Bicelles: Lipid Nanostructured Platforms with Potential Dermal Applications

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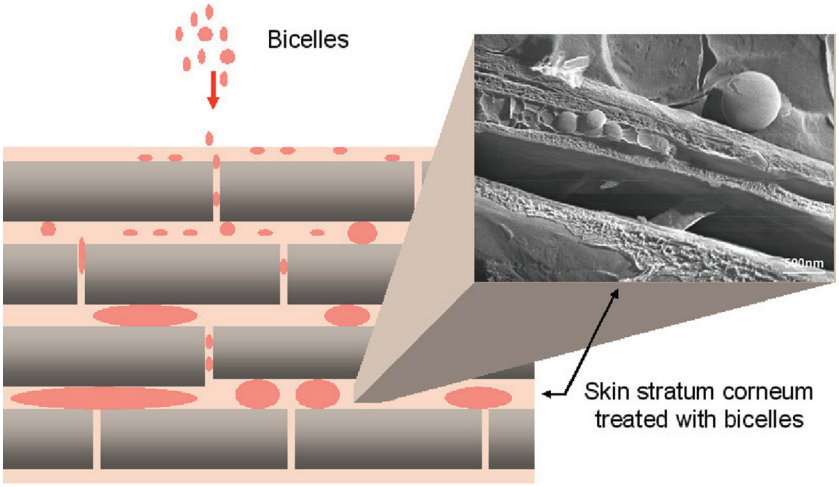
Lipid Nanosystems

Bicelles: Lipid Nanostructured Platforms with Potential Dermal Applications

Lucyanna Barbosa-Barros, Gelen Rodríguez, Clara Barba, Mercedes Cócera, Laia Rubio, Joan Estelrich, Carmen López-Iglesias, Alfonso de la Maza, and Olga López*



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***B**icelles emerge as promising membrane models, and because of their attractive combination of lipid composition, small size and morphological versatility, they become new targets in skin research. Bicelles are able to modify skin biophysical parameters and modulate the skin's barrier function, acting to enhance drug penetration. Because of their nanostructured assemblies, bicelles have the ability to penetrate through the narrow intercellular spaces of the stratum corneum of the skin to reinforce its lipid lamellae. The bicelle structure also allows for the incorporation of different molecules that can be carried through the skin layers. All of these characteristics can be modulated by varying the lipid composition and experimental conditions. The remarkable versatility of bicelles is their most important characteristic, which makes their use possible in various fields. This system represents a platform for dermal applications. In this review, an overview of the main properties of bicelles and their effects on the skin are presented.*

1. Introduction

Bicelles are a fascinating category of versatile and robust lipid assemblies that have been increasingly utilized in several research fields. They consist of nanostructures formed by long- and short-chain phospholipid molecules dispersed in aqueous solution.[1,2] These assemblies have been described as discoidal nanostructures of approximately 15–50 nm in diameter, in which a long-chain phospholipid, usually dimyristoyl-phosphatidylcholine (DMPC), forms a flat bilayer, and a short-chain phospholipid, normally dihexanoyl-phosphatidylcholine (DHPC), stabilizes the rim of the structure (**Figure 1**). Although these characteristics have been observed in several bicellar systems, bicelles are known to display different morphologies depending on the sample preparation and experimental conditions. The remarkable versatility of this system is its most important characteristic and allows its use in several different fields.[3–5]

The bicellar structure was developed by Sanders and Schwonek in 1992 to solve experimental problems associated with the use of lipid vesicles and micelles as membrane models for NMR studies of the characteristics of protein.[6] These systems were not suitable for high-resolution NMR[7–9] studies because the overall reorientation rates of lipid vesicles are too low, leading to significant line broadening and extreme micelle curvature. These characteristics are dramatically different from those of membranes, indicating that these systems are poor models of membrane environments. Further, micellar environments alter molecular activities, as is the case with enzymes. Bicelles, in turn, exhibit an intermediate morphology between lipid vesicles and micelles, combining some of the more attractive properties of both systems. Similar to micelles, bicelles are noncompartmentalized, optically transparent, and effectively monodisperse. As in lipid vesicles, bicelles are formed by a bilayer, which allows for the encapsulation of lipophilic molecules in their structure.[10]

These systems are replacing micelles and lipid vesicles in NMR studies of membrane proteins[11–13] because of their ability to spontaneously align in magnetic fields that are higher than 1 T. Their versatility permits the preparation of systems with different ratios of long- to short-chain phospholipids, giving rise to aligned or isotropic fast-tumbling bicelles that can be used in solid and/or solution NMR experiments to obtain information regarding conformations, interactions, and locations of molecules encapsulated in bicelles.

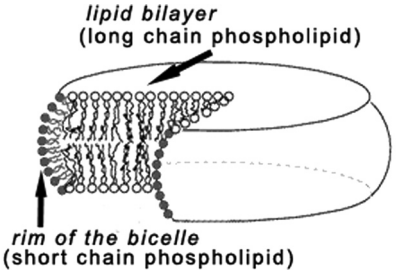

Figure 1. Schematic representation of a bicelle showing the flat bilayer region that is composed of long-chain phospholipids surrounded by a rim of short-chain phospholipids. Reproduced with permission.[40] Copyright 2008 Elsevier.

This development was received with great enthusiasm by the NMR community and is changing the way membrane-associated molecules are studied.[14,15]

Other techniques, such as electron microscopy, dynamic light scattering, small-angle X-ray scattering, electron paramagnetic resonance, electrokinetic chromatography, and neutron diffraction, have also been used to characterize bicelles.[16–18] Since their introduction, bicelles have opened new fields of study, and their exemplary utility suggests their potential use in other research fields.

The interesting combination of lipid composition, small size, and morphological versatility has made bicelles new targets for skin-related studies. Their lipid bilayer structure, with diameters in the range of 15–50 nm and thicknesses of 4–6 nm, leads to optimal conditions for applications related to skin research. Bicelles are well suited for such applications because of their structural resemblance to the lipid layers of the skin stratum corneum (SC), the absence of surfactants in their composition and the possibility of encapsulating different molecules in the their structure.

