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Topical Delivery of Interferon Alpha by Biphasic Vesicles: Evidence for a Novel Nanopathway across the Stratum Corneum

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Abstract: Noninvasive delivery of macromolecules across intact skin is challenging but would allow for needle-free administration of many pharmaceuticals. Biphasic vesicles, a novel lipid-based topical delivery system, have been shown to deliver macromolecules into the skin. Investigation of the delivery mechanism of interferon alpha (IFN α), as a model protein, by biphasic vesicles could improve understanding of molecular transport through the stratum corneum and allow for the design of more effective delivery systems. The interaction of biphasic vesicles with human skin and isolated stratum corneum membrane was investigated by confocal microscopy, differential scanning calorimetry (DSC) and small- and wide-angle X-ray scattering (SAXS and WAXS). Confocal microscopy revealed that biphasic vesicles delivered IFN α intercellularly, to a depth of 70 μm, well below the stratum corneum and into the viable epidermis. DSC and SAXS/WAXS data suggest that the interaction of biphasic vesicles with SC lipids resulted in the formation of a three-dimensional cubic *Pn*3*m* polymorphic phase by the molecular rearrangement of intercellular lipids. This cubic phase could be an intercellular permeation nanopathway that may explain the increased delivery of IFN α by biphasic vesicles. Liposomes and submicrometer emulsion (the individual building blocks of biphasic vesicles) separately and methylcellulose gel, an alternative topical vehicle, did not induce a cubic phase and delivered low amounts of IFN α below the stratum corneum. Molecular modeling of the cubic *Pn*3*m* phase and lamellar-to-cubic phase transitions provides a plausible mechanism for transport of IFN α. It is hypothesized that induction of a *Pn*3*m* cubic phase in stratum corneum lipids could make dermal and transdermal delivery of other macromolecules also possible.

Keywords: Biphasic vesicles; dermal and transdermal delivery; interferon alpha; protein delivery; *Pn*3*m* cubic phase

Introduction

Dermal and transdermal delivery of large molecules has remained a significant challenge, although several novel physical and electrically driven technologies, such as microneedles,[1−3] iontophoresis,[4,5] and ultrasound,[6−8] are

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expanding the ability to administer not only small compounds but also larger molecules such as peptides, proteins, and DNA into and through the skin. In many therapeutic applications, however, noninvasive dermal delivery of macromolecules via a topical cream would have distinct advantages, including needle- or device-free delivery of drugs, antigens and other desired compounds; direct and painless delivery into the skin without inducing tissue injury; and the potential to deliver dermal therapeutics to a larger skin surface area.

Studies using biphasic vesicles in a topical cream preparation have demonstrated their potential for dermal delivery of macromolecules such as insulin[9−11] and vaccine antigens[12,13] in animal models, and interferon alpha (IFN α) in healthy human subjects and patients with HPV infection.[14] Biphasic vesicles are related structurally to liposomes and form upon mixing a phospholipid phase with a submicrometer emulsion phase, creating a multicompartmental delivery system that contains phospholipid bilayers, aqueous compartments, oil droplets and micellar domains.[14] The structure of biphasic vesicles and the specific combination of permeation-enhancing excipients used to formulate them are selected with the aim to both facilitate drug encapsulation and transport large molecules through the skin.

Chemical permeation enhancers (CPEs) are molecules that have the ability to interact with either the intercellular lipids or the corneocytes of the stratum corneum (SC). The more than 300 known CPEs are classified into three main groups based on their mechanism of permeation enhancement.[15,16] Group 1 enhancers, which include solvents such as ethanol and organic acids such as salicylic acid, extract skin lipids or damage the SC, thereby weakening the barrier. Group 2 enhancers, of which polyols such as propylene glycol are an example, increase drug solubility within the skin. Group 3 enhancers disorder the intercellular lipids. Examples include terpenes, surfactants, fatty acids, fatty acid esters, Azone (1-dodecylazacycloheptan-2-one) and its derivatives, amides (e.g., dimethylformamide) and sulfoxides (e.g., DMSO).[17−20] Many of these molecules can be incorporated into the multiple compartments of biphasic vesicles; thus this delivery system can be considered a type of combination enhancer mixture.

