotes different compartments across the sample (thread, stratum corneum and viable skin layers) and $k_1^{(j)}$ and $k_2^{(j)}$ are the rate constants for reduction of ASL and for liposome decay, $D^{(j)}$ and $D^{(j)(L)}$ represent the corresponding diffusion coefficients for free ASL and for entrapped ASL, respectively. This system of equations was solved numerically using the algorithm described previously (Schara, 1990) and integrated using the Runge-Kutta method. From the best fit with the experimental reduction kinetic curves, the concentration profiles $C(x,t)$ for free and entrapped ASL molecules in the skin were obtained.

The measurements were performed on a Varian E-9 X-band spectrometer at room temperature with simultaneous measurement of intensity

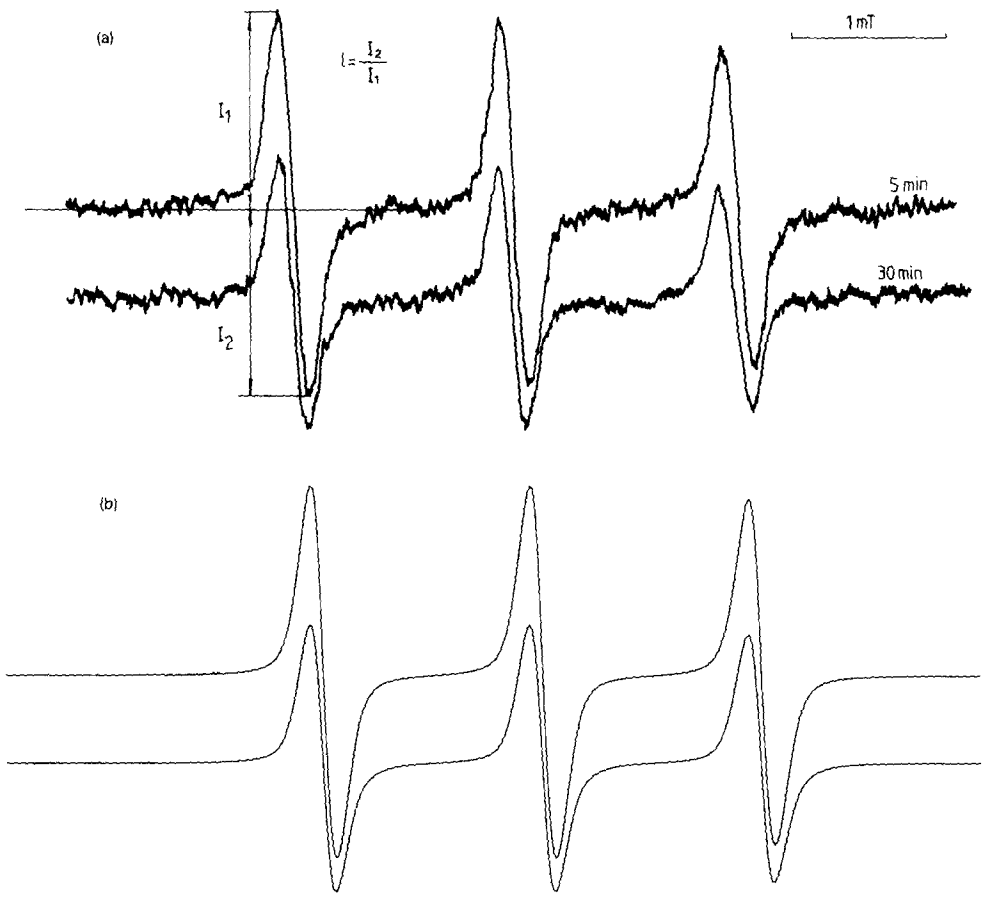

Fig. 1. 1D-EPRI spectra of ASL in the sample of pig ear skin 5 and 30 min after application of REV (EL:Chol = 7:3) with entrapped 0.01 mol/l ASL. (a) Experimental spectra; parameters used: magnetic field gradient, 0.25 T/m; modulation amplitude, 0.1 mT; microwave power, 10 mW; sweep, 10 mT. The spectra were measured at room temperature. (b) Calculated spectra: concentration distribution function ($C(x,t)$) of ASL obtained from the reduction kinetic experiment (Fig. 4) was used. Spectra are a superimposition of the spectra of released ASL (represented by a composite spectrum, Gaussian lineshape 95%) and the entrapped ASL (Lorentzian lineshape 70%, due to the spin-spin interaction between ASL molecules) (the lineshapes for different concentrations of ASL have been evaluated for water solution in the concentration range from 10^{-4} to 10^{-2} mol/l).

decay and of the EPR spectral lineshape in the presence of the magnetic field gradient. The concentration distribution function evaluated from the kinetic imaging experiment $C(x,t)$ was used to calculate the lineshape of the 1D-EPRI spectrum (Eqn 1) and to compare it with the experimental one. Herewith the model used and the concentration distributions obtained were checked by two independent experiments.