The SC is a bilayered lipid-rich matrix with embedded keratinocytes that forms the upper layer of skin.[19] One of the SC's key functions is to control permeability, making it the main target and the main barrier for transdermal drug delivery.[20–22] Perturbations in the structure and lipid composition of the SC are associated with various diseases.[23–25] For instance, there is a marked decrease in ceramides in patients with atopic dermatitis, suggesting that an insufficiency of this lipid is an etiologic factor in atopic dry and barrier-disrupted skin.[26,27] In addition, cholesterol and cholesteryl sulfate may play important roles in properties associated with SC stability properties, such as cohesion, desquamation, and the regulation of barrier functionality.[28–30] Alterations in epidermal cholesteryl sulfate (due to gene mutations in steroid sulfatase) result in the accumulation of this amphipathic lipid in the outer epidermis. This effect provokes a typical scaling phenotype and a permeability barrier dysfunction termed x-linked ichthyosis.[31]

To replace SC lipids or deliver drugs or other substances that are required to restore skin functionality, several systems have been designed as skin carriers, delivery systems, and penetration enhancers. Micelles and liposomes are some of the

Dr. L. Barbosa-Barros, Dr. G. Rodríguez, Dr. C. Barba, Dr. M. Cócera, Dr. L. Rubio, Dr. A. de la Maza, Dr. O. López
Dept. of Chemical Technology and Surfactants
Institut de Química Avançada de Catalunya (I.Q.A.C.)
Consejo Superior de Investigaciones Científicas (C.S.I.C.)
C/Jordi Girona 18-26, 08034 Barcelona, Spain
E-mail: l.barbosa.barros@ub.edu
Dr. J. Estelrich
Dept. of Physicochemistry
Faculty of Pharmacy, Barcelona University
Av. Joan XXIII s/n, 08028, Barcelona, Spain
Dr. C. López-Iglesias
Scientific and Technological Centers
Barcelona University
C/Baldiri i Reixac 10, 08028 Barcelona, Spain

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most commonly used systems for these purposes. However, despite their demonstrated beneficial effects, there are some limitations to their application.[32,33] Liposomes that are normally used for skin-related treatment are typically too large (usually 200–500 nm) to effectively penetrate into the skin because the thickness of SC intercellular spaces is approximately 6–10 nm.[34,35] Micelle surfactants normally produce skin irritation.[36,37] Here, again, bicelles present advantages over these two classical systems; they are small enough in size to pass through the narrow SC lipid lamellae, they contain a bilayer for molecular encapsulation, and they are composed entirely of lipids.[38,39]

These interesting features have been the basis for new bicelle research. In recent years, bicelle structure, function and interaction with skin tissue has been investigated. This research includes the formation and characterization of bicelles with new compositions, the study of the interaction of these systems with skin both in vitro and in vivo, the encapsulation of pharmaceutical and cosmetic compounds in bicelle structures, and the development of strategies to stabilize these systems. Some reports suggest that bicelles may permeabilize skin SC or reinforce the SC lipid structure. As drug delivery systems, bicelles retard percutaneous absorption when encapsulating anti-inflammatory drugs and enhance percutaneous absorption when applied to the skin before drug application.

The potential of these nanostructures for skin research is outstanding; their applications could range from model membranes for the study of SC lipid behavior to their use as SC lipids regenerators, skin carriers, penetration enhancers or retardants, and drug delivery systems.[40] This is in large part because of their versatility and ability to penetrate into the SC, depending on the system design. These properties have led to the study of bicelles as nanostructured platforms for dermal applications.

The following topics are reviewed in the present work: 1) the morphological versatility of bicelle systems, 2) the advantages of using bicelles for skin-related applications, 3) the interaction of different bicellar systems with skin tissue in vitro and in vivo, and 4) new strategies to improve bicelle effectiveness. The collected studies focus on two systems: the classical bicelle formed by DMPC and DHPC and another bicelle, which is more compatible with skin characteristics, formed by DPPC (dipalmitoyl-phosphatidylcholine) and DHPC. In light of the characteristics of bicelles and their promising skin-related applications, this review suggests a new strategy for the improvement of skin structure research, skin therapies, pharmacological applications, and clinical use.

2. Morphological Versatility of Bicellar Systems

Depending on their composition, lipid concentration (c_L), and the long-/short-chain phospholipid molar ratio (q), bicelles display various morphologies. In general, bicelle size increases with the value of q, but decreases with increasing values of c_L. Temperature also exerts significant effects on bicelles. At temperatures higher than the transition temperature (T_m) of the long-chain phospholipid, bicelles undergo morphological transitions and may align in magnetic fields. Bicelle composition is a key factor in determining the aggregates' morphologies at different temperatures and in the design of systems for use under specific conditions.

Characterization studies have shown the morphology and phase diagram of DMPC/DHPC bicelles to be very complex. The first temperature–lipid composition phase diagram of bicellar mixtures was constructed by Raffard et al.[41] They determined the phase diagram of the lamellar and isotropic phases, but the precise morphologies of aggregates within the isotropic phase remained unresolved. Luchette et al.[42] (2001) observed that the isotropic phase was consistent with bicelle morphology in specific cases reported by Bolze et al.[43] and Sternin et al.,[44] in which magnetic alignment was observed to be a function of temperature, lipid concentration, and lipid distribution. Nieh et al.[45] constructed the bicellar temperature–lipid concentration phase diagram. At low temperatures, the isotropic phase was an isotropic suspension of monodisperse bicelles that persisted until 25 °C, the temperature at which they transformed into magnetically alignable, extended perforated lamellae. It is generally accepted that bicelles with q values between 2.8 and 6.5 and lipid concentrations in the range of 3–40% w/w spontaneously align in magnetic fields such that the bilayer plane is parallel to the magnetic field, giving rise to a ^{31}P NMR spectrum showing two well-resolved resonances. The high-field resonance corresponds to DMPC localized in the planar surface of the aggregate, while the low-field resonance is attributed to DHPC distributed in the torus.[14,46,47] The ^{31}P NMR spectra are used as a first approach to detect bicelle formation and verify the quality of sample orientation. The samples with low q ratios do not align in magnetic fields and are normally used in high-resolution NMR studies.

Lucyanna Barbosa-Barros received her B.S. in Pharmacy from the Federal University of Ceara, Brasil (2000) and, her PhD from the University of Barcelona, Spain (2006). She has worked for several companies and, currently, she is an associate professor at the Faculty of Pharmacy, University of Barcelona. Her research interests are focused on the study and characterization of lipid nanostructures and on the biomedical application of these nanoaggregates.

Olga López Serrano completed her B.S. in chemistry and her PhD (1999) at the University of Barcelona. She was a staff Scientific Researcher at the Spanish Nacional Research Council (2001). She has published more than 100 papers in prominent international journals focused on the biophysics of lipids and other amphiphilics, self-assembled nanostructures, and the study of the skin barrier function.