The design, formulation and characterization of biphasic vesicles, the delivery of IFN α by biphasic vesicles through intact skin in human volunteers, and the first biphasic IFN α proof-of-concept study in patients with human papillomavirus infection (condylomata acuminata) were reported in a previous study.[14] Here, the results of an investigation of the mechanism of interaction between biphasic vesicles and SC lipids is presented. It was of interest to evaluate whether delivery of IFN α by biphasic vesicles was attributable to a novel mechanism or mechanisms described previously for liposomes or CPEs, which enhance absorption through interaction with intercellular lipids in the SC and creation of a perturbed microenvironment based on various possible means[21−23] such as (1) alteration of lipid phase fluidity; (2) enhancement of solubility characteristics of the skin for the drug to be delivered; (3) creation of a disordering

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effect among the alkyl chains of skin lipids; and (4) localized separation of lipid domains to create hydrophilic pores.

Experimental Section

Encapsulation of IFN α in Biphasic Vesicles, Liposomes and Methylcellulose Gel. IFN α-2b (specific activity 2.24×10^8 IU/mg, Schering-Plough) was encapsulated into biphasic vesicles (Code F#6) as described previously.[14] Briefly, the phospholipid phase contained the following (all ingredients % w/w): hydrogenated soya phosphatidylcholine (Phospholipon 90H) 10%; cholesterol 2%; monolauroyl lysine 2%; and propylene glycol 7%. The aqueous phase for biphasic vesicles is a submicrometer emulsion, which was prepared from olive oil 3%; phospholipid EFA (linoleamidopropyl-PG-dimonium chloride phosphate) 5%; glyceryl monostearate 1%; cetyl alcohol 0.6%; beeswax 0.28%; methyl paraben 0.15%; propyl paraben 0.05%; and sterile distilled water q.s. to 100%. Biphasic vesicles were prepared by hydrating the phospholipid phase with the submicrometer emulsion at 57 ± 3 °C by low-shear mixing.[14] The submicrometer emulsion (average particle size 200−300 nm) was prepared using a Microfluidizer M-110 homogenizer (Microfluidics). An aliquot of IFN α stock solution corresponding to 20 MIU/g formulation was added to the submicrometer emulsion before the hydration step. Liposomes were prepared from the same phospholipid phase as that used for preparing biphasic vesicles, except the aqueous phase contained IFN α solution adjusted to correct volume with distilled water. The gel vehicle was prepared from methylcellulose 1500 cps, 2%, hydrated with sterile distilled water.

Labeling IFN α with Alexa 594 Dye. Alexa 594-labeled IFN α for confocal microscopy studies was prepared using an aliquot of stock solution containing 1 mg of IFN α, which was lyophilized using a Labconco CentriVap concentrator. The lyophilized IFN α was reconstituted in 250 μL of sterile 0.1 M sodium bicarbonate buffer solution. While vortexing, 20 μL of the Alexa dye solution (10 mg/L DMSO) was added to the protein solution, followed by stirring for 1 h. Unreacted dye was separated from IFN α using a Micro Bio-Spin 6 column, from which the purified labeled protein fraction was collected for encapsulation in biphasic vesicles, liposomes and incorporation into methylcellulose gel.[14]

***In Vitro* Diffusion Cell Studies and Confocal Microscopy.** The collection of human breast skin samples from elective mammoplasty surgeries were conducted with permission of the University of Saskatchewan Committee on Ethics in Human Research. *In vitro* absorption studies were performed in flow-through diffusion cells. Full thickness human skin samples ($n = 4$) were set up in diffusion cells (9 mm diameter) and maintained at 32.0 °C. The perfusion buffer (0.01 M Na-phosphate buffer containing 100 μg/mL bovine serum albumin and 0.26 mg/mL L-methionine, pH 7.2) was circulated under the skin at 37 °C. The skin samples were treated with 0.1 g of various formulations containing Alexa 594-labeled IFN α (biphasic vesicles, liposomes or submicrometer emulsion) for 24 h. After treatment the remaining formulations were recovered from the surface of the skin, skin samples were cleansed by washing with 3 × 15 mL Millipore water and blotted with tissue paper.