Results

In Fig. 1 typical 1D-EPRI spectra are shown for REV, 5 and 30 min after application of liposomes to the skin surface. The corresponding values of parameter l, which are related to the lineshape asymmetry caused by the penetration of ASL (entrapped and free ASL) into the skin, are also shown. After 30 min l is lower than after 5 min ($\Delta l = l_5 - l_{30} = 0.021$), indicating significant transport of ASL into the skin. For the applied ASL entrapped in MLV or free ASL, no difference in l was observed with time (even after 60 min) in accordance with our previous results (Gabrijelčič et al., 1990).

Fig. 2 shows the kinetics of spin probe reduction in the same samples as described above: for ASL applied in solution, entrapped in MLV, and in REV.

Kinetic curves have been adequately fitted using a model where the skin is treated as a single homogeneous compartment in contact with the other compartment, the thread. In the thread there is no reduction of ASL and no decay of liposomes ($C(x,t)^{(R)}$, k_1 and k_2 are 0); the diffusion of liposomes in the thread and in the skin differs from the diffusion of free ASL. However, when the distribution function $C(x,t)$ obtained by this model was used to calculate the lineshape of the 1D-EPRI spectra the calculated differences in l (Δl) are negligible for all three samples, in contrast to the experiments where $\Delta l = 0.03 \pm 0.01$ (mean value of three experiments) was measured for REV. To match the lineshapes of the experimental spectra with those calculated from the reduction kinetic imaging, in addition to the different concentration of reducing agents

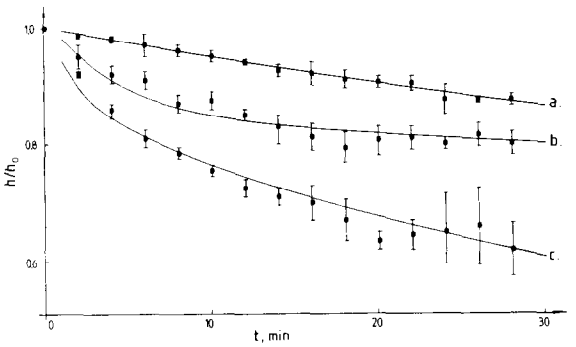

Fig. 2. Kinetics of the EPR spectral amplitude decrease of ASL applied on the surface of the pig ear skin in: (a) water solution; (b) entrapped in MLV (EL: Chol = 7:3); (c) entrapped in REV (EL:Chol = 7:3). The dots are the measured amplitudes of $m = 1$ spectral component (mean values of three experiments, bars denote the standard error). Solid lines are the best fits of the experimental kinetic curves, which also fulfil the condition that the calculated distribution function of ASL ($C(x,t)$), which is used for the EPR spectral lineshape calculation, gives the best fit of the experimental 1D-EPRI spectra. The parameters used in the calculation are presented in Table 1; those which should not depend on the form of preparation are kept the same for all three samples.

(Gabrijelčič et al., 1990), the different diffusion rates for liposomes and free ASL were used as well as the different liposome decay rates in stratum corneum and viable skin layers. Through this approach the calculated spectra, corresponding to the spectra measured 5 and 30 min after application of liposomes to the skin, show a good fit (the calculated Δl for REV is 0.02, the same as the experimental value) (Fig. 1b). The best fits of the reduction kinetic curves, with the condition that the calculated concentration profiles matches best with the experimental 1D-EPRI spectra, are represented in Fig. 2 as solid lines. The corresponding parameters are shown in Table 1. The set of parameters used in the calculation, which should not depend on the form in which the ASL is applied to the skin (rates of reduction and permeability constants for released or free ASL), were kept constant for all three examples.