Electron microscopy techniques have proven useful as complementary techniques to determine sample morphology. Van Dam et al. applied cryotransmission electron microscopy (cryo-TEM) techniques to characterize DMPC/DHPC, with $q = 3.2$ and $c_L = 5\%$ w/w doped with dimyristoyl phosphatidylglycerol (DMPG) and cetyl trimethyl ammonium bromide (CTAB).[47] With this technique, they observed disk-shaped structures, thread-like cylindrical micelles, and branched toroidal structures at 20, 25, and 30 °C, respectively. In another study, these authors used the same technique to investigate the morphologies of DMPC/DHPC samples over a wide range of c_L and q values, observing isotropically tumbling bicelles and larger aggregates.[48] They concluded that the temperature and the ratio q largely determine the sample morphology, while c_L affects the aggregate structure to a lesser extent. At $q = 0.5$, they observed small, possibly disk-shaped aggregates with a diameter of approximately 6 nm. At higher q values, they observed distorted discoidal micelles that tended to sort cylindrical micelles. Upon increasing either q or the temperature, they described long, slightly flattened cylindrical micelles that eventually branch. Freeze-fracture electron microscopy (FFEM) has also been applied for bicelle characterization with great success. Barbosa-Barros et al. used this technique and Cryo-SEM to visualize DMPC/DHPC bicelles with $q = 2$ and 3.5 and $c_L = 20\%$ at 20 and 40 °C. In this study, they observed small, rounded bicelles (at $q = 2$) and disk-shaped bicelles (at $q = 3.5$) at 20 ºC and elongated branched aggregates (at $q = 2$) and stacked bilayer sheets (at $q = 3.5$) at 40 °C.[49] Another interesting aspect of their work is the use of the high-pressure freezing technique (HPF).

In working with bicellar structures, it is important to choose techniques that require minimal sample pretreatment. This should be specifically addressed in case of EM techniques, which frequently require dilution. The use of these procedures would cause variations in bicelle lipid concentrations and consequently change the system morphology, leading to mistakes in data interpretation. This problem is circumvented with techniques that avoid sample modification, such as HPF fixation, where the sample is quickly frozen under high-pressure conditions without any pretreatment.

Small-angle X-ray spectroscopy (SAXS) is another technique that is frequently used to characterize bicelle structure, which allows for the determination of bilayer thickness that accounts for the thickness of the edge of bicelles.[17] Small-angle neutron scattering (SANS) are also used to identify bicellar structural phases and the existence of long-range order.[45] Dynamic light scattering can provide information on the aggregate's hydrodynamic diameter, which can be compared to the information obtained by EM. Fluorescence spectroscopy is usually applied to detect the formation of DHPC micelles and to monitor lipid mixing as an indicator of bicelle structure and aggregation.[50]

Using a combination of polarized light microscopy (POM) and SANS,[4,51] it was concluded that the phase space previously thought to be composed of bicelles[41] or extended lamellar sheets[45] is in fact a chiral nematic phase made up of worm- or ribbon-like, micelles. This result was verified by van Dam et al.,[48] who observed quasi-cylindrical, elongated micelles using cryo-TEM. This worm-like morphology is consistent with the frequent observations of increased viscosity in the temperature range, within which magnetic alignment is known to occur,[1] and is the result of these elongated structures becoming entangled.

DMPC/DHPC bicelles are classically described as disk-shaped objects formed by a DMPC bilayer and closed on the edges by DHPC molecules. Although this description is well-accepted for bicelles with $q < 2.8$ at temperatures below the main DMPC T_m, in the last decade, this characterization has been extensively debated for bicelles with $q > 2.8$ and temperatures above the T_m.[4,46,47]

The disk-shaped model is not consistent with the mechanism of bicellar alignment as a function of temperature in the magnetic field. According to Ottiger and Bax, disk fusion would be necessary to reach an appropriate size for co-operative alignment.[52] The changes in viscosity and sample transparency that occur under temperature variations corroborate these hypotheses. Rowe and Neal added that the classical model does not explain the increase of viscosity at temperatures where alignment begins, which suggests the formation of large aggregates.[50]

Based on their observations, Nieh et al. proposed a model for bicelles with $q = 3.2$, both with and without lanthanide cations (Ln^{3+}).[4,16,53] At temperatures below the T_m of DMPC, the bicelles were disk-shaped. As the temperature rose and the systems changed from the gel to liquid-crystalline phase in the presence of Ln^{3+}, the bicelles fused together in an end-to-end manner to form lamellar sheets with perforated holes that were lined with DHPC. Further increases in temperature caused phase separation, with the formation of DHPC-rich mixed micelles and DMPC-rich oriented lamellae that incorporated the DHPC-rich mixed micelles, even at higher temperatures.[45] In the absence of Ln^{3+}, bicelles were disk-shaped in the gel phase and chiral nematic or "worm-like micelles" in the liquid-crystalline phase. These bicelles became multilamellar vesicles at higher temperatures.

While some authors have proposed that the aligned samples correspond to elongated aggregates, which are present when samples become viscous,[51] others claim that the aligned bicelles are perforated lamellar sheets, which are present when the sample viscosity drops and its appearance becomes milky.[47,52,54] This latter model is known as the "Swiss cheese model" (**Figure 2**). In a comprehensive study, Triba et al.[46] found that both elongated and perforated lamellar structures are compatible with the NMR aligned spectra, although they did not disprove the possibility of discoidal structure in the aligned phase. The presence of biologically interesting cations (from salts of KCl, NaCl, $CaCl_2$, and $MgCl_2$) in the preparation of bicelles has been shown to modulate their size and to improve their magnetic alignment. The optimum salt concentration for such an effect ranges from 50 to 200 mM. Then, care should be taken when adding negatively charged lipids or proteins that include counterions. The optimum salt concentration may thus already be reached with the endogenous salts in bicelles, including components other than the usual neutral phospholipids.[18]

Recently, a comparative study related the morphologies of alignable ($q = 3.5$) and non-alignable ($q = 2$) bicellar systems,

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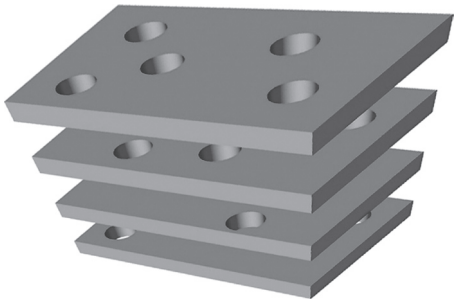

Figure 2. Representation of the Swiss cheese model, which consists of flat bilayer sheets of long chain phospholipids containing perforations lined with short chain phospholipids.

c_L = 20%, to temperature changes.[49] ^{31}P-NMR spectra indicated that the q = 2 bicelles exhibited isotropic behavior at all temperatures (20–60 °C), while the q = 3.5 bicelles varied considerably with temperature. Even at 20 °C, a low-field resonance peak and a broad higher-field signal suggested partial orientation of the sample with the magnetic field. Just above the DMPC T_m, at 25 °C, the spectrum showed two broad resonances near the positions of those observed at 20 °C. At higher temperatures, the lines became more intense, indicating increased magnetic alignment that reached a maximum at 40 °C. These peaks disappeared at higher temperatures and were replaced by a broad signal at higher-field resonances, which are characteristic of a phase transition from bicellar aggregates to larger, slow-moving structures, such as vesicles. There was also a gradual upfield shift of both peaks with increasing temperature, which represents an increase in DMPC bilayer order and the gradual incorporation of DHPC molecules into this bilayer. This study suggested that increasing temperatures promote the migration of DHPC molecules from the edges of the structures to the bilayer area of the alignable bicelles. Depletion of DHPC molecules at the edges leads to bilayer fusion, which increases the bicellar diameters and improves the alignment up to a certain point, at which larger and/or nonflat structures are formed, and the alignment is lost.

