Physicochemical Characteristics of Liposomes Formed with Internal Wool Lipids

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ABSTRACT: The bilayer-forming capability of internal wool lipids and their physicochemical properties were studied in an attempt to enhance our understanding of the lipid structure present in wool and other keratinized tissues. Internal wool lipids were extracted and analyzed, and the mixture obtained [sterol esters (10%), free fatty acids (24%), sterols (11%), ceramides (46%), and cholesteryl sulfate (9%)] was shown to form stable liposomes. A phase-transition temperature of 60°C was obtained from nuclear magnetic resonance spectra for this lipid mixture. The spontaneous permeability of these vesicles was lower than that of phosphatidylcholine liposomes but slightly higher than that of the vesicles formed with lipids extracted from other keratinized tissues with higher amounts of cholesterol. The transmission electron micrographs showed large vesicular aggregates of approximately 300 nm, which seem to be made up of smaller structures of approximately 20 nm in size. This particular structure could account for the large diameters and small internal volumes found by dynamic light-scattering and spectrofluorometric measurements.

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KEY WORDS: Liposome, NMR, TEM, TLC/FID, wool lipids.

Fine wool fibers are composite materials that consist of two types of cells: cortical cells, which make up the bulk of the fiber, and overlapping cuticle cells, which enclose the cortex. Cuticle cells are separated from the underlying cortical cells by a cell membrane complex (CMC), which also surrounds individual cortical cells.

The CMC of wool plays an important role in adhesion between the cells of both the cuticle and the cortex in the keratinized fiber, in the transport of dyestuffs and processing chemicals into the fiber, and in determination of the surface properties of the fiber (1).

It is possible to distinguish the different CMC regions according to their dyeability. There are two resistant membranes, two unstained layers, called the β-layers, and a dark stained central layer, the δ-layer. Chemically, the CMC is mainly made up of proteins, except the β-layers, which are made up of lipids and are assumed to form a bilayer structure. Merino wool fibers contain about 1% by weight of lipids. The major lipids present in the CMC of wool (sterols, fatty acids, and ceramides) (2,3) do not resemble those normally found in viable cells (such as phospholipids) but resemble those found in the membranes of the stratum corneum (SC) of skin.

Despite recent advances in characterizing the lipid components of the CMC (1–4), little progress has been made in furthering our understanding of the structure and arrangement of these components within the CMC. The β-layers of the CMC are assumed to be attributed to lipid bilayers, derived from the plasma membranes of living cells. The types of lipids, low levels of phospholipids found in the CMC, and observations that phospholipids are lost in keratinization of skin (5) suggest, however, that the lipid bilayers could be fairly different from those in plasma membranes.

The lipids of the SC are capable of forming liposomes (6), even when phospholipids are not present. Thus, the β-layers in the CMC might contain a lipid bilayer similar to the multiple intercellular lipid lamallae formed in the stratum corneum of mammals (7). In fact, liposomes have been formed from hair and wool lipids (8).

Much work has been performed in our laboratories on the effect of surfactants on phosphatidylcholine liposomes (9–12), and liposomes formed with lipids from keratinized tissues have aroused our interest because of their possible mimetic behavior. In fact, recent work, based on the formation of stratum corneum lipid liposomes, involved the study of the influence of different synthetic lipid mixtures (approximating the composition of the stratum corneum) on bilayer formation and their physicochemical properties (13,14). Furthermore, liposomes were prepared from a mixture of lipids extracted from pig SC, and their physicochemical properties were also determined (15).

Hence, in the present work, we investigated the bilayer-forming capability of internal wool lipids and their physicochemical properties, especially their permeability. Comparison of these results with the findings obtained with the bilayers formed with SC lipids and with phospholipids could improve our understanding of the lipid structure present in keratinized tissues.

EXPERIMENTAL PROCEDURES

Materials. Raw industrially scoured Spanish Merino wool, supplied by Corcoy S.A. (Tarrasa, Spain), was used to obtain

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the internal wool lipids (IWL). Prior to the extraction, wool was equilibrated in a conditioned room (20°C, 60% relative humidity).