The calculated distribution functions of the total amount of ASL (free and entrapped) in the skin 30 min after application of liposomes to its surface are shown in Fig. 3a (for all three samples). Fig. 3b shows separately the corresponding

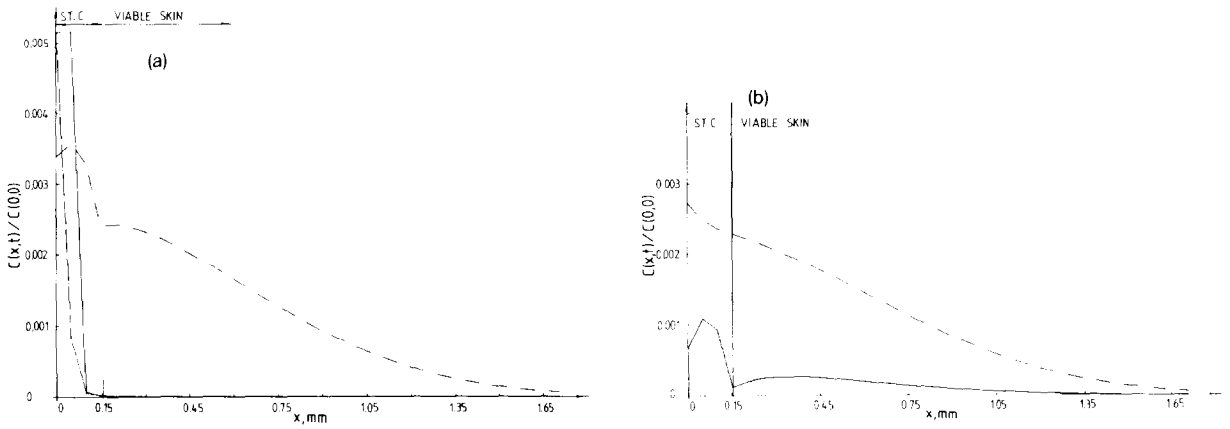

Fig. 3. (a) The total distribution function of ASL in pig ear skin 30 min after application of ASL: in water solution (solid line), entrapped in REV (EL:Chol = 7:3) (dashed line) or entrapped in MLV (EL:Chol = 7:3) (dotted line). The distributions were calculated from the described model (Eqns 2–4), with the parameters presented in Table 1. (b) Distribution functions of ASL applied in REV separately for the released (solid line) and liposome entrapped ASL (dashed line). The concentration of ASL in tissue $C(x,t)$ is normalized to the total concentration of ASL ($C(0,0)$) in the volume element used in the calculation and was calculated from the known concentration of ASL in liposomes (10^{-2} mol/l) and the internal volume of the liposome dispersion. $C(x,t)/C(0,0) = 0.001$ corresponds to $C(x,t)$ which is: for REV, 8×10^{-7} mol/l; MLV, 2×10^{-7} mol/l; ASL solution, 10^{-5} mol/l.

TABLE 1

The parameters used in the calculation of the kinetics of the EPR spectra amplitude decrease (Eqns 2–4) by which the best fits of the experimental curves (Fig. 2) were obtained

Type of preparation with ASL		Water solution	Liposomes	
			MLV	REV
Decay rate constant (k_2) (s^{-1})	thread	–	0	0
	s.c.	–	0.08	0.02
	v.skin	–	0.0002	0.0002
Diffusion coefficient (D) ($\times 10^{-7}$) ($cm^2\ s^{-1}$)	thread	1	0.05	0.5
	s.c.	0.15	1	100
	v.skin	1.5	0.1	10
Entrapped volume (V_n) (ml ml^{-1})		–	0.02	0.08

For calculation the three-compartment model was used: thread, stratum corneum and viable skin: k_2, rate constant for liposome decay; D, diffusion coefficient of ASL; V_n, entrapped volume of liposomes measured by EPR (as described in Materials and Methods). The rate constant of ASL reduction, $k_1 = 5$ l mol^{-1} s^{-1}, is taken to be the same in the stratum corneum and in viable skin, while the concentration of reducing agent in the stratum corneum is 10^{-2} mol/l, and in viable skin 3×10^{-4} mol/l, as was calculated from the best fit with the reduction kinetic curve for ASL solution. s.c., stratum corneum; v.skin, viable skin layers.

distribution functions of entrapped ASL and of ASL released from REV. In Fig. 4 the time development of the concentration profiles of the total amount of ASL (entrapped and released) for REV is represented and the corresponding calculated 1D-EPRI spectra (5 and 30 min after application) are in Fig. 1b.