EM studies of the samples found round aggregates of approximately 20 nm for bicelles with q = 2 below T_m and elongated aggregates of approximately 2000 nm, similar to those reported by van Dam et al.[48] above T_m. These larger aggregates do not align in magnetic fields, although their size and morphology explains the increase in viscosity with temperature. At q = 3.5, discoidal bicelles of approximately 40 nm were observed below T_m, and extended areas of stacked lamellar sheets were observed above T_m. The authors considered these aggregates to be the most ordered phase because the best alignments in the ^{31}P-NMR experiments were obtained at the same temperatures.

Extensive and comprehensive studies have been reported on bicelle morphology and phase behavior.[5,15,45,55,56] Throughout this work, researchers have reported a) that bicelles are disk-shaped nanoaggregates at temperatures below the T_m of the long-chain phospholipid; b) that increases in temperature cause an initial increase and subsequent drop in viscosity; and c) that when the temperature is raised above the long-chain phospholipid T_m, bicelles with $q > 2.8$ undergo morphological transitions from disks to elongated micelles, perforated lamellar sheets, and mixed multilamellar vesicles.

However, the studies disagree on the exact morphology of structures with $T \geq T_m$ that exhibit magnetic alignment. Works describing the morphology of bicelles with $q < 2.8$ at temperatures above the long-chain phospholipid T_m are scarce. For these reasons, most recent reports use the term "bicelle" to refer only to the sample composition and not the disk-shaped morphology. This nomenclature is used in this review. Clearly, and as Katsaras et al. concluded in their 2005 review article,[15] the phase behavior of bicellar systems is much richer than previously assumed. The bicellar composition, net charge, and lipid concentration all play significant roles in determining the variety of phases and morphologies observed. As applications of these mixtures expand and further compositions are required and explored, there is a clear need for continued investigation and characterization of the phase behavior and morphologies of these enigmatic lipid mixtures.

3. Bicelle Suitability for Skin-Related Applications

In the last decade, liposomes have been intensively studied as drug carriers for topical delivery because of their capacities to enhance drug penetration into the skin,[34] their ability to improve therapeutic effectiveness,[57] and a decrease in their drug side effects.[58] However, although several mechanisms of vesicle–skin interactions have been described, no evidence of intact vesicle penetration into the deeper skin layers has been found.[59] It was suggested that vesicles likely disintegrate in the skin's surface and their components disperse into the intercellular lipid matrix, where they mix with the SC lipids, modifying the lipid lamellae or inducing new vesicle-like structures.[60] Recently, Karande et al. published a review of the enhancement of transdermal drug delivery via synergistic chemical action, employing solvent mixtures, microemulsions, eutectic mixtures, complex self-assembled vesicles, and the inclusion of complexes.[61] Currently, new delivery systems are being developed to improve aggregate surfaces and their effects on skin. Dragicevic-Curic et al.[62] reported that loaded liposomal hydrogels were able to deliver active compounds in efficient doses into the SC and deeper skin layers. Alves et al.[63] reported a new drug model for in vitro skin penetration that consisted of semisolid topical formulations containing nanocarriers (nanospheres, nanocapsules, and nanoemulsions). These authors demonstrated the influence of polymers and different types of nanocarriers (matricial, vesicular, or emulsion) on drug penetration through human skin. Küchler et al.[64] similarly reported that nanoparticulate carrier systems, solid lipid nanoparticles, and dendritic core–multishell nanotransporters were suitable skin drug delivery systems for hydrophilic agents.

Bicelles are novel nanostructured platforms that promote skin surface modification and enhance drug penetration.[65] Because of their lipid composition, bicelles also promote skin layer reinforcement, and most importantly, due to their

particular structure, bicelles penetrate into the skin; this feature has been convincingly shown.[66]

The motivation for using bicelles in the skin arises from the resemblance of these structures to micelles and liposomes. Bicelles combine some of the most attractive characteristics of these systems and therefore present several advantages for skin applications compared to either micelles or liposomes. Bicelle structures contain a bilayer that allows for the incorporation of different molecules, but the are much smaller (approximately 15–40 nm) than a regular liposome (~200 nm). This is due the presence of DHPC molecules on the edges of the structures, which control the diameter of the assembly. These molecules are responsible for discoidal-shaped bicelles and the formation of other structures, such as small vesicles. In fact, systems formed by long-alkyl-chain phospholipids and DHPC have been reported to produce reasonably monodisperse unilamellar vesicles that are thermodynamically stable, with radii ranging from 10 to 40 nm.[67–70] Several kinetic studies have shown that discoidal bicelle morphology is a precursor to small-vesicle morphology.[70,71] DHPC molecules solubilize the DMPC bilayer similar to a surfactant; however, DHPC is a phospholipid with the same polar head group as DMPC. These two lipids differ only in the lengths of their hydrophobic chains. The use of systems composed only of lipids avoids damage to the skin barrier function caused by surfactants, which is characterized by breaking of the corneocyte envelopes and disorganization of the intercellular lipid structures.

Bicellar systems have been adapted to better mimic membranes and to suit other tissues. Bicelles with acidic phospholipids or positively charged lipids that are doped with small amounts of charged amphiphiles and that incorporate other components, such as cholesterol, ceramides, cardiolipin, and polyethylene glycol (PEG) lipids, have been reported.[55,72–75] These compositional changes provide these systems with different charges, better resemblances to biological structures, and better stability and adaptability to the target tissues.

Bicelle compositions have also been adapted for use in the skin. As already mentioned, above the DMPC T_m (approximately 23 °C), DMPC/DHPC bicelles undergo phase transitions, changing from small aggregates of approximately 15–20 nm to structures larger than 500 nm. Because skin intercellular spaces lie in the range of 6–10 nm and physiological skin temperatures are near 37 °C, this phase transition would present a handicap for skin penetration. DMPC/DHPC bicelles are likely not able to penetrate into SC intercellular spaces, and their effects are limited to the skin's surface.

A different scenario is obtained if the long-chain phospholipid DMPC is substituted by another phospholipid with higher T_m, such as DPPC. This phospholipid has two additional carbons in its hydrophobic chain and a T_m of 41 °C. At physiological temperatures, bicelles composed of DPPC and DHPC are still small aggregates. Some studies have found that at 37 °C, these structures have dimensions of approximately 15 nm in diameter and 5.4 nm in thickness. These structure have been shown to penetrate through the intercellular spaces of the skin SC and affect deeper internal layers.[66]

By varying bicelle lipid compositions and/or lipid ratios, novel systems with unique physicochemical characteristics can be obtained. The morphologies and sizes of bicellar structures may be varied to obtain a given effect, which may be superficial or profound depending on the particular application, allowing the modulation of bicelle penetration into the SC. These features provide bicelles with the potential to become new multifunctional, skin-compatible platforms.