Confocal microscopy of skin treated with the various formulations (biphasic vesicles, liposomes and methylcellulose gel) was carried out using a Zeiss LSM 410 confocal microscope equipped with a HeNe 594 laser excitation source and a 635 ± 55 nm emission filter. The image size was 512 × 512 pixels, where 1 pixel is 0.625 μm for the 40× Plan-NeoFluar oil-immersion objective. Software used for image acquisition was Zeiss LSM 410. A minimum of 120 0.5 μm sequential horizontal optical sections were taken either of intact skin, starting with the skin surface, or of 80 μm cross sections. Three-dimensional images were generated using the WCIF Image J software package (NIH, public domain software).

Isolation of Stratum Corneum from Human Skin. Stratum corneum sheets were isolated from human breast skin samples as described previously.[24] Briefly, the epidermis was separated from the dermal layers by immersion in 60 °C water for 2−3 min followed by incubation of the epidermis, with SC side facing up, on filter paper saturated with 1% w/v trypsin solution at 4 °C overnight. The digested epidermal layers were removed, and the remaining SC sheets were rinsed with distilled water and stored in the fridge until used. Skin hydration for the DSC experiments was estimated in a small study ($n = 3$) by weighing the fully hydrated stratum corneum before and after blotting with tissue paper. After weighing the blotted SC, it was freeze-dried to obtain the anhydrous weight. Hydration was determined as the difference in weight of (blotted SC − freeze-dried SC)/blotted SC × 100. Blotting for 5 s was found to give 20−40% water content.

Differential Scanning Calorimetry Measurements. Differential scanning calorimetry (DSC) measurements of SC were performed with a Calorimetry Sciences Corporation model 4215 differential scanning calorimeter (TA Instruments, Lindon, UT). SC sheets were isolated from human skin as described previously[24] and were treated with various formulations by applying the formulation to a piece of SC and incubating at 4 °C for 24−48 h. Before loading into sealed DSC cells, SC samples were equilibrated at room temperature for 1 h and then rinsed 5 times with filtered, degassed Nanopure (Barnstead) water. Samples were then

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blotted dry with tissue paper to approximately 20−40% hydration. Background excess thermal power scans were obtained with the Nanopure water (15−90 mg) in the sample cell; these were subtracted from the scans for each sample (80−120 mg) by choosing the water power scan that most closely matched the sample power scan. All sample and background scans were done using an empty reference cell. All scans were from 1 to 110 °C at a scan rate of 1 °C/min.

SAXS and WAXS Measurements. Small- and wide-angle X-ray scattering (SAXS and WAXS) measurements of isolated SC treated with various formulations for 24−48 h at 4 °C were made using beamline X21 at the National Synchrotron Light Source at Brookhaven National Laboratory, Upton, NY. Beamline X21 is based on a wiggler source, with a helium gas-cooled Si(111) monochromator selecting the photon energy. The scattering pattern was recorded using a 13 cm CCD detector (Mar USA) at 1.26 m (calibrated with the scattering pattern of silver behenate) downstream of the sample. The measurements were performed with 12 keV X-rays, and data covered a q-range from 0.008 to 0.5 Å^{-1}. All beam paths were under vacuum, except at the sample position, where the sample was in air. Kapton windows terminated the vacuum beam paths. Fully hydrated samples were loaded into 1.5 mm capillaries or round cavities of in-house fabricated 1.5 mm thick aluminum sample holders and data recorded at room temperature. All spectra were processed to remove background contributions by subtracting the scattering profile obtained for an empty capillary or round cavity sealed with Kapton tape. SAXS studies have been carried out with SC samples from three different patients for each formulation. Treatments with biphasic vesicles were additionally extended to evaluate reproducibility of the effect using 6 different laboratory batches and 5 different scale-up batches of biphasic formulations. SC samples were treated with various formulations by applying 100 mg of the formulations to approximately 1 × 1 cm square pieces of SC. The formulations were then rinsed from the SC with 3 × 2 mL volumes of Millipore purified water. The rinsed samples were placed in the sample holder, and the scattering profiles were measured as above. In each SAXS profile peak positions were assigned and changes to the untreated SC as a baseline were identified. Polymorphic phase change was assigned based on the largest number of identifiable peaks patterns.[25]

Variations in peak intensity and width in all samples were noted and attributed to inherent variations in biological samples and the random orientation of samples within the sample holder.