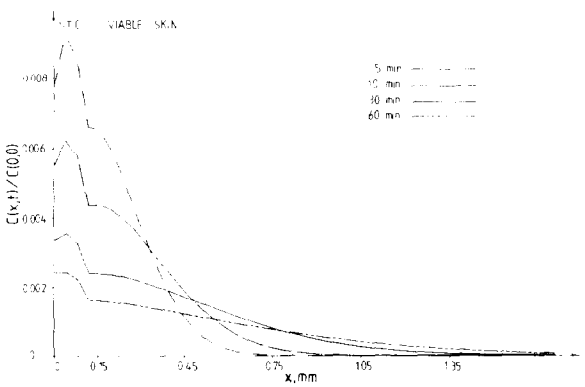

Fig. 4. The time evolution of the distribution of the total amount of ASL in pig ear skin $C(x,t)/C(0,0)$, calculated with the parameters in Table 1. 5 min (– – –), 10 min (— · —), 30 min (—) and 60 min (– · · –) after application of REV with entrapped ASL to the pig ear skin surface.

Discussion

The observed development of the concentration profiles of the substance applied to the skin entrapped in liposomes, provided by kinetic and 1D-EPR imaging, is instructive. Firstly, it reproduces the enhanced pharmacological effect of liposome entrapped drugs, proven by physiological tests (Mezei, 1988). On the other hand, the role of nonhomogeneous tissue structure, such as epidermis, is directly reflected in the transport rate, which can be evaluated from the measured concentration profiles. In the model the penetration barrier (stratum corneum) is included. According to our results (Table 1), the concentration of reducing agent in the stratum corneum is about 30-times greater than in the inner layers. This is in close agreement with our previous measurements of the nitroxide reduction rate of ASL in skin, which is larger for ASL in the surface layer than in inner skin layers (Gabrijelčič et al., 1991). The diffusion constants in the stratum corneum are higher than in the inner skin layers and are 1000-times higher for entrapped ASL than for released or free ASL. From Table 1 it also follows that the rate of liposome decay in the stratum corneum is two orders of magnitude larger than in inner layers of the skin. In the barrier this rate is larger for MLV than for REV. Irrespective of the rapid liposome decay in the stratum corneum, one can estimate from Fig. 2 and from Fig. 3b that after 30 min about 5% of the ASL molecules which were delivered into the skin remain in the form which protects them from reduction (entrapped ASL). The concentration profiles in Fig. 3a for REV, MLV and ASL solution show that after 30 min free and MLV entrapped ASL remain in the stratum corneum, while for ASL applied in REV the concentration profile develops deep into the skin, in agreement with the experimental results (Cevc and Blume, 1991; Gabrijelčič et al., 1990; Hofland et al., 1991). One possibility is that some liposomes persist during their penetration through the stratum corneum. However, it seems more probable that liposomes are damaged on the surface of the skin, but their lipids still remain in a form which constitutes some kind of envelope which protects ASL from reduction. This is corroborated with some other suggestions, that after the destruction of liposomes on the surface of the skin, a significant amount of water and entrapped substances remain associated with the liposome bilayer and are carried into the deeper layers of the skin (Egbaria and Weiner, 1990), where liposomes can be re-formed (Hofland et al., 1991). The mechanism of this phenomenon is not clear and is a matter of investigations in several laboratories, including ours.

The time evolution of concentration profiles for entrapped ASL, which was applied to the skin entrapped in REV, is presented in Fig. 4. It gives a clear illustration of the rapid transport of entrapped ASL for this particular type of liposomes into the deeper skin layers. In contrast, the penetration rate of ASL released in the barrier is slower than in inner layers, as was expected due to the hydrophilic nature of this molecule, which could not penetrate the lipophilic membranes easily (Magin and Morse, 1983).

The calculated parameters are qualitatively in close agreement with the observed pharmacological effects of liposome entrapped drugs (Schäfer-Korting et al., 1989). They support the proposed mechanism of liposome penetration through the skin by a transdermal hydration gradient in the stratum corneum (Cevc and Blume, 1991) which represents the driving force for the transport. On passing the barrier, liposomes rapidly decay, probably because they have to penetrate through the narrow maze-like passages between corneocytes filled with lamellar lipid structures. These passages are even more unsuitable for larger and more rigid MLV than for REV. Therefore, the decay rate of MLV is greater.

Conclusion

The combination of reduction kinetic imaging and 1D-EPRI experiments, together with the described model furnished a detailed insight into some processes in the skin after the application of liposomes on the skin surface. With this method, the time evolution of the concentration profiles in different skin layers can be obtained

separately for entrapped and released substances. Our observations support the idea that in the stratum corneum a larger amount of liposomes rapidly decay. However, for some types of liposomes a definite amount can penetrate deeper into the skin or at least their lipids protect the entrapped marker substance from chemical transformation induced by metabolic agents in the skin.

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