All chemicals were of analytical grade, and the standards used were supplied by Sigma Co. (St. Louis, MO) (Ceramides type III and cholesterol sulfate) and by Fluka (Buchs, Switzerland) (cholesteryl palmitate, palmitic acid, and cholesterol).

The nonionic surfactant Triton X-100, octylphenol polyethoxylated with 10 units of ethylene oxide and active matter of 100%, was purchased from Rohm and Haas (Lyon, France). *Tris*-(hydroxymethyl)-aminomethane (TRIS) was supplied by Merck (Darmstadt, Germany). Fluorescent 5(6)-carboxyfluorescein (CF) (Eastman Kodak, Rochester, NY) was purified by a column-chromatographic method (16). The buffer used was 5.0 mM TRIS with 100 mM NaCl (reagent-grade), adjusted to pH 7.50, and supplemented with 10 mM CF when studying bilayer permeability of liposomes. Water was purified by a Milli-Ro system (Millipore, Madrid, Spain).

Isolation and analysis of internal wool lipids. The internal lipids were Soxhlet-extracted from cleaned wool (4 g) with chloroform/methanol azeotrope (250 mL, 79:21, vol/vol) for 5 h. The lipid extracts were concentrated down to 10 mL under a stream of dry nitrogen and stored in chloroform/methanol (2:1, vol/vol) at 6°C. Aliquots were dried and weighed, and the lipid extraction percentages were determined (3,17).

Qualitative lipid analysis was performed as described in an earlier work (3). The quantitative analysis was performed by thin-layer chromatography (TLC), coupled to an automated ionization detection (FID) system (Iatroscan MK-5; Iatron Lab. Inc., Tokyo, Japan) (18,19).

The lipid fractions were directly spotted (1 μL) with a Sample Spotter SES 3202/IS-01 (SES GmbH, Nieder-Olm, Germany) on Silica Gel SIII Chromarods and developed with the first solvent system (i) *n*-hexane/ethyl ether/formic acid (50:20:0.3, vol/vol/vol) to separate the nonpolar lipids. After partial scan of 80% to quantitate and eliminate the apolar lipids, the redevelopment of Chromarods with (ii) chloroform/methanol/ammonia (58:10:2.5, vol/vol/vol), twice for 7 cm, leads to the separation and quantitation of the polar lipids (3,20). The same procedure was applied to the standards cholesteryl palmitate, palmitic acid, cholesterol, ceramide III, and cholesteryl sulfate to determine their calibration curves for quantitation of each compound.

Preparation of liposomes. Liposomes were formed by IWL that were extracted and analyzed as previously described. IWL solution (0.5 mL, ≈10 mg/mL chloroform/methanol 2:1) was taken to dryness in culture tubes with a stream of nitrogen. Buffer (4 mL) that contained 100 mM NaCl, 5 mM TRIS and was supplemented with 10 mM CF dye to provide the final concentration of approximately 1 mg lipid per mL at pH 7.50, was added. Suspensions were then sonicated in a Labsonic 1510 (B. Braum, Melsungen, Germany) sonicator at 100 Watt with a Ultraterm 6000383 (Selecta, Barcelona, Spain) thermostated bath at a temperature of 65°C (5°C higher than that corresponding to the phase transition temperature of IWL, see the Results and Discussion section) for about 15 min until the suspensions became clear. The preparations were then annealed at the same temperature for 30 min and incubated at 37°C under nitrogen atmosphere.

Characterization of liposomes. Phase transition temperature was determined by nuclear magnetic resonance (NMR) spectrometry. IWL were left to hydrate for 10 min at 80°C in 1 mL deuterated water. The suspensions were then sonicated in a bath sonicator at 80°C for 10 min. Proton magnetic resonance spectra were obtained every 5 degrees ranging from 25–95°C on a 300-MHz Varian Unity (Palo Alto, CA) to determine the phase-transition temperatures of the lipid dispersions forming liposomes. The line widths of the CH_2 band at 1.3 ppm were measured after 1024-scan accumulation. The representation of the different line widths vs. the temperatures gave an inflection point that can be taken as the phase-transition temperature for the lipid mixture.