4. Application of Bicelles to the Skin

The structure of the SC is very specific in composition and behavior. The lipid matrix is formed by bilayers composed mainly of ceramides, cholesterol, fatty acids, and small amounts of cholesterol sulfate and cholesterol esters. This structure ensures cohesiveness between the corneocytes and accounts for the permeability of the SC.[76,77] This exceptional composition and organization is not observed in other biological membranes, which in general consist predominantly of phospholipids as bilayers. Because the SC is remarkably devoid of phospholipids, its ability to form bilayers is somewhat surprising. Systems' interactions with the SC are key factors in determining their potential for skin delivery and other skin-related applications.

4.1. In Vitro Interaction of Bicelles with the Skin

4.1.1. Microstructural Studies

Experiments have been reported in which human and pig SC samples were incubated with bicelles to study the effects of these systems on the SC microstructure. In one of these studies, fresh human SC samples were incubated with DMPC/DHPC bicelles with c_L = 20% and q = 2 for 18 h at 25 °C. Treated and untreated SC samples were cryofixed, cryosubstituted, and visualized with TEM. No differences, including microstructural alterations and/or apparent damage, were observed in images of the treated SC compared with untreated samples (**Figure 3**A,B). Given these bicelles' small size (20 nm in diameter and 4.5 nm in thickness) and bilayered structures, lipid dispersion through the SC lamellae and the reinforcement of the SC bilayer area would be expected. However, this was not observed, suggesting that these bicelles were not able to penetrate or disperse through the SC lipid area.[40] This result was clarified by Rodriguez et al.[78] using attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscopy. These authors reported that the application of DMPC/DHPC q = 2 bicelles caused phase transitions in the SC lipid conformation from the gel state to the liquid-crystalline state. This transition would be promoted by the incorporation of phospholipids from bicelles in the SC lipid lamellar structure. This process involves an increase in the fluidity and/or disorder of the lipids. An analysis of phosphate vibrations only detected effects from DMPC/DHPC bicelles on lipids of the outer layer of the SC, suggesting that the majority of DMPC/DHPC bicelles remained in the outermost part of the tissue. This is likely why Barbosa-Barros et al.[40] found no microstructural differences between untreated and treated SC; transitions occurring mainly on the SC surface do not imply structural modifications that can be visualized in EM experiments.

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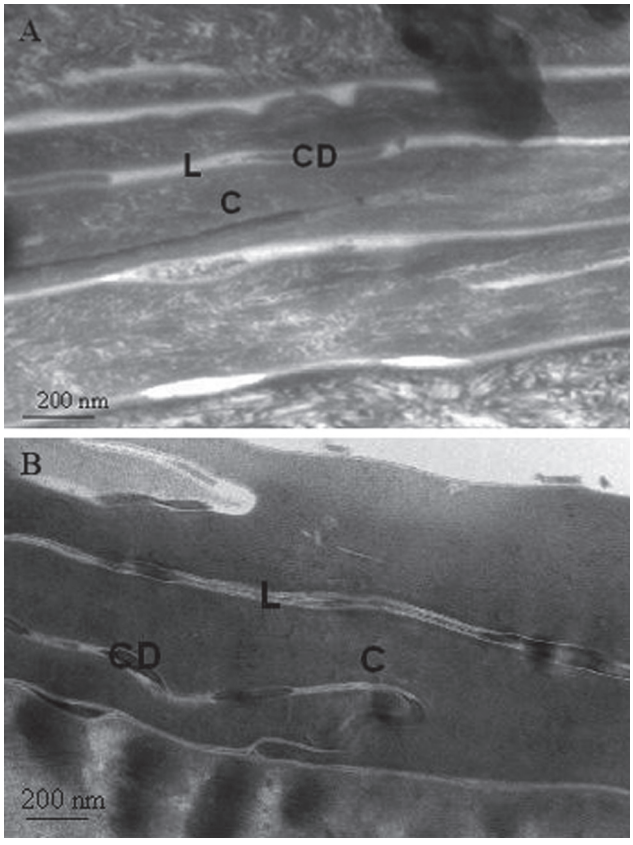

Figure 3. Freeze substitution transmission electron microscopy (FSTEM) image of A) native SC and B) SC treated with DMPC/DHPC bicelles. Both images show regular areas of corneocytes (C), lipid intercellular spaces (L), and corneodesmosomes (CD). Reproduced with permission.[40] Copyright 2008 Elsevier.