Lattice constants (α) were calculated for the cubic phase (Q224) based on

$$s_{h,k,l} = (1/\alpha)(h^2 + k^2 + l^2)^{0.5}$$

where h, k and l are the Miller indices.

Results

Confocal Microscopy. Comparative confocal microscopic studies were conducted of skin samples treated with biphasic vesicles and their subcomponents (i.e., liposomes and submicrometer emulsion). Whole skin (cleansed after treatment) from both the SC horizontal and cross sections, precut with a blade into approximately 2−300 μm thickness, were optically sectioned. Three-dimensional reconstruction of horizontal and cross-sectional skin slices treated with biphasic vesicles encapsulating Alexa 594-labeled IFN α indicated an intercellular distribution with bright spotted pattern to a depth of 70 μm, into the viable epidermis, well below the 10−15 μm depth of the SC (Figure 1a,b). In contrast, fluorescence was mainly on the surface of the skin, with limited fluorescence observed around superficial SC cells, after topical treatment with Alexa 594-labeled IFN α encapsulated in liposomes (Figure 1c,d) or mixed into the submicrometer emulsion (Figure 1e,f) or methylcellulose gel vehicle (Figure 1g,h). Cross-sectional views in Figure 1 demonstrate the greater depth and distribution of labeled IFN α in biphasic vesicle-treated skin (Figure 1b, INF α in viable epidermal layer) relative to the superficial depth and distribution of labeled IFN α in liposome-, submicrometer emulsion-, and methylcellulose gel-treated skin samples, which show INF α distribution only in the SC. Figure 1b,d,f,h show the orthogonal-cut image of the cross-sectional image series for the four formulations. Biphasic vesicles promoted permeation of IFN α into the viable skin layers, whereas treatment with liposomes and submicrometer emulsion localized IFN α in the SC. To model the permeation of free IFN α, this protein was incorporated into a low viscosity methylcellulose (MC) gel, which allowed easier dosing onto the skin surface. However, subsequent analysis of IFN α permeation was more difficult because the MC gel adhered strongly to the surface of the skin and was not removable by the skin-cleansing protocol that was suitable for the other three formulations. Nevertheless, it was evident that IFN α from the MC gel did not penetrate into the skin beyond the superficial layers of the SC.

Differential Scanning Calorimetry. DSC measurements indicated changes in thermotropic behavior of SC lipids after topical treatment. After biphasic vesicle treatment, the T1, T2, and T3 transitions decreased and Tx (at about 55 °C) increased in all replicate samples ($n = 4$) (Figure 2). This peak is not always detectable in human SC (in 5/10 samples in this study); however, its amplification is attributed to changes in the structural arrangement of the covalently bound lipids outside the corneocyte membranes.[26,27] A decrease in

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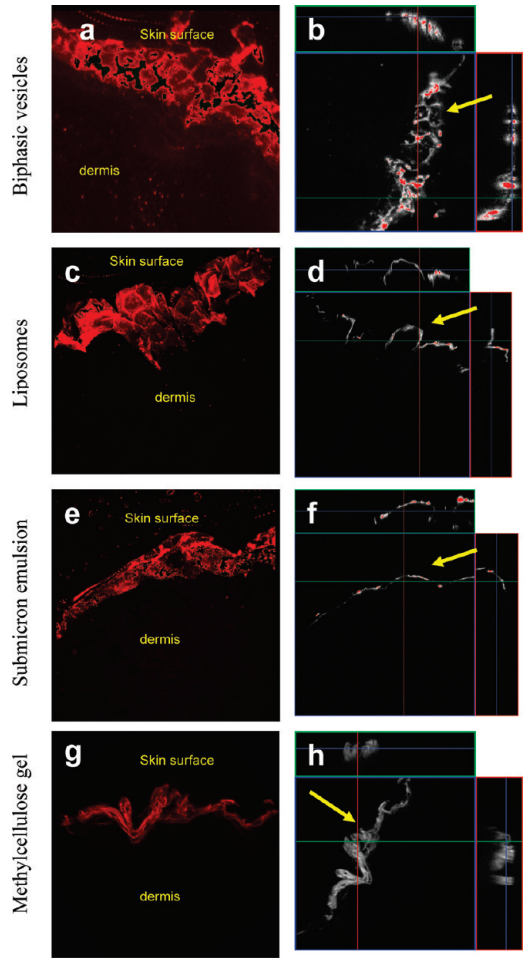