Permeability alterations, encapsulation efficiency of bilayers, and internal volumes were determined as follows: the complete liposomes suspensions were chromatographed through Sephadex G-50 medium resin (Pharmacia Uppsala, Sweden) with TRIS buffer, and the liposome fraction that was freed of unencapsulated material was made up to 100 mL with TRIS buffer to measure the liposome permeability spectrofluorimetrically by means of CF release.

The spontaneous permeability alterations of the IWL liposomes vs. time were determined by monitoring the release of the CF from these structures (21). Fluorescence measurements were made at different times with a Shimadzu RF-540 (Kyoto, Japan) spectrofluorophotometer, equipped with a thermoregulated cell compartment (λ_{ex}495 nm, λ_{em}515.4 nm).

The proportion of the CF released was calculated by means of the following equation (16):

$$\%\text{CF release} = \frac{I_t - I_0}{I_\infty - I_0} \cdot 100 \qquad [1]$$

where I_0 is the initial fluorescence intensity of the CF-loaded liposome suspension at 515.4 nm, and I_∞ is the fluorescence intensity at 515.4 nm after destroying the liposomes by the addition of Triton X-100 (Rohm and Haas) [60 μL of 10% (vol/vol) solution]. I_t corresponds to the fluorescence intensity at different time intervals.

The encapsulation efficiency, defined as the fraction of the aqueous compartment sequestered by bilayers and expressed in percentage, was calculated from the I_∞ value.

The internal volume of liposomes, defined as the volume enclosed by a given amount of extracted material and expressed as μL/mg, was calculated from the ratio between the encapsulation efficiency value and the lipid amount in the liposome suspension.

Mean vesicle size distribution and polydispersity indexes of the IWL liposomes were determined after preparation of liposome suspensions using a Photon correlator spectrometer (Malvern Autosizer 4700c PS/MV, Malvern, England) by particle number measurement at 37°C with a lecture angle of 90°.

Electron micrographs were obtained by two different

methodologies: freeze-fracturing and negative staining. For freeze-fracturing, liposome suspensions were placed on thin copper specimen carrier plates and frozen in liquid propane at −190°C. Freeze-fracturing was carried out in a Balzers 301 apparatus (Balzers AG, Balzers, Liechtenstein), and the specimen was shadowed with platinum and coated with carbon. The replicas were then coated with a support film of Parlodion (SPI Supplies, West Chester, PA), applied in amyl acetate, and air-dried before the copper carriers were dissolved by floating in an acid mixture (orthophosphoric/sulfuric/glacial acetic, 1:1:1). The replicas were then washed in distilled water, cleaned in Clorox bleach for 2–3 h, and rinsed several times in distilled water before being picked up on Formvar-coated grids. The Parlodion support film was dissolved by standing in methanol for 30 min. The cleaned replicas were examined in a Hitachi H-600 AB transmission electron microscope (Hitachi, Mito, Japan) at 75 kV.

For negative staining, carbon-coated/palladium grids G-400 mesh, 0.5 Taab with 0.5% E 950 collodium films in *n*-amyl acetate were employed. A drop of the vesicular solution was sucked off the grid, and after 1 min, it was blotted with filter paper down to a thin film. Negative staining with a drop of a 1% solution of uranyl acetate was performed. After 1 min, this drop was again removed with filter paper, and the resulting stained film was dried in a dust-free place. Samples were also examined in a Hitachi H-600 AB transmission electron microscope at 75 kV.

RESULTS AND DISCUSSION

Lipid analyses. IWL were obtained by extraction in a Soxhlet with chloroform/methanol azeotrope. The yield of lipids was 1.2% on wool weight. IWL have been reported at similar weight percentages: 0.8 (3), 1.2% (22) and 1.5% (1).