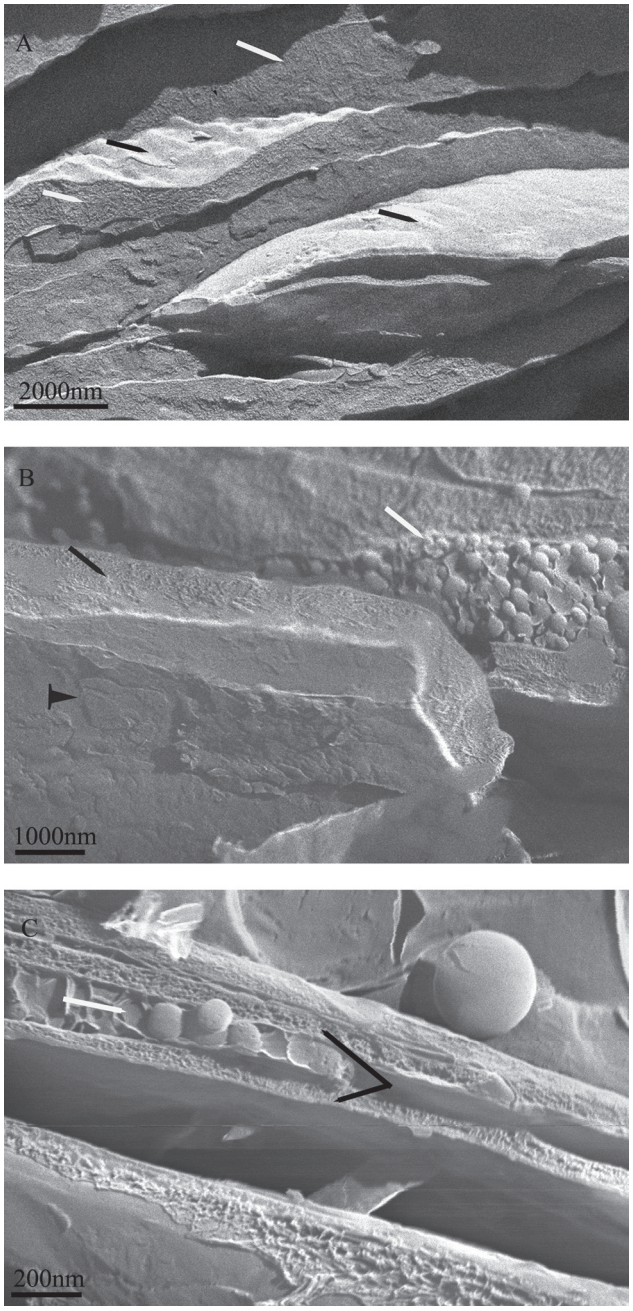

Figure 4. Cryo-SEM images of A) native SC and B,C) SC treated with DPPC/DHPC bicelles. In A, the white arrow indicates the corneocyte area, and the black arrow indicates the intercellular lipid area. In B, the white arrow shows vesicle structures with sizes of approximately 200 nm, and the arrowhead indicates the lamellar-like structures in the intercellular lipid areas. The black arrow shows a corneocyte area. C displays a magnification of vesicles in the intercellular lipid area (white arrows) between two cross-fractured corneocytes (black arrows). Reprinted with permission.[66] Copyright 2008 American Chemical Society.

In another study, DPPC/DHPC bicelles with $c_L = 10\%$ and $q = 3.5$ were incubated with fresh pig skin SC samples for 18 h at 37 °C. As mentioned in Section 3, this DPPC/DHPC system was specially developed to obtain improved effects on the SC microstructure and better skin penetration at physiological conditions. Because the DPPC T_m is 41 °C, the structures do not undergo phase transitions at physiological temperatures (approximately 37 °C), and small bicelle structures are favored during the incubation process. After incubation, both treated and untreated SC samples were HPF treated from an initial temperature of 37 °C, freeze fractured, and observed using cryo-SEM.[66] These bicelles produced quite different results in the SC than the DMPC/DHPC $q = 2$ system. The DPPC/DHPC system penetrated and interacted with the SC, forming lipid vesicles and new lamellar-like structures observed by cryo-SEM (**Figure 4**). To study this phenomenon, Rodriguez et al.[79] studied the effects of DPPC/DHPC on SC lipids using ATR-FTIR spectroscopy coupled with a tape-stripping methodology. Analysis of the lipid organization in terms of chain conformational order and lateral packing showed that bicelles hampered the temperature-dependent fluidization of SC lipids in the most superficial layers of the SC and led to a lateral packing corresponding to a stable hexagonal phase. CH_2 stretching and phosphate vibrations in the ATR-FTIR spectra of subsequent stripping indicated that DPPC/DHPC bicelles penetrated into and were widely distributed in deep layers of the SC. These results corroborate the presence of different DPPC/DHPC structures inside the SC layers, and bicellar reinforcement of the SC structures was observed.

The appearance of vesicles inside the SC lipid layers was investigated. Various authors have reported studies of bicellar state-transitions from disks to vesicles that were induced by dilution or temperature changes. As mentioned in Section 2, there is a general consensus that these transitions occur in progressive steps, implying the coexistence of different aggregates in the medium.[43,47,55] To study the bicelle-to-vesicle transition, dynamic light scattering (DLS) studies were reported by Barbosa-Barros et al. in 2008.[66] A DPPC/DHPC sample with q = 3.5 and c_L = 10% was sequentially diluted with water in seven steps, and each diluted sample was measured using DLS at 37 °C to imitate physiological conditions. The DLS curves indicated that the hydrodynamic diameter (HD) of the structures increased upon dilution from 11.3 nm (assigned to bicelle disks) to aggregates larger than 1 μm. The morphologies of the aggregates were analyzed using EM, which confirmed the DLS results, indicating a dilution-induced transition from disks to vesicles.

The transitions that disk-shaped bicelles undergo by the variation in lipid concentration, temperature, or lipid molar ratio[80] are very similar to those involved in the reconstitution of surfactant–lipid mixed micelles in vesicles. This phenomenon, which also occurs via dilution, has been discussed in a number of works.[81,82] A resemblance between surfactant–lipid micelles and phospholipid bicelles explains their similar behavior. In phospholipid bicelles, the DHPC molecules solubilize the DPPC bilayer, forming disk-shaped structures similar to surfactant-solubilized liposomes forming surfactant–lipid micelles. In bicellar systems, the DHPC molecules are found mainly on the edges of the disk structures and in the water (as monomers). With increasing dilutions, the DHPC concentration in water decreases, and DHPC is transferred from the bicelle edges into solution, maintaining monomeric equilibrium. Hence, disk diameters increase, and high dilutions lead to the fusion and closure of large bilayered disks, forming vesicles.

This bicelle-to-vesicle transition explains the presence of vesicles in the SC intercellular spaces treated with DPPC/DHPC.[66] This process would have been promoted by the dilution of aggregates because the SC pieces were washed with water after incubation. Bicelles that have been transformed into vesicles presumably follow a process similar to that observed in the DLS observations of diluted bicellar solutions outside of the skin. A similar process has been reported by López et al., who applied octylglucoside–phosphatidylcholine mixed micelles to the SC.[83]

4.1.2. Percutaneous Penetration Studies

Rubio et al.[65] performed percutaneous penetration studies with DMPC/DHPC and DPPC/DHPC systems with c_L = 10% and q = 2 to evaluate their effects on the skin penetration of diclofenac diethylamide (DDEA). The authors reported that the incorporation of DDEA in the bicelles led to markedly decreased bicelle sizes, indicating that DDEA tends to be located at the bicelle edges, similar to DHPC. **Figure 5** shows Cryo-TEM images of DPPC/DHPC bicelles with and without DDEA. Both systems decreased the percutaneous absorption of the DDEA compared to an aqueous solution of DDEA,

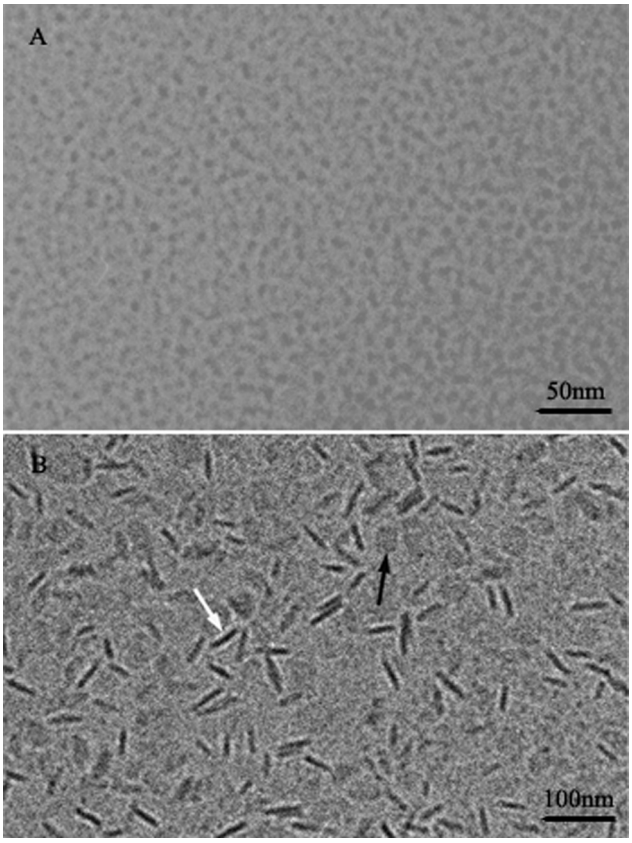

Figure 5. Cryo-TEM images of DPPC/DHPC bicellar systems with reduced sizes due to a) diclofenac diethylamide (DDEA) or b) bicellar structures of regular size without DDEA. Reprinted with permission.[65] Copyright 2010 Elsevier.

