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Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements

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Abstract

The composition of vesicles determines the physical state and elasticity of their bilayers. Fatty acid spin labels were incorporated into vesicles, composed of the single chain non-ionic surfactant octaoxyethylenelaurate-ester (PEG-8-L), the sucrose laurate-ester L-595 and cholesterol sulfate (CS) to monitor local dynamic properties of lipid molecules in vesicle bilayers and to study the elasticity of vesicle bilayers. Studies with the spin label probes 5-, 12- and 16-doxyl stearic acid (DSA) indicated that both the order parameter and the rotational correlation times increased when the doxyl group was positioned closer to the headgroup region. These findings indicate that the fluidity of membranes decreased near the headgroup region. Comparing 16-DSA incorporated in vesicle formulations with either 30 or 70 mol% showed no difference in alkyl chain mobility as was reflected by the order parameter. The rotational correlation times, however, showed a slowdown from 0.38 to 0.71 and 1.13 ns when the PEG-8-L molar content was decreased from 100 to 70 and 30 mol% for PEG-8-L:L-595:CS vesicles, respectively. Extrusion measurements indicated an increase in elasticity of vesicle bilayers as the molar content of PEG-8-L was increased from 10 to 90 mol%. Incorporation of cholesterol sulfate stabilizes vesicles and thereby, decreases the elasticity. The increased elasticity correlated excellent with a reduction in the rotational correlation times observed. In conclusion, these results demonstrate that when the molar content of the single chain non-ionic surfactant PEG-8-L in vesicles is increased the elasticity is enhanced and the rotational correlation time is reduced. The enhanced elasticity might contribute to an optimal design of vesicles as drug carriers for transdermal application.

Keywords: Vesicles; Electron spin resonance; Electron microscopy; Extrusion; Fluidity; Elasticity

Abbreviations: PEG-8-L, octaoxyethylenelaurate-ester; L-595, sucrose laurate-ester L-595; CS, cholesterol sulfate; EggPC, egg phosphatidylcholine; ESR, electron spin resonance; DSA, doxyl stearic acid.

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1. Introduction

Liposomes, spherical bilayers prepared from lipids, were discovered in the early 1960s by Bangham et al. (1965) and used as models for membranes in order to study the physiological functions of biological membranes in relation to the physicochemical properties of membranes. The nature and the state of lipids are often important regulators in membrane functions and properties.

Since the 1980s, vesicles have received widespread attention as drug carriers for transdermal drug delivery due to their biocompatibility and capability to incorporate both hydrophilic and lipophilic drugs. The major problem in transdermal drug delivery is the low permeability of the most apical layer of the skin, the stratum corneum. The stratum corneum is regarded as a heterogeneous two-compartment system composed of keratin-filled corneocytes, embedded in a lipid-enriched intercellular matrix. The permeability barrier is located within lipid bilayers in the intercellular spaces of the stratum corneum (Elias and Friend, 1975; Landmann, 1986). Several classes of vesicles have been designed in an attempt either cross the skin barrier or increase diffusion of drugs through the stratum corneum. For example, studies of Knepp et al. (1990), Hofland et al. (1991) showed an increase in transport of progresterone and estradiol across the stratum corneum using liquid-state vesicles compared with gel-state vesicles. From these observations, it was concluded that only liquid-state vesicles might be able to enhance the permeation rate of drugs across the skin.

Vanlerberghe et al. (1978) broadened the type of vesicle by using non-ionic surfactants. Since then various types of surfactants have been used for the preparation of non-ionic vesicles, such as polyglycerol alkyl ethers (Handjani-Vila et al., 1979; Baillie et al., 1985), glucosyl dialkyl ethers (Kiwada et al., 1985) and polyoxyethylene alkyl ethers (Hofland et al., 1991).

The most recent development in vesicle design for (trans)dermal drug delivery is the use of elastic vesicles, differing from conventional liquid-state niosomes and liposomes by their characteristic fluid membrane with high elasticity. These features should enable vesicles to squeeze themselves through intercellular regions of the stratum corneum under the influence of the transepidermal water-activity gradient (Cevc et al., 1998) and increase the drug transport across skin further compared with rigid liquid-state vesicles. The elasticity of these vesicles is caused by the simultaneous presence of different stabilizing and destabilizing molecules, and their tendency to redistribute in bilayers. Very recently, elastic vesicles were designed based entirely on surfactants. After application onto human skin in vivo arrays of vesicular structures were observed in deeper layers in the stratum corneum (Honeywell-Nguyen et al., 2000). These arrays of vesicular structures were not observed in human skin after application of rigid liquid-state vesicles. These results were in agreement with earlier observations that only after application of elastic vesicles, channel-like permeation patterns of a lipophilic fluorescent label were observed in human stratum corneum in vitro (van den Bergh et al., 1999).

In an earlier study, we investigated the effect of incorporating a single chain non-ionic surfactant on vesicle structure by examining the ultrastructure of these vesicles with cryo and freeze fracture electron microscopy and size by dynamic light scattering (van den Bergh et al., 2000, submitted). Often a charge inducer is incorporated, such as cholesterol sulfate, in order to stabilize the vesicles by electrostatic repulsion.

In this study, the physical properties of several liquid-state vesicles, in which a single chain surfactant or cholesterol was combined with bilayer forming surfactants has been investigated using various techniques. Spin label techniques have been widely used to investigate lipid mobility in biological membranes and vesicles (Korstanje et al., 1989, 1990; Hatfield and Fung, 1995; Gershfeld and Ginsberg, 1997). Using spin label techniques, information can be obtained about the influence of for example hydration state, lamellarity, curvature and physical state of vesicles. The general hypothesis tested in the present study has been that surfactants influence fluidity of the vesicle bilayers and its elasticity. Electron spin resonance (ECR) was used to gain information on the

fluidity of the vesicle bilayers using the spin labels 5-, 12-, and 16-doxyl stearic acid (5-, 12-, and 16-DSA) and among vesicle suspensions by using 16-DSA. The extrusion rate of vesicles was measured to estimate values of elasticity. In addition, transmission and scanning electron microscopy studies were carried out to examine the penetration of vesicle suspensions into filters, which is in fact also a measure for the elasticity of the vesicles.