Figure 1. Confocal microscopic images of IFN α delivery into human skin. 3D-reconstructions of horizontal *z*-sections (left column; reconstructed from 120 0.5 μm optical sections) and orthogonal display of cross sections (right column) (arrows indicate fluorescence distribution due to IFN-α) of human skin treated *in vitro* with biphasic vesicles, their components (liposomes, submicrometer emulsion) and a reference topical vehicle (methylcellulose gel): (a, b) treatment with Alexa 594-IFN α encapsulated in biphasic vesicles; (c, d) treatment with Alexa 594-IFN α encapsulated in liposomes; (e, f) treatment with Alexa 594-IFN α mixed into submicrometer emulsion; (g, h) treatment with Alexa 594-IFN α mixed into methylcellulose gel. Image sizes: 512 × 512 pixels, where each pixel = 0.625 μm for the 40× objective.

the T1 peak at 38−42 °C (lipid packing) indicates a lipid disordering effect. A reasonable interpretation is that the originally orthorhombic lattice changes to a fluid or a hexagonal phase.[28] The propylene glycol component in the biphasic formulation may have contributed to this lowering of enthalpy at 40 °C. Additionally, a noticeable decrease was

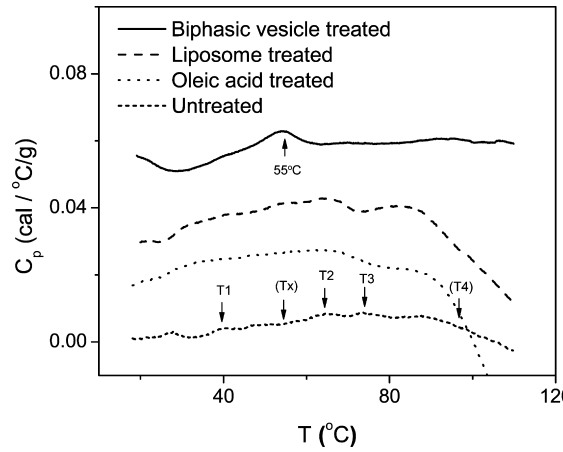

Figure 2. Differential scanning calorimetry thermograms of untreated SC and SC samples treated with biphasic vesicles, liposomes and oleic acid. Arrows indicate major transitions (T1, 35−42 °C; Tx [if present], *ca.* 55 °C; T2, 60−77 °C; T3, 70−90 °C; T4 [if present], 95−120 °C[76,77]) in untreated hydrated SC. Scan rate was 1 °C/min. Thermograms have been shifted on the *y*-axis for clarity and normalized to the sample mass.

observed on the other thermal transitions, T2 and T3, detected typically at 69−77 °C (reversible lipid transition) and 78−92 °C (lipids bound to protein) in biphasic vesicle-treated SC. A very small peak at 95−120 °C (protein denaturation) was apparent only on the biphasic vesicle thermogram, possibly because of the increased hydration level in these samples. A DSC scan of the biphasic vesicles (results not shown) indicated major transitions at around 80 °C. This further supports that the main transition seen in biphasic vesicle-treated SC can be attributed to the internal lipid-phase change in this layer.

Liposome treatment decreased the T1 and T3 thermotropic transitions, indicating that liposomes can disorder intercellular lipids and induce some degree of phase change. Oleic acid, a known permeation enhancer for small molecules, decreased the endothermic transitions at all thermal transition temperatures (Figure 2), which is consistent with previous data.[29,30]

SAXS and WAXS Analysis. Comparative SAXS data of untreated SC and SC treated with the biphasic vesicle formulation indicated a strong interaction with SC lipids (Figure 3). Untreated SC showed a scattering profile similar to previous patterns of lamellar structure, with a periodicity