suggesting a retarded effect after treatment with bicelles. This effect was more marked for the DMPC/DHPC bicelles. This could be related to the different T_m values for the two systems. At 37 °C, DMPC/DHPC ($T_m \approx 23$ °C) bicelles were in the liquid-crystalline phase, in contrast to the gel phase SC lipids ($T_m \approx 60$ °C). However, DPPC/DHPC bicelles ($T_m \approx 41$ °C), were in gel phase, similar to the SC lipids. Different mixing behavior of lipids from bicelles with SC lipids could induce different effect on the retention of DDEA in the upper layers of the skin. This retarded effect was ascribed in part to rigidity in the head groups of bicelle phospholipids caused by the carboxyl groups of DDEA. This rigidity would hinder the penetration of DDEA through the skin. However, DDEA was likely unable to diffuse out of the bicellar systems because of its high affinity for its vehicle.

A second percutaneous penetration assay was also performed by these authors to evaluate the potential of bicelles as penetration enhancers. In this experiment, the skin was pretreated with the bicellar systems (DMPC/DHPC and DPPC/DHPC systems with c_L = 10% and q = 2) before the application of aqueous DDEA solution. The global results obtained showed that pretreatment of the skin with bicelles promotes the percutaneous absorption of DDEA, with no significant differences between DMPC/DHPC or DPPC/DHPC systems. These studies suggest that treatment with these bicelle

systems prior to drug treatment can enhance drug absorption. This enhancement is ascribed to an initial interaction of bicelles with the SC that causes some disorganization of the intercellular lipids, which are responsible for the SC barrier functionality. These results suggest a route to aid the absorption of DDEA through the skin.

4.2. In Vivo Interaction of Bicelles with the Skin

To estimate the effects of bicelles on the skin in vivo, non-invasive biophysical studies were performed with healthy volunteers. These studies report mainly on measurements of skin hydration, elasticity, erythema, and transepidermal water loss (TEWL). Skin hydration and elasticity are useful measures of skin water content, distensibility, extensibility, and tonicity.[84,85] Erythema indicates skin tolerance,[86] while TEWL indicates barrier function integrity.[87,88]

4.2.1. Effect of Bicelle Composition on the Biophysical Parameters of the Skin

The application of DMPC/DHPC bicelles with $c_L = 20\%$ and $q = 2$ to the skin of healthy volunteers has been reported.[40] In this experiment, intra-individual comparisons of three test areas on the volar forearms of 6 healthy Caucasian female (ages 25–38) volunteers with no visible skin abnormalities were performed. In the test areas, bicelles, deionized water and control (nontreated) areas were randomized regarding the test sites on each subject. The solutions were applied daily over a period of 10 days, and the skin properties were measured each day before application.

The authors observed that successive bicelle applications led to an increase in TEWL from day 0 to 11. This increase was moderate and did not reach pathological levels, which are from 25 to 40 g m^{-2} h^{-1}.[89,90] Decreasing skin hydration was also observed. Elasticity, in turn, showed improvement with the application of bicelles. The changes in the erythema of the skin, considering inter- and intra-individual variability, were not indicative of an irritation process.[91] The bicelles were found to promote increases in TEWL and skin elasticity and harmless decreases in skin hydration.

Bicelles act to enhance penetration, causing phase transformations in lipid domains that may be relevant to skin permeation.[92] As reported by Rodriguez et al.,[78] the phase transition of the SC lipid conformation from the gel state to the liquid-crystalline state, which causes the fluidity of these lipids, explains the increase of TEWL in vivo. Compared to other enhancers, bicelles would have the additional advantage of not causing skin irritation.[93]

Another study reported the effects of the application of DPPC/DHPC bicelles with $c_L = 20\%$ and $q = 2$ on the skin of healthy volunteers. The results were similar to those obtained with the DMPC/DHPC system, although they were more discrete. In a similar way, this system led to an increase in TEWL and a decrease in skin hydration. However, the effects were approximately 75% and 50% less intense, respectively, than those obtained with the DMPC/DHPC system.[94] This result is not unexpected because the DPPC/DHPC system contains less DHPC, so the aggregates formed are slightly bigger and have longer bilayer areas, which would exert a protective effect on the SC. In addition, as observed in the in vitro studies, this system penetrates into the skin SC and reconstitutes its lipids in lipid vesicles inside of the skin lamellae, which reinforce the lipid structure of the tissue.

4.3. Effects of Lipid Self-Assembly on the Biophysical Parameters of the Skin

As with other lipid systems, bicelles are expected to exert different effects on the skin depending on their composition. Recently, Barbosa-Barros et al. evaluated the relevance of lipid self-assembly and the composition of phospholipid nanoaggregates to skin property effects.[94] Liposomes made of DMPC and DPPC (LipDMPC and LipDPPC), micelles of DHPC (MicDHPC), bicelles of DMPC/DHPC ($q = 2$) and DPPC/DHPC ($q = 3.5$) (BicDMPC/DHPC and BicDPPC/DHPC) and liposomes formed by the dilution of the bicelles (LipBicDMPC/DHPC and LipBicDPPC/DHPC) were studied. The results are shown in **Table 1** (adapted from Barbosa-Barros et al., 2009).[94]

This investigation showed that consecutive applications of the samples containing bicelles to the skin increases TEWL, but the effect is more drastic when the bicelles consisted of DMPC/DHPC ($q = 2$). The samples containing DMPC promoted higher barrier impairment than the samples containing DPPC. Liposomes or vesicular samples generally did

Table 1. Skin biophysical parameter values (F, Final Value) after the in vivo treatment with the different phospholipid systems and standard deviation (SD) reflecting the inter-individual variation.