2. Materials and methods

2.1. Materials

The sucrose-ester Wasag-7™ was purchased from C.M. Schmidt (Amsterdam, The Netherlands), whereas the sucrose-ester L-595™ was a gift from Mitsubishi Kasei (Tokyo, Japan). Wasag-7™ consists of 70% stearate-ester and 30% palmitate-ester (40% mono, 60% di/tri-ester) (HLB, 7); L-595™ consists of 100% laurate-ester (30% mono, 40% di, 30% tri-ester) (HLB, 7). The polyoxyethylene ester PEG-8-L was a gift from Lipo Chemicals (Paterson, NJ, USA). Cholesterol (chol), cholesterol sulfate (CS) and stearic acid spin labels were purchased from Sigma chemicals. Egg phosphatidylcholine was obtained from Avanti Polar Lipids (Pelham, AL, USA). The buffer used was a phosphate buffered saline (PBS) pH 7.4 (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 139 mM NaCl, 2.5 mM KCl in millipore water). Polycarbonate membrane filters were obtained from Nucleopore (Costar Europe, Badhoevedorp, The Netherlands).

2.2. Preparation of vesicle suspensions

Vesicles were prepared by a modification of the sonication method described by Baillie et al. (1985). The surfactants, cholesterol and cholesterol sulfate were dissolved in chloroform/methanol (3:1 v/v). The solvent was evaporated overnight in a vacuum centrifuge and the remaining surfactant film was hydrated with PBS pH 7.4. The final suspension contained 5% (w/w) of lipids.

Vesicle suspensions composed of PEG-8-L:L-595:CS and PEG-8-L:EggPC were sonicated for 15 s at room temperature, whereas vesicles composed with Wasag-7 and L-595 in combination with cholesterol and cholesterol sulfate were heated to 80°C to dissolve the lipid film and sonicated for 45 s. The sonicator used was a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT) with an 1/8 inch microtip at 60 Watt energy output. The suspensions were cooled down to room temperature and pre-examined for phase separation, vesicle formation and crystal formation using a polarization microscope.

Table 1
Composition vesicle suspensions, calculated order parameters (S) and rotational correlation times (τ_0 (ns)) from the ESR spectra

Vesicle suspensions (molar ratio%)		Label	*S* value	τ_0 (ns)
Variation in composition				
PEG-8-L	100	16-DSA	0.609	0.383
PEG-8-L:L-595:CS	70:30:5	16-DSA	0.628	0.709
PEG-8-L:L-595:CS	30:70:5	16-DSA	0.610	1.13
L-595:chol.:CS	50:50:5	16-DSA	0.800	1.42
Wasag-7:chol.:CS	50:50:5	16-DSA	0.817	1.29
Variation in localization label				
PEG-8-L:L-595:CS	70:30:5	5-DSA	0.878	2.40
		12-DSA	0.668	1.15
Wasag-7:chol.:CS	50:50:5	5-DSA	1.040	17.0
		12-DSA	0.980	4.93

2.3. Electron spin resonance (ESR) measurements

For ESR measurements, the composition and molar ratio of vesicle suspensions are shown in Table 1. The vesicles were prepared as described above, except that 0.1 mol% of stearic acid spin label was added prior to the evaporation step. In this way, the label is incorporated in the vesicle bilayers. 16-DSA was used for each vesicle suspension (Table 1), as well as for micelles composed of 100 mol% PEG-8-L.

To compare the fluidity across the bilayer, 5-DSA, 12-DSA and 16-DSA were incorporated into bilayers of 70 mol% PEG-8-L:L-595:CS and Wasag-7 vesicles. In bilayers, the spin labels are oriented with their longest axis parallel to the surfactant chains, yielding information on the environment of the C_5, C_{12} and C_{16} carbons, respectively.

Aliquots of suspensions were packed in the tip of a Pasteur pipet and electron spin resonance spectra were determined with a Brucker model ESP 300 EPR spectrometer equipped with a temperature control accessory. Since the vesicles are designed for application to skin, the ESR-spectra of vesicles were measured at a temperature of 32°C, the approximate temperature of the surface of human skin.

Typical parameters by which vesicle bilayer dynamics and ordering are characterized are the order parameter S and the rotational correlation time τ_0. The order parameter S is a measure of the average angular deviation of the fatty acid acyl chain of the spin label probe at the nitroxide group from the average orientation of the fatty acids in the membrane (Seelig, 1970; Hubbel and McConnel, 1971). The order parameter is calculated from the ESR-spectra (Fig. 1, example ESR-spectrum) as a quantitative indicator of membrane fluidity. Since the outer features of $T'_{\parallel}$ of the ESR spectra of a few samples were not resolved, we decided to take the approach of Bales et al. (1977), and an estimation of S was obtained using the relationship $S = [(43.7\ \text{G} - T'_{\perp})/46.1\ \text{G}] \times 1.723$. $2'_{\perp}$ is the separation between inner hyperfine lines (Fig. 1) and is obtained from the corresponding ESR spectra. Although the numerical value of S might deviate somewhat from

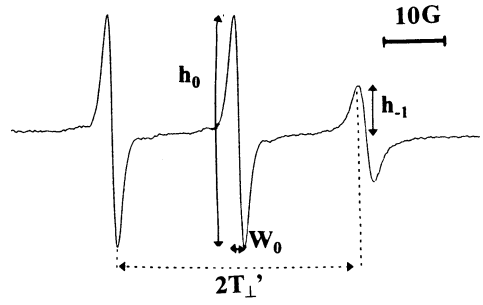

Fig. 1. Representative ESR-spectrum of 16-DSA incorporated into PEG-8-L:EggPC vesicles, measured at 32°C. $2T'_{\perp}$ is the separation between the inner hyperfine lines; W_0 and h_0 are the width and height, respectively, of the midfield line of the spectrum and h_{-1} is the height in Gauss of the high-field line.