Application of the TLC/FID technique to the extract enabled us to quantitatively determine its composition. The method consists in a multiple development of chromarods with solvent systems described in the experimental part by using a partial scan to resolve the nonpolar lipids and a total scan for the polar lipids. The same procedure was applied to standard compounds, which resulted in the following response factors: SE, $0.4 \cdot 10^{-3}$; FFA, $0.35 \cdot 10^{-3}$; CHOL, $0.24 \cdot 10^{-3}$; CA, $0.30 \cdot 10^{-3}$; and CHOL-S, $0.92 \cdot 10^{-3}$.

The percentages on total lipid extract were obtained from the amount of each compound after multiplying each area by the corresponding response factor. The mean value of five lipid analyses of the lipid wool extract gave the lipid percentages of the major lipid classes expressed as sterol esters (SE), 9.7; free fatty acids (FFA), 23.6; cholesterol (CHOL), 11.5; ceramides (CA), 46.4; cholesteryl sulfate (CHOL-S), 8.8 wt%.

Similar values were obtained before for the composition of IWL (3,19), even though higher cholesterol percentages have been obtained by other authors (2,18). The lipids from other keratinized tissues, such as stratum corneum, also consisted of the same kind of compounds, the main difference being the higher amount of cholesterol (≈25%) (7,15).

Liposome formation and characterization. Liposomes were prepared with the IWL composition by following the method described in the experimental part. Liposome formation is not surprising because the percentages of free fatty acids at 23.6% and cholesteryl sulfate at 8.8% fall in the range that is capable of forming vesicles (6,13).

The lipid mixture chosen as a model of lipids of another keratinized tissue, such as SC (6), has a similar composition in free fatty acids (25%), ceramides (40%), and cholesteryl sulfate (10%), even though with a higher amount of free cholesterol (25%). The presence of either free fatty acids or cholesteryl sulfate, which are ionized at physiological pH, is considered to be essential for bilayer formation (6). Furthermore, lipid mixtures with the same compounds but at different relative concentrations, as indicated below, were also capable of forming liposomes (13,14): palmitic acid (21.25 to 28.75%), cholesterol (21.25 to 28.75%), ceramides (34 to 46%), and cholesteryl sulfate (0.25 to 19.75). All lipid mixtures reported led to bilayer formation, even though their physicochemical characteristics varied with the lipid composition used. Differences in the permeability of the vesicles are expected because of the smaller amount of cholesterol, which is known to affect the fluidity of the membrane (14,23).

The phase transition temperature of the IWL liposome suspension was determined as described in the experimental part. The different line widths of the CH_2 band of the NMR spectra were obtained at the thirteen temperatures investigated.

The different line widths are presented vs. the temperature in Figure 1. They give an inflection point at about 60°C for IWL, which can be taken as the phase-transition temperature for this lipid mixture.

This value is much higher than the phase-transition temperature of lipid mixtures that are usually used to form liposomes, such as phosphatidylcholine (PC), which is below 0°C. However, it is slightly lower than the value obtained for

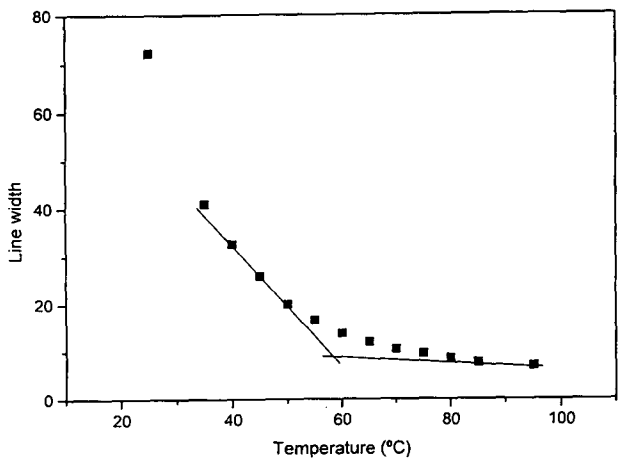

FIG. 1. Phase-transition temperature of internal wool lipids, obtained by the inflexion point of the representation of line widths of the CH_2 band of ^1H nuclear magnetic resonance spectra vs. temperature.

pig stratum corneum lipids [65°C (15)] or human horny layer lipids [75°C (6)]. This difference could be due to the different lipid composition, especially the smaller amount of cholesterol in the IWL liposomes.