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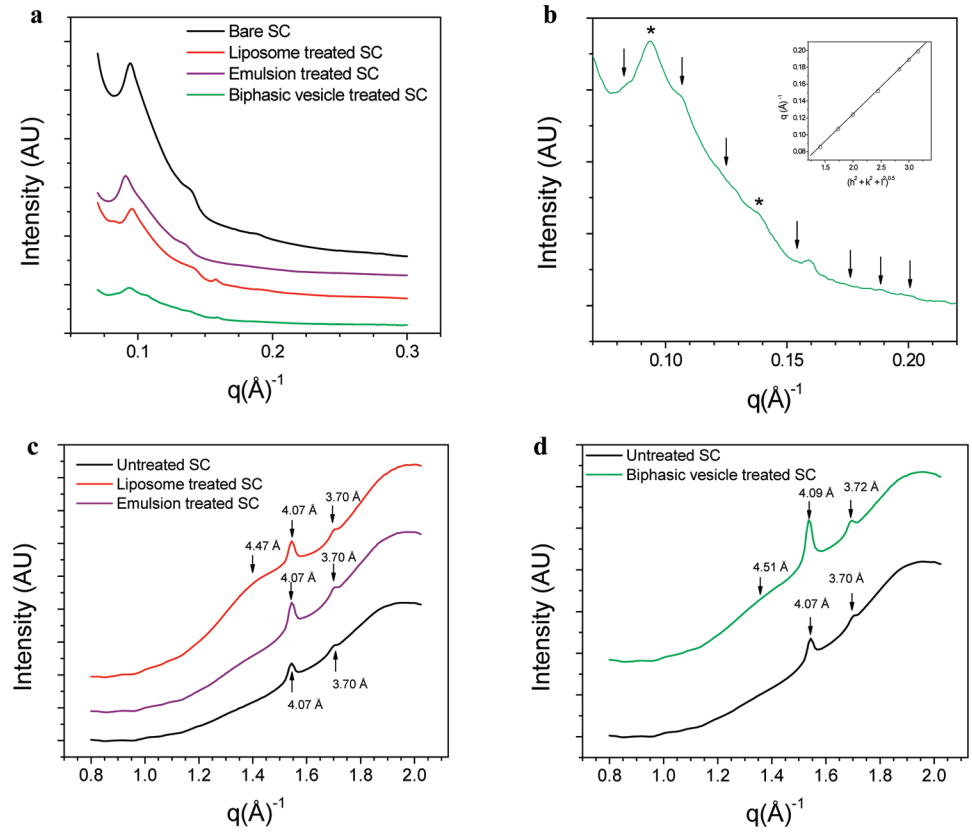

Figure 3. SAXS and WAXS patterns showing the effect of biphasic vesicles and their components (submicrometer emulsion and liposomes) on the lipid organization structure of human SC. The intensity is plotted as a function of q (Å^{-1}), the scattering vector, where q is defined as $4\pi \sin \theta/\lambda$; θ is the scattering angle and λ is the wavelength of the X-rays. SAXS curves of untreated SC and SC treated with biphasic vesicles, liposomes and submicrometer emulsion (a); and a scaled graph with an insert of the Miller plot for SC treated with biphasic vesicles showing the reflections ($\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$, $\sqrt{8}$, $\sqrt{9}$, and $\sqrt{10}$) representative of Q^D cubic phase 224, *Pn*3*m* (b). The reflection observed at 0.158 Å^{-1} is due to crystalline monolauroyllysine, a suspended excipient in the formulation. WAXS curves of untreated SC and SC treated with liposomes and submicrometer emulsion (c), and SC treated with biphasic vesicles (d).

of approximately 14 nm.[31,32] Examination of 22 SC samples treated with biphasic vesicles (half treated with biphasic vesicles with IFN α and half with placebo biphasic vesicles) indicated a pattern consistent with a polymorphic change within the otherwise lamellar SC lipid phase (Figure 3a shows a representative SAXS profile). A Miller plot analysis of the position of the reflections as a function of the Miller indices ($\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, and $\sqrt{6}$ reflections) resulted in the assignment of a *Pn*3*m* bicontinuous cubic phase (space group No. 224)[33] with a calculated lattice constant of 101 and 106 Å, in the absence and presence of IFN α (treatment with biphasic vesicles made without or with IFN α), respectively.