that calculated from $T'_{\parallel}$ and $T'_{\perp}$, the relative values of S, in which we are interested in this study, are still the same (Bales et al., 1977). An S value of 1.0 is characteristic for a rigid lipid environment and a reduction in this value means an increase in the fluidity of the membranes. In the rapid motion regime the calculation of S is less reliable. For this reason we also calculated the rotational correlation time, τ_0. τ_0 reflects the dynamics of the lipid acyl chains and is calculated by the following equation Keith et al. (1970) $\tau_0 = 6.5 \times 10^{-10} W_0[(h_0/h_{-1})^{1/2}\text{-}1]$, where W_0 and h_0 are the width and height, respectively, of the midfield line of the spectrum and h_{-1} is the height in Gauss (G) of the high-field line (Fig. 1). A lower value of τ_0 reflects an increased label rotational motion.

2.4. Transmission electron microscopy (TEM)

Vesicle suspensions (150 μl/cm^2) were applied to Millipore filters with a pore size of 100 nm. The filters were placed in diffusion cells in which the upper side of the filter was exposed to the air, whereas the lower side, facing the acceptor chamber, was kept hydrated with PBS buffer. The suspensions were thus applied non-occlusively and in addition to vesicle suspensions, PBS was applied to Millipore filters as a control. After an incubation period of 2 h, the filters were removed, divided into two pieces and prepared for both transmission (TEM) and scanning electron microscopy (SEM). For TEM, samples were fixed at

4°C in Karnovsky's fixative overnight, 1% osmium tetroxide for 1 h and 0.2% Ruthenium tetroxide + 0.25% $K_3Fe(CN)_6$ for 20 min. Subsequently, the samples were dehydrated with graded ethanol solutions (30, 50, 70, 90, 95, 98 and 100%) and embedded in Spurr's resin. Ultrathin sections were cut (Ultracut E, Reichert-Jung, Austria), collected on formvar coated grids and examined in a Zeiss EM10 (Oberkochen, Germany) electron microscope.

For SEM, samples were fixed at 4°C in Karnovsky's fixative overnight, dehydrated with graded ethanol solutions (30, 50, 70, 90, 95, 98 and 100%) and coated with gold prior to examination in an Amray 1820 SEM.

2.5. Dynamic light scattering (DLS)

The *z*-average diameters of vesicles used for extrusion were determined DLS using a Malvern 4700 (Malvern Ltd., Malvern, UK) with a 25 mW He–Ne laser and the Automeasure (version 3.2) software. The samples were diluted in order to avoid multiple scattering. As a measure of the particle size distribution, the polydispersity index is calculated which ranges from 0.0 (monodisperse) to 1.0 (very heterogeneous). The measurements were performed at 27°C at an angle of 90° between laser and detector. For each vesicle composition, four suspensions were measured in triplicate by DLS.

2.6. Extrusion measurements

The elasticity of bilayers is directly proportional to $j*(r_v/r_p)^2$. j is the rate of penetration through a permeability barrier, r_v is the size of vesicles and r_p is the pore size of the barrier (Cevc, 1995). To measure j the vesicles were extruded (Sartorius Instrumenten, Nieuwegein, The Netherlands) through polycarbonate filters with a pore size of 30 nm (r_p), at a pressure of 2.5 bar. The amount of suspension, which was extruded during 10 min, was weighed (j) and plotted against PEG-8-L molar content. The following molar ratio's for PEG-8-L:L-595 and PEG-8-L:EggPC vesicles were examined: 10:90, 30:70, 50:50, 70:30 and 90:10. The incorporation of 5 mol% cholesterol sulfate into PEG-8-L:L-595 vesicles was investigated for the same molar ratio's.

3. Results

3.1. ESR measurements

3.1.1. Comparison of lipid environment and molecular motion in various vesicle suspensions and micelle solution

Table 1 shows the estimated S and τ_0 values of 16-DSA incorporated in vesicles and micelles and measured at 32°C. The S value of gel-state Wasag-7 vesicles (see Table 1) is slightly higher than that of liquid-state L-595 vesicles. A decrease in S value was observed between L-595 in the absence of the single chain surfactant PEG-8-L and the vesicles containing 30% PEG-8-L. This indicates that in the absence of PEG-8-L (but in the presence of cholesterol) the vesicles are more rigid than in the presence of PEG-8-L. However, increasing the molar content of PEG-8-L in L-595 bilayer from 30 to 70 mol% and in the micelles (100 mol% PEG-8-L) did not decrease the S value further.

From the calculated τ_0 values (Table 1) a difference is observed between micelles and liquid-state vesicles. The τ_0 value of the 16-DSA spin label in micelles is decreased compared with 70 and 30% mol PEG-8-L containing vesicles (0.709 and 1.13 ns, respectively) indicating an increased label rotational motion in micelles. A further reduction of PEG-8-L content or a replacement of L-595 by Wasag-7 surfactant does not change the τ_0 value significantly.

3.1.2. Position of the spinlabel

The PEG-8-L:L-595:CS (70:30:5) and Wasag-7:chol.:CS (50:50:5) vesicles were labeled with 5-, 12-, and 16-DSA. For both vesicle suspensions, the high field line extreme of the spectra were markedly decreased as the position of the nitroxide group changed from the C_{16} to the C_5 position, which is closer to the polar headgroup region of the bilayer. Consequently, this increased the order parameter S values (Table 1), reflecting a more restricted mobility of the fatty acid acyl

chains closer to the headgroup region of the membrane (Gaffney, 1975; Hatten et al., 1978). For cholesterol-containing Wasag-7 vesicles the mobility of fatty acid acyl chains of 5-, 12-, and 16-DSA is lower compared with those of spin labels incorporated in 70 mol% PEG-8-L vesicles.