The particle size distribution of the IWL liposome suspensions was determined at 1 h and 24 h after vesicle preparation. The results are expressed in Table 1. A particle size average mean distribution of about 270 nm and a polydispersity index of around 0.2 was found at the two different times, which translates to a size range of 150–500 nm. The stability of this suspension was longer than a week.

A smaller size range has been found by other authors who formed liposomes with lipids from keratinized tissues: 20–150 nm with SC lipids (6,13,15), 20–90 nm with human hair lipids (8), and 20–80 nm with wool lipids (8). The larger size obtained by us vs. the results of other authors could be attributed to the lower temperature of 65°C and the shorter sonication time, 15 min, at which IWL liposomes were formed. However, we cannot discard the presence of small particles that are undetected by this technique because they could be masked by the large particles, which have greater intensity.

The encapsulation efficiency and internal volume of the IWL vesicles (Table 1) are much lower than those published for PC vesicles of similar size, irrespective of whether they are multilamellar vesicles (MLV) or large unilamellar vesicles (LUV) (24). Surprisingly, the values of encapsulation efficiency and the internal volume corresponded to the values obtained for small unilamellar vesicles of PC with diameters that are ten times smaller than those obtained for IWL liposomes.

Similar results were obtained for vesicles formed with pig SC lipids, even though a higher internal volume was obtained in that study (15).

Spontaneous permeability and transmission electron micrographs (TEM) can shed light on the nature of the structural associations that lead to the formation of different types of liposomes, which may be correlated with the nature of the component-building bilayers.

The bilayer permeability of the IWL liposome suspensions were followed by kinetic studies on the release of the fluorescent dye 5-(6)CF, encapsulated in the interior of bilayers, as a function of time in the absence of any surfactant. The results of this spontaneous permeability are compared with those obtained from PC and SC lipids as presented elsewhere (15) in Figure 2.

TABLE 1
Particle Size Distribution, Encapsulation Efficiency, and Internal Volume of Internal Wool Lipids (IWL) Vesicles

Sample	Particle size distribution: Average mean (nm)	Particle size distribution: Polydispersity	Encapsulation efficiency (%)	Internal volume µL/mg
IWL				
1 h	265	0.173	0.076	0.4483
24 h	282	0.276		

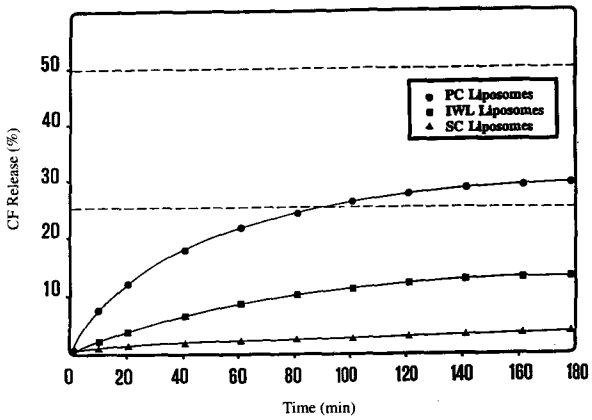

FIG. 2. Spontaneous permeability of liposome suspensions of internal wool lipids (IWL), phosphatidylcholine (PC), and stratum corneum lipids (SC) as a function of time.