Treatment with the liposome phase of the formulation resulted in a shift in the SC peaks, corresponding to the short and long periodicity phases[32] (q = 0.094 and 0.099 Å^{-1}, respectively) to smaller q and a decrease in the intensity of the scattering (Figure 3a). Such a decrease in scattering intensity has previously been attributed to a disruption of the lipid bilayers in the SC. Treatment with the submicrometer emulsion phase of the formulation showed no apparent change compared with untreated SC (Figure 3a). Two peaks at 0.084 and 0.165 Å^{-1}, appearing only in the liposome treated SC, and the peak at 0.165 Å^{-1} in the biphasic vesicle treated SC were assigned to a lamellar phase that may result from the adherence or some degree of incorporation of the lipids from the liposome or biphasic vesicle formulations within the upper SC layers. Control experiments on SC without removing the formulations after incubation clearly indicated these formulation-related peaks (results not shown). These peaks are distinct from the pattern of the peak set

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indicating the presence of the cubic phase in the biphasic vesicle treated SC.

Additional WAXS analysis of untreated SC indicated characteristic chain packing peaks at ~4.1 and 3.7 Å, corresponding to an orthorhombic lateral packing of the lipid chains (Figure 3c,d). No effect was observed after treatment with the submicrometer emulsion of the formulation. Small variations in the interchain spacings were observed for SC treated with liposomes or biphasic vesicle formulations. In both cases reflections corresponding to orthorhombic (3.7, 4.1, 4.1 Å), hexagonal (4.1, 4.1, 4.1 Å), and liquid (4.6, 4.6, 4.1 Å) packing could be detected. Interestingly, the ratio of the intensities of the peaks at 4.1 and 3.7 Å also increased upon treatment with biphasic vesicle formulations, from ~4.3 to 4.9, increasing the probability of a hexagonal packing arrangement contributing to the peak at 4.1 Å. As such, the increase in the ratio of intensities may indicate that the biphasic formulation increased the portion of lipid molecules arranged in a lateral hexagonal packing, corresponding to a reduced packing density of lipids and an increase in permeability.[25] Therefore, it is possible that liposome treatment, because of the higher intensity of the 4.6 Å reflection, induced more of an orthorhombic-to-liquid rearrangement (with a smaller proportion of hexagonal), whereas biphasic vesicle treatment induced an orthorhombic-to-hexagonal rearrangement (with a smaller proportion of liquid state).

Discussion

The development of noninvasive dermal and transdermal delivery systems suitable for the next generation of therapeutic agents (proteins, peptides, and oligo- and polynucleotides) is challenging, as these molecules have molecular weights typically well above the generally accepted transdermal delivery limit of 500−1000 Da. Developing needle-free methods for protein and gene delivery through difficult barriers such as intact skin will provide urgently needed alternative administration methods for drugs and vaccines, as well as increase drug safety and compliance.

Evidence is mounting that combining different CPEs can cause synergistic enhancement. For example, when two types of enhancers are combined, where one increases the diffusion coefficient and the other the partition coefficient (e.g., Azone and propylene glycol or Azone and Transcutol), a synergistic effect is obtained.[21,22] More recent efforts are attempting to identify synergistic combinations of permeation enhancers (SCOPE) by high-throughput screening.[34,35] Although these enhancer combinations were tested mostly for small drug molecules, it is expected that the concept can be applied to macromolecules as well.

Liposomes were first applied as a topical delivery system in the early 1980s by Mezei and colleagues[36,37] and were subsequently used by many researchers to deliver different drug molecules.[38] Dermal and transdermal delivery by liposomes was successful for small lipophilic molecules, but was limited for hydrophilic and larger compounds. The mechanism of delivery is explained by the structural similarity of the liposome bilayers to those of the intercellular lipids, which are also arranged in a bilayer in the SC, making interaction between liposomes and the SC feasible. The intermixing of these lipids would explain the greater enhancement of delivery of lipophilic drugs trapped in the liposome bilayer into the skin, compared with delivery of hydrophilic drugs. However, the overall degree of permeation enhancement by simple liposomes is limited.[13] Modified forms of liposomes that include additional components indicate enhanced drug delivery properties and can be considered synergistic mixtures. Examples include ethosomes, a combination of phospholipids and ethanol,[39] and transfersomes, a combination of phospholipids and sodium cholate.[40] Another example is lipid vesicles that consist of soybean phosphatidylcholine and polyoxyethylene(20) oleyl ether (C(18:1)EO(20)).[41]

Additionally, there is evidence to support the importance of the type and phase properties of lipids used for liposome construction. For topical as well as intracellular delivery, fluid phase phospholipids were shown to be superior to gel phase lipids.[32,42−44] The molecular organization of the lipids may also be critical. El Maghraby and colleagues demonstrated