The calculated τ_0 values show a strongly reduced rotational motion of spin-label closer to the headgroup region of the membrane for both 70 mol% PEG-8-L:L-595:CS and Wasag-7 vesicles and the τ_0 values calculated for Wasag-7 vesicles are increased compared with those of 70 mol% PEG-8-L:L-595:CS vesicles.

Comparing the S and τ_0 values of 12- and 16-DSA incorporated in both 70 mol% PEG-8-L:L-595:CS and Wasag-7 vesicles, there is a larger increase in both the mobility of the acyl chain and spin label rotational motion upon moving the spin-label form the C_{12} to the C_{16} position in Wasag-7 vesicles ($S = 0.980$ and 0.817; $\tau_0 = 4.39$ and 1.29 ns, respectively) compared with 70 mol% PEG-8-L:L-595:CS vesicles ($S = 0.668$ and 0.628; $\tau_0 = 1.15$ and 0.71 ns, respectively). Considering the calculated distance of the nitroxide spin label to the head group region; 17.0 Å for 12-DSA and 21.5 Å for 16-DSA (Hoebeke et al., 1993), both nitroxide spin-labels extend further than the C_{12} chain attached to PEG-8-L and L-595 molecules (14.4 Å) when assuming a fully extended chain in PEG-8-L:L-595:CS vesicles and, therefore, exhibit less difference in their mobility. For Wasag-7 vesicles containing C_{18} chains (21.6 Å), only 16-DSA is close to the center of the vesicle bilayer.

3.2. Transmission electron microscopy

Representative electron micrographs of Millipore filters, which were treated non-occlusively with PBS or vesicles and examined with TEM and SEM, are shown in Figs. 2 and 3, respectively. Fig. 2a and b show transmission electron micrographs of cross-sections at the surface and within a Millipore filter treated with PBS, respectively. Large cavities, represented by electron lucent areas, are present below the surface of the filter and they can be distinguished from the filter material itself by the difference in electron density. After 2 h application with liquid-state vesicles, no residual vesicle material was present at the surface of filters, indicating that vesicles penetrated into the 100 nm pores. Fig. 2c shows an overview of how PEG-8-L:L-595:CS (70:30:5) vesicles have penetrated the 100 nm pores and filled up cavities inside the filter. At higher magnification, electron dense material derived from vesicle material is visualized inside cavities (Fig. 2d). For all three liquid-state vesicle suspensions, the filters were completely filled with vesicles or vesicle material and no residual vesicle material was present on the filter surface.

After treatment with L-595 and Wasag-7 chol.-containing vesicles, however, a large amount of vesicle material was still present on the filter surface. Vesicles penetrated into the 100 nm pores only to a small extent and appear to have fused to form large multilamellar structures within the filter pores. Fig. 2e shows an overview of a filter treated with Wasag-7 vesicles. Only just below the surface of the filters, vesicle bilayers were visualized within filter cavities (Fig. 2f).

The results obtained with SEM are consistent with the TEM pictures. Fig. 3a and b show the upper and lower side of a control filter, respectively. The upper side is characterized by the presence of the 100 nm pores, whereas cavities within the filter can be observed examining the lower side of the filter. Fig. 3c–f show the surface of filters treated with PEG-8-L:L-595:CS, PEG-8-L:EggPC, Wasag-7:chol:CS and L-595:chol.:CS, respectively. Again PEG-8-L:L-595:CS and PEG-8-L:EggPC vesicles penetrated into filters, whereas cholesterol-containing vesicles are visualized on the surface of the filter.

3.3. Dynamic light scattering

The average size of vesicles that were used for extrusion experiments as measured by DLS are summarized in Table 2. The polydispersity indexes ranged from 0.12 to 0.25.

3.4. Extrusion

The calculated $j^*(r_v/r_p)^2$ values for the various vesicle suspensions are plotted against PEG-8-L molar content in Fig. 4. The average size are