The spontaneous permeability of IWL is extremely low when compared with PC liposomes, but it is not as negligible as for SC liposomes (14,15). The higher permeability, at room temperature, of the membranes of viable cells, mainly composed of PC, with respect to the membranes from keratinized tissues, such as SC or wool, could be due to the different phase-transition temperatures of egg PC (below freezing) and of SC and IWL (between 60 and 65°C). Furthermore, the slightly higher spontaneous permeability of the IWL liposomes with respect to those of SC could be attributed to the differences in lipid composition, especially to the lower amount of cholesterol in IWL. These findings are in line with those reported by Kibat and Stricker (25), who investigated the influence of cholesterol and other factors on the permeability of soy lecithine liposomes.

Samples that correspond to the IWL liposome suspensions were examined by the TEM technique with freeze-fracturing and negative-staining methodologies to determine the type of structures formed and to confirm the influence of the IWL on liposome structure and vesicle size. Two representative pictures with freeze-fracturing and negative-staining are shown in Figures 3 and 4, respectively.

There is agreement between the microphotographs obtained by the two methodologies. There are large aggregates, approximately 300 nm in size, and much smaller structures, approximately 20 nm in size. These two sharply contrasting sizes and the small ratio in number of small particles with respect to the big ones (less than 100) could explain the nondetection of the small vesicles by light scattering (which for particles with a diameter 10 times smaller would need a ratio of at least 1000 to obtain the same intensity). A striking feature is the "blackberry-like structure," which is more clearly shown in Figure 3. This seems to be made up of smaller vesicles.

The controversial results discussed above, big vesicle size distribution and a small internal volume, could be explained by this unusual structure in which small vesicles with a small internal diameter form bigger structues, which leads to a large size distribution.

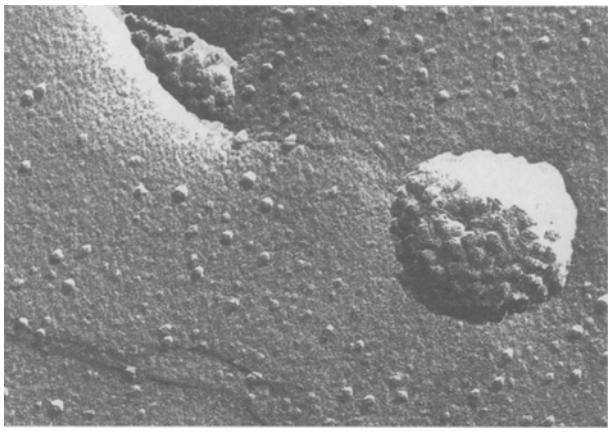

FIG. 3. Transmission electron micrograph photomicrograph of internal wool lipids liposomes, obtained by freeze-fracturing. Bar represents 100 nm.

Summarizing, IWL were extracted, quantitated, and shown to form stable liposomes with a phase-transition temperature of about 60°C. The spontaneous permeability of these vesicles was low but slightly higher than that of the vesicles formed with lipids from other keratinized tissues. A particle size average mean distribution of 270 nm was controversial, given the low encapsulation efficiency and small internal volume. However, the TEM micrographs show large aggregates, which are approximately 300 nm in size and are made up of smaller structures of approximately 20 nm in size, which could account for these results.

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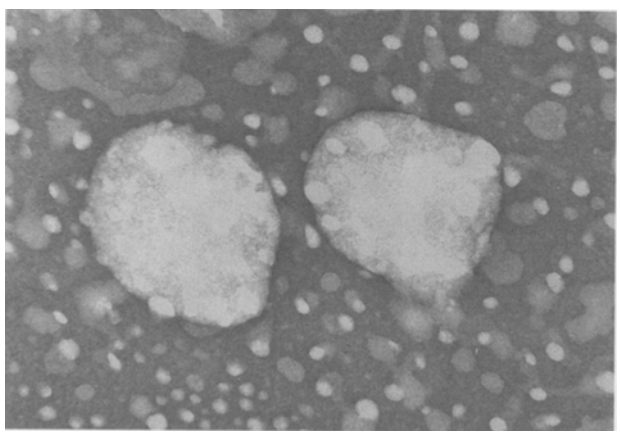

FIG. 4. Transmission electron micrograph photomicrograph of internal wool lipids liposomes, obtained by negative staining. Bar represents 100 nm.

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