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that bilayer vesicle structure was essential to achieving increased estradiol delivery.[45] When the drug and lipid ingredients were mixed without liposome formation, skin penetration was about five times lower than with liposome formation. Interestingly, liposomes made from SC ceramide lipids interacted with corneocytes and transformed into lamellar sheets, indicating that these types of lipids migrate into the skin.[46] However, by integrating into the skin, these ceramide-based lipid vesicles improved barrier strength rather than enhanced permeability.[47,48]

The importance of formulation colloidal structure in increasing skin permeation was demonstrated by adding increasing concentrations of isopropyl myristate to a lamellar liquid crystal phase lecithin/water mixture, which resulted in an increased permeability coefficient of encapsulated fenoprofen acid.[49] This effect was attributed to the formation of a hexagonal phase lipid structure. In a recent paper by Barbosa-Barros et al.[50] the effect of structure/morphology and the composition of the lipid aggregates were shown to determine the interaction with stratum corneum. It was demonstrated that formulations with the same lipid composition but different self-assembly or different lipid compositions arranged in the same self-assembled particle (liposomes) caused different degrees of transepithelial water loss (TEWL) *in vivo*.

If further permeation enhancement is to be achieved, delivery systems need to be designed where the combination of components provides permeation enhancement in two ways: (1) through selection of the right synergistic mixtures of materials and (2) through assembly of the right polymorphic molecular arrangements of the building blocks. Thus, the basis for biphasic vesicles design was that they should be built from excipients that provide synergistic skin permeation enhancement for macromolecules. The vesicles are made from phosphatidylcholine that forms concentric bilayers that entrap an aqueous submicrometer emulsion phase containing the drug to be encapsulated. Relevant properties of the vesicles for skin interaction are mixed lipid membrane characteristics, multicompartmental structure and cationic charge, all of which could contribute to enhanced drug delivery by any of the previously described mechanisms in the literature. For example, the mixed bilayer lipids of the vesicles could cause disordering of the lipid channels and modify lipid phase fluidity in the intercellular spaces of the skin, enhancing the lipoidal pathway of penetration; encapsulation of the drug into the multicompartments of the vesicle could create a stable supersaturated system, thereby maximizing thermodynamic activity (drug diffusion driving force into the skin); and the cationic charge of the vesicles could facilitate an attraction to the negatively charged interior of skin layers through a micro-osmotic process. The effect of biphasic vesicles on the structural order of the SC lipids and the induction of a polymorphic rearrangement of SC lipids are observations not reported previously for any dermal or transdermal delivery system or permeation enhancer.

The results in this study indicate a complex mechanism of delivery. Biphasic vesicles appear to interact with the intercellular lipids of the SC, which may be responsible for enhancing the lipoidal pathway of penetration. The interaction causes the original orthorhombic lipid packing configuration in the SC (which provides a strong barrier to permeation of substances) to assume a hexagonal configuration or liquid state, thereby increasing permeability (Figure 4a,b). The interaction with intercellular lipids appears to be more evident among the lipids most closely surrounding the corneocytes. To this point biphasic vesicles appear to behave similarly to other lipid vesicles on the skin, although DSC data indicate that a different type of interaction also occurs. SAXS and WAXS data demonstrated that biphasic vesicles induce the formation of a three-dimensional bicontinuous *Pn3m* cubic phase in the SC. Formation of this cubic phase was unique to biphasic vesicles, as their subcomponents — liposomes and submicrometer emulsion — did not induce such a polymorphic phase change in the SC. The cubic phase is a three-dimensional network of aqueous channels within a lipid matrix on an infinite periodic minimal surface (Figure 4c, right illustration; also see ref 51). The aqueous channels of the cubic phase could be conduits for hydrophilic molecules[52] and appear to be compatible with accommodating various proteins and enzymes.[53−55] The transport of proteins through a cubic phase structure made from hydrated monoacylglycerols was shown to depend on the diameter of the aqueous channel.[56] In monoolein systems the diameter of the aqueous channels was estimated between 69.8 Å and 112.8 Å, depending on the water content.[57] Further, the physical properties, including aqueous channel diameter of

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