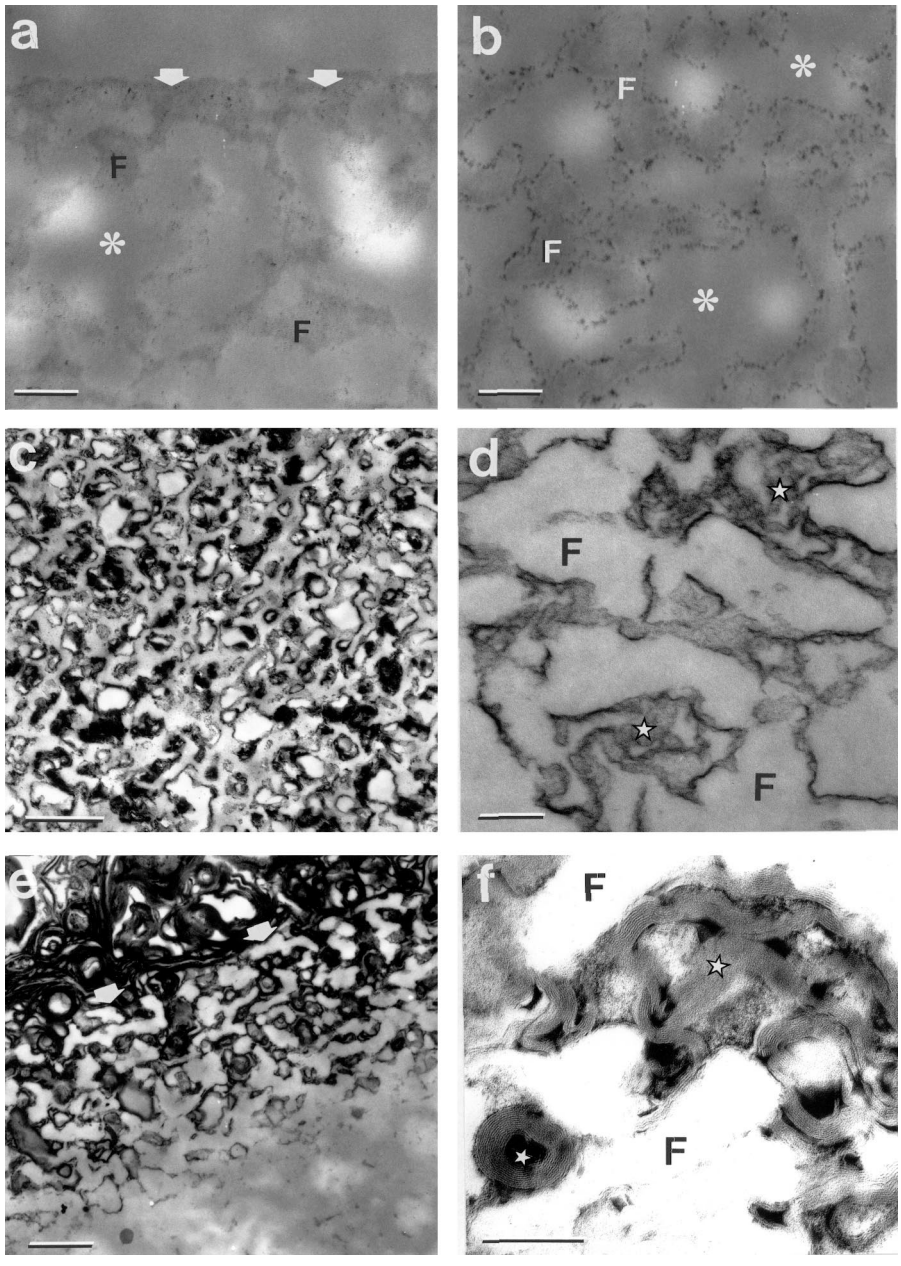

Fig. 2. TEM photographs of Millipore filters, treated non-occlusively with PBS and vesicle formulations for a period of 2 h. (a) Surface of a PBS treated filter. Bar corresponds to 200 nm. (b) Center of a PBS treated filter. Bar corresponds to 200 nm. (c) Overview of a filter treated with liquid-state vesicles PEG-8-L:L-595:CS (70:30:5). Cavities of the filter are filled with electron dense vesicle material. Bar corresponds to 1 μm. (d) Detail electron micrograph of a filter treated with liquid-state vesicles PEG-8-L:L-595:CS (70:30:5). Bar corresponds to 200 nm. (e) Overview of a filter treated with Wasag-7:chol.:CS (50:50:5) vesicles. Bar corresponds to 1 μm. (f) Filtercavity filled with L-595:chol.:CS (50:50:5) vesicle material. Lipid bilayers are clearly visualized. Bar corresponds to 200 nm. (a–f) Arrows indicate surface of the filter. * represents electron lucent cavity within the filtermaterial; F represents electron dense filtermaterial. ☆ represents filter cavity filled with vesicle material.