	Trans Epidermal Water Loss		Hydration		Melanin		Erythema	
	F	SD	F	SD	F	SD	F	SD
BicDMPC/DHPC	161.84	27.20	71.70	9.62	100.5	0.41	100.8	0.72
LipBicDMPC/DHP	118.1	30.83	92.18	21.20	94.87	0.31	101.3	0.68
BicDPPC/DHPC	115.8	31.44	87.18	21.14	100.6	0.97	100.3	1.38
LipBicDPPC/DHPC	102.1	29.06	110.2	26.46	100.0	0.39	99.49	1.41
LipDMPC	109.4	26.48	88.14	21.40	99.80	0.41	101.3	0.84
LipDPPC	93.59	20.01	90.96	15.30	100.1	0.59	99.22	1.67
MicDHPC	124.7	22.04	83.64	21.93	100.6	0.76	100.8	1.28

not significantly modify TEWL values; however, the system formed by dilution, LipDMPC/DHPC, induced a remarkable increase in this parameter, whereas the LipDPPC system decreased it. The systems organized as discoidal bicelles increased TEWL values, while those organized as vesicles mostly negated (in the case of LipDMPC/DHPC) and even reversed (in the case of LipDPPC/DHPC) this increase. The authors ascribed this effect to the self-assembly of lipids in different nanostructures, resulting in aggregates of different sizes and morphologies. The small bicelle size compared to that of vesicles seems to be a relevant factor in barrier modification. Other factors are involved, however, because systems with similar size induced different modifications in skin parameters. Moreover, the increase in TEWL due to DHPC micelles was less than that due to the BicDMPC/DHPC system, which contained the same amount of DHPC. This highlights the importance of self-assembly in these interactions. In addition, hydration values decreased after treatment with all of the samples, except LipDPPC/DHPC. As expected, the lowest values of hydration were in the same areas in which the highest TEWL values were detected. The hydration values also showed differences between the samples of identical composition but different self-assembly (compare BicDMPC/DHPC to LipDMPC/DHPC and BicDPPC/DHPC to LipDPPC/DHPC).

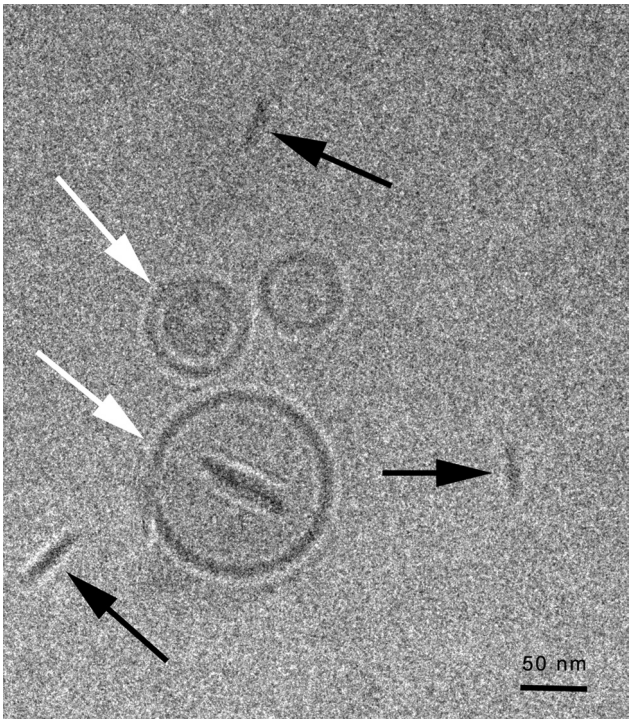

Figure 6. Cryo-TEM image of a bicosome sample showing two projections of bicelles (face on and edge on) inside the lipid vesicles (white arrows) and free unencapsulated bicelles (black arrows). Reproduced with permission.[95] Copyright 2010 Elsevier.

5. Strategies to Stabilize Bicelles in High-Water-Content Environments

Although their structural and morphological dependence on environmental conditions allows bicelles to be used in several areas, this variability may limit its application, for example, in environments with high water content. As discussed above, bicelles are good skin carriers. However, the addition of bicelles to vehicles with higher water content and the administration of bicelles via a systemic route are challenging because, under diluted conditions, bicelles present variable morphologies and effects.

To address this limitation, Rodriguez et al. have recently proposed a strategy to preserve discoidal bicelles for use in high-water-content environments.[95] In their strategy, the bicelles are first prepared and the aggregates are subsequently encapsulated in liposomes. These new structures were termed "bicosomes" (**Figure 6**). Bicosomes would protect bicelles from dilution, and for delivery via a systemic route, bicosomes would preserve the aggregates until the target tissue is reached. To show this, Rodriguez et al. performed dilution studies using DLS and Cryo-TEM. In addition, bicelles and bicosomes were stereotactically injected into the ventricular systems of rat brains and visualized by magnetic resonance imaging (MRI) to verify the bicosome protective effect in a diluted in vivo environment. They observed that the bicosome exterior lipid membrane ensured the isolation and stability of the encapsulated bicelles in vitro, protecting them from the effects of dilution. The liposome capsule is biocompatible and stable with temperature change and has a controllable size that is not altered by dilution. In the in vivo study, the morphology of the aggregates after injection was not verified. However, the injection of free bicelles was lethal to the animals, whereas the injection of bicosomes was not, corroborating the protective effect of the bicosomes.

Other methods have been used to stabilize the morphology of discoidal bilayers, such as using bicelles with charged amphiphiles[55,74] or disks formed by mixtures containing polyethylene-glycol–lipid conjugates (PEG–lipids).[75] However, the PEG lipids may negate some of the bicelle properties related to structural versatility, such as their enhancement of permeability through physiological barriers, because bicelles formed with PEG lipids are sterically stabilized and contain lipids with the same or very similar alkyl chain lengths.

6. Conclusion

The use of bicelles in skin is a scientific novelty. This relatively new lipid system represents a unique versatile structure that has different effects on the skin depending on the self-assembly adopted. Control over bicelle physicochemical properties is required for their use in biomedical applications. Hydration and temperature determine their self-assembly parameters (size, morphology, and structure), while selection of an appropriate bicelle composition, for instance, the use of lipids with appropriate transition temperatures to yield a specific particle size, is a key factor to make more efficient the use of these lipid nanostructures.

The application of bicelles to the skin modifies its biophysical parameters without affecting SC lipid microstructure

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or promoting irritation. The penetration and growth of DPPC/DHPC bicelles inside the SC opens up new avenues for the treatment of these systems. Bicelles are an effective skin carrier due to their size, structure, and composition. Although bicelles have no aqueous internal compartment for encapsulating drugs, their bilayered structure allows for the encapsulation of lipophilic and amphiphilic compounds. Because of their ability to increase the permeability of the SC, these structures enhance the penetration of hydrophilic components dissolved in aqueous medium. Further, the conversion of bicelles into vesicles inside the SC hinders their migration outside of the tissue and allows a lipid reinforcement effect on the skin. This property could be very useful to intensify the effects of specific compounds carried by bicelles into the SC layers. By modulating their physical and chemical characteristics, bicelles may be useful for a wide range of applications. Bicelles are therefore promising nanostructures that represent new platforms for skin-related applications.

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