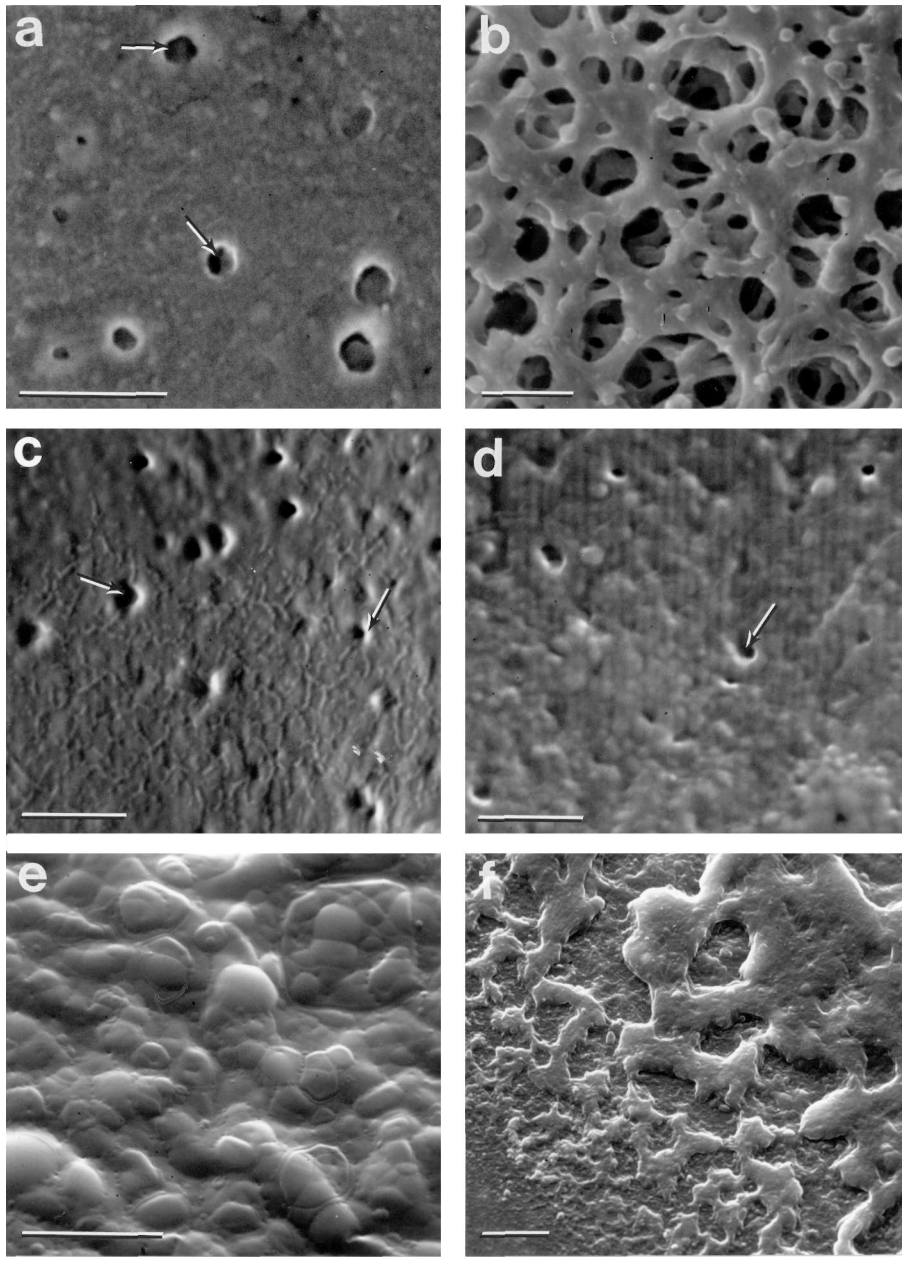

Fig. 3. SEM photographs of Millipore filters, treated non-occlusively with PBS and vesicle formulations for a period of 2 h. (a) Surface of a PBS treated filter with a 100 nm pore size. Bar corresponds to 1 μm. (b) Lower side of a PBS treated filter. Bar corresponds to 1 μm. (c–d) Filters treated with liquid-state vesicles PEG-8-L:L-595:CS (70:30:5) and PEG-8-L:EggPC (70:30), respectively. Liquid-state vesicles have penetrated through the 100 nm pores into the filter cavities. Bar corresponds to 1 μm. (e–f) Filters treated with cholesterol-containing vesicles (e) Wasag-7:chol.:CS (50:50:5) and L-595:chol.:CS (50:50:5), respectively. Vesicles remain on the surface of the filters. Bar corresponds to 10 μm. (a–f) Arrows indicate pores on the surface of the Millipore filters.

Table 2
The average sizes (r_v) of the various vesicle suspensions

Molar ratio (%)	PEG-8-L:L-595	PEG-8-L:L-595[a]	PEG-8-L:EggPC	Wasag-7:chol.[a]	L-595:chol.[a]
10:90	156.9 ± 21.7	141.9 ± 12.5	150.5 ± 26.5	–	–
30:70	130.7 ± 18.3	122.8 ± 13.8	97.4 ± 9.7	–	–
50:50	134.3 ± 28.2	122.7 ± 18.4	139.7 ± 5.9	176.7 ± 3.0	155.8 ± 2.8
70:30	189.1 ± 33.0	148.5 ± 23.3	104.0 ± 22.0	–	–
90:10	258.0 ± 66.5	180.4 ± 12.2	201.8 ± 10.1	–	–

[a] Sizes are given as mean ± S.D. ($n = 4$). These suspensions contain 5 mol% CS.

provided in Table 2. For liquid-state suspensions, PEG-8-L:L-595:CS molar ratio 10:90:5 and PEG-8-L:EggPC molar ratio 10:90 and 30:70 could not be extruded through 30 nm pores. Both cholesterol-containing L-595 and Wasag-7 suspensions could not be extruded either. Increasing the PEG-8-L content in all three liquid-state vesicle suspensions increased the elasticity value of the membranes. Incorporating 5 mol% CS in PEG-8-L:L-595 (90:10 mol%) vesicle bilayers decreased the elasticity values significantly ($P < 0.05$), whereas combining PEG-8-L with EggPC instead of L-595 decreased elasticity values significantly at a PEG-8-L molar content of 50, 70 and 90 mol% ($P < 0.01$).

4. Discussion

In an earlier study (van den Bergh et al., 2000, submitted) we described the ultrastructural changes of liquid-state vesicles when a single chain non-ionic surfactant was incorporated into vesicle bilayers and the molar content was varied. Initially, increasing the amount of the single chain non-ionic surfactant in vesicle bilayers gives rise to a growth in vesicle size and vesicle aggregation or fusion. However, further increasing the content of the single chain non-ionic surfactant leads to pore-formation in bilayers and when the concentration of surfactant reaches a certain threshold, micelles or mixed micelles are starting to be formed. In the current study, we have examined the effect of incorporating a single chain non-ionic surfactant or cholesterol into vesicle bilayers on the bilayer elasticity. Studies of Cevc et al. (1993) revealed that the simultaneous presence in one membrane of different stabilizing/destabilizing molecules, and their tendency to redistribute in the bilayers, enables vesicles to be elastic (van den Bergh et al., 2000, submitted; Cevc et al., 1993).

Fatty acid spin labels were used to monitor local dynamic properties of lipid molecules in the vesicle bilayer. 5-DSA reports on mobility and rotational motion near the polar headgroup region, whereas 12- and 16-DSA describe mobility and rotational motion closer the center of the bilayer (Jost et al., 1971; Smith, 1972; Zhang and Fung, 1994). The calculated S-values from our ESR results demonstrate that the mobility of the acyl chain near the polar headgroup was more restricted than the mobility in the acyl chain region in the center of the vesicle bilayer for liquid-state vesicles composed of PEG-8-L:L-595:CS (70:30:5), as well as for cholesterol-con-

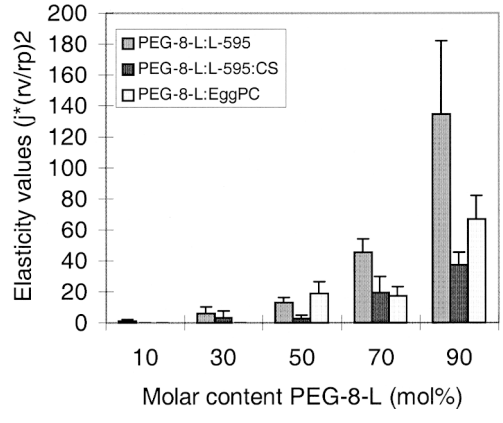

Fig. 4. Calculated elasticity values for extruded vesicle suspensions in relation to the vesicle composition and PEG-8-L content.

taining Wasag-7 vesicles. From ESR spectra of the spin-labeled fatty acids incorporated in bilayers, a decrease is observed in the hyperfine splitting as the nitroxide group was inserted deeper in the hydrophobic core of membranes.

The mobility of the fatty acid acyl chain of the spin label, reflected by the order parameter S of 16-DSA, did not alter the fluidity of the membranes when increasing the alkyl chain length from C12 to C18 of sucrose ester (L-595 to Wasag-7). In liquid-state vesicles with 30% mol PEG-8-L the order parameter S is reduced compared with that in the absence of PEG-8-L, reflecting the increased fluidity in the former. In liquid-state vesicles and micelles with 70% mol PEG-8-L content and micelles (100% PEG-8-L) the order parameter S was shown to vary only slightly. The rotational motion of the spin-label, however, reflected by the rotational correlation time τ_0, showed a systematic change in rotational motion when the molar content of PEG-8-L is increased from 30 to 70 mol% (0.71 and 1.13 ns, respectively) and to 100 mol% ($\tau_0 = 0.383$ ns). A decrease in τ_0 indicates an increase in rotational motion of the spin label. Although this is a direct measure for the rotational mobility of the label and thus the fluidity of the membrane, it is not a direct measure for the elasticity of the bilayers. However, an increase in elasticity and thus a reduction in the interfacial tension is expected to be correlated with a reduced restriction of the surfactant molecules to move (increased fluidity) in order to be able to easily redistribute in the bilayers.

Cholesterol has been shown to affect a variety of membrane properties (Oldfield and Chapman, 1972; Chapman and Benga, 1984); incorporation of cholesterol in gel-state bilayers can induce a continuous transition to an ordered liquid-crystalline state in gel-state bilayers and an increase in ordering in liquid-state bilayers. Korstanje et al. (1990) have studied the effects of steroids on the dynamic structure of liquid-state vesicles and found that incorporation of steroids in bilayers enhances the orientational order S of the spin label molecules and thus the degree in molecular ordering.

Increasing the molar PEG-8-L content in liquid-state vesicle suspensions, increased bilayer elasticity as shown in Fig. 4. Although vesicles become larger in size (Table 2a), the amount of extruded material through 30 nm pores increases when the PEG-8-L content is changed from 10 to 90 mol%. However, according to earlier ultrastructural studies (van den Bergh et al., 2000, submitted), micelles are starting to be formed in suspensions composed of 60 mol% PEG-8-L and are abundantly present in 90 mol% PEG-8-L suspensions. The presence of micelles may result in an enhanced amount of extruded material and, therefore, contribute to increased elasticity values. The vesicle size distribution measured by DLS did not show the presence of micelles, however, the size of the micelles might have been too small (< 10 nm) to be detected by the DLS. Therefore, it is important to establish an extensive characterization of the ultrastructure of vesicle suspensions in addition to DLS to detect micelles.

Incorporating CS in PEG-8-L:L-595 vesicles decreases the elasticity significantly when the PEG-8-L molar content ≥ 50 mol%. The CS induces a charge in bilayers thereby limiting fusion and/or aggregation of the vesicles. The decrease in elasticity is in accordance with ultrastructural changes we observed with cryo-TEM (van den Bergh et al., 2000, submitted) when CS is incorporated in PEG-8-L:L-595 vesicles. The threshold concentration at which micelle-formation occurs is shifted towards a higher amount of PEG-8-L, which can be incorporated in vesicles.

The visualization of the extrusion of vesicles into the filters by TEM and SEM confirm the difference in penetration for liquid-state vesicles and cholesterol-containing vesicles and thus the difference in elasticity of these vesicles. Using these visualization techniques, it is not possible to quantify differences in elasticity between suspensions. However, the micrographs give an indication of vesicle penetration into the filters.

The results in this study demonstrate that increasing the molar content of a single chain nonionic surfactant in vesicle bilayers increases fluidity and elasticity of these bilayers. However, at a certain single chain surfactant/double chain surfactant ratio, micelles are starting to be formed

(van den Bergh et al., 2000, submitted) and the stability of the vesicles decreases As demonstrated by vesicles containing 70% PEG-8-L and 30% L-595, which increased in size during a time course of 7 days. To stabilize vesicles, a charge inducer such as cholesterol sulfate can be incorporated. In order to design vesicles for topical drug delivery in which vesicle bilayers elasticity is thought to be an advantage, an optimum should be found between stability and elasticity of vesicle bilayers.

References

Baillie, A.J., Florence, A.T., Hume, L.R., Muirhead, G.T., Rogerson, A., 1985. The preparation and properties of niosomes-non-ionic surfactant vesicles. J. Pharm. Pharmacol. 37, 863–868.

Bales, B.L., Lesin, E.S., Oppenheimer, S.B., 1977. On cell membrane lipid fluidity and plant lectine agglutinability. Biochim. Biophys. Acta 465, 400–407.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Cevc, G., 1995. In: Lipowsky, R., Sackmann, E. (Eds.), Material Transport in Across Permeability Barriers by Means of Lipid Vesicles in Handbook of Biological Physics. Elsevier, Amsterdam, pp. 465–490.

Cevc, G., Schätzlein, A., Gebauer, D., Blume, G., 1993. Ultrahigh efficiency of drug and peptide transfer through intact skin by means of novel drug carriers, transfersomes in Prediction of Percutaneous Penetration, 3rd International Conference; 14–16 April 1993 (Brain, K.R., James, V.J., Walters, K.A., Eds.), pp. 226–236, STS Publishing, Cardiff.

Cevc, G., Gebauer, D., Stieber, J., Schatzlein, A., Blume, G., 1998. Ultraflexible vesicles, transfersomes, have an extremely pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. Biochim. Biophys. Acta 1368, 201–215.

Chapman, D., Benga, G., 1984. In: Chapman, D. (Ed.), Biological Membranes, vol. V. Academic Press, New York, pp. 1–56.

Elias, P.M., Friend, D.S., 1975. The permeability barrier in mammalian epidermis. J. Cell Biol. 65, 180–191.

Gaffney, B.J., 1975. Fatty acid chain flexibility in the membranes of normal and transformed fibroblasts. Proc. Natl. Acad. Sci. USA 72, 664–668.

Gershfeld, N.L., Ginsberg, L., 1997. Probing the critical unilamellar state of membranes. J. Memb. Biol. 156, 279–286.

Handjani-Vila, R.M., Ribier, A., Rondot, B., Vanlerberghe, G., 1979. Dispersions of lamellar phases of no-ionic lipids in cosmetic products. Int. J. Cosmet. Sci. 1, 303–314.

Hatfield, R.M., Fung, L.W.M., 1995. Molecular properties of stratum corneum lipid model system: large unilamellar vesicles. Biophys. J. 68, 196–207.

Hatten, M.E., Scandella, C.J., Horwitz, A.F., Burger, M.M., 1978. Imilarities in the membrane fluidity of 3T3 and SV 101-3T3 cells and its relation to concanavalin A-and wheat germ agglutin-induced agglutination. J. Biol. Chem. 253, 1972–1977.

Hoebeke, M., Seret, A., Piette, J., Van de Vorst, A., 1993. Destruction of stearic acid notroxyl radicals mediated by photoexcited merocyanine 540 in liposomal and micellar systems. Biochemistry 32, 2730–2736.

Hofland, H.E.J., Bouwstra, J.A., Geest van der, R., Boddé, H.E., Spies, F., Junginger, H.E., 1991. Transdermal delivery of estradiol from non-ionic surfactant vesicles and visualization of effects these vesicles have on human skin in vitro. Proc. Int. Symp. Contr. Rel. Bioact. Mater. 18, 155–155.

Honeywell-Nguyen, P.L., Graaff, de A., Junginger, H.E., Bouwstra, J.A., 2000. Interactions between elastic and rigid vesicles with human skin in vivo. Proc. Int. Symp. Contr. Rel. Bioact. Mater. 27, 237–238.

Hubbel, W.L., McConnel, H.M., 1971. Molecular motion in spin-labeled phospholipids and membranes. J. Am. Chem. Soc. 93, 314–326.

Jost, P., Libertini, L.J., Herbert, V.C., Griffith, O.H., 1971. Lipid spin labels in lecithin multilayers: a study of motion along fatty acid chains. J. Mol. Biol. 59, 77–98.

Keith, A.D., Bulfield, G., Snipes, W., 1970. Biophys. J. 10, 618–626.

Kiwada, H., Niimura, H., Kato, Y., 1985. Tissue distribution and pharmacokinetic evaluation of the targetting efficiency of synthetic alkyl glycoside vesicles. Chem. Pharm. Bull. 33, 2475–2482.

Knepp, V.M., Szoka, F.C., Guy, R.H., 1990. Controlled drug release from a novel liposome delivery system. II. Transdermal delivery characteristics. J. Contr. Rel. 12, 25–30.

Korstanje, L.J., van Faassen, E.E., Levine, Y.K., 1989. Reorientational dynamics in lipid vesicles and lipsomes studied with ESR: effect of hydration, curvature and unsaturation. Biochim. Biophys. Acta 982, 196–204.

Korstanje, L.J., van Ginkel, G., Levine, Y.K., 1990. Effect of steriod molecules on the dynamic structure of dioleylphosphatidylcholine and digalactosyldiacylglycerol bilayers. Biochim. Biophys. Acta 1022, 155–162.

Landmann, L., 1986. Epidermal permeability barrier: transformation of lamellar granular disks into intercellular sheets by a membrane-fusion process. J. Invest. Dermatol. 87, 202–209.

Oldfield, E., Chapman, D., 1972. Molecular dynamics of cholesterol and sphingomyelin-cholesterol interactions: in myelin membrane structures. FEBS Lett. 21, 303–306.

Seelig, J., 1970. Spin label studies for oriented smectic crystals: a model system for bilayer membranes. J. Am. Chem. Soc. 92, 3881–3887.

Smith, I.C.P., 1972. In: Swartz, H.M., Bolton, J.R., Borg, D.C. (Eds.), The Spin Label Method in Biological Applications of Electron Spin Resonance. Wiley, New York, pp. 483–539.

van den Bergh, B.A.I., Vroom, J., Gerritsen, H., Junginger, H.E., Bouwstra, J.A., 1999. Interactions of elastic and rigid vesicles with human skin in vitro: electron microscopy and two-photon excitation microscopy. Biochim. Biophys. Acta 1461, 155–173.

van den Bergh, B.A.I., Frederik, P.M., Bomans, P.H.H., Junginger, H.E., Bouwstra, J.A., 2000 submitted for publication to Biochim. Biophys. Acta, in press.

Vanlerberghe, G., Handjani-Vila, R.M., Ribier, A., 1978. Les 'niosomes' une nouvelle famille de vesicules a base d'amphiphiles non ioniques. Colloques Nationaux du C.N.R.S. 304–304.

Zhang, Y., Fung, L.W.M., 1994. The roles of ascorbic acid and other antioxidants in the erythrocyte in reducing membrane nitroxide radicals. Free Rad. Biol. Med. 16, 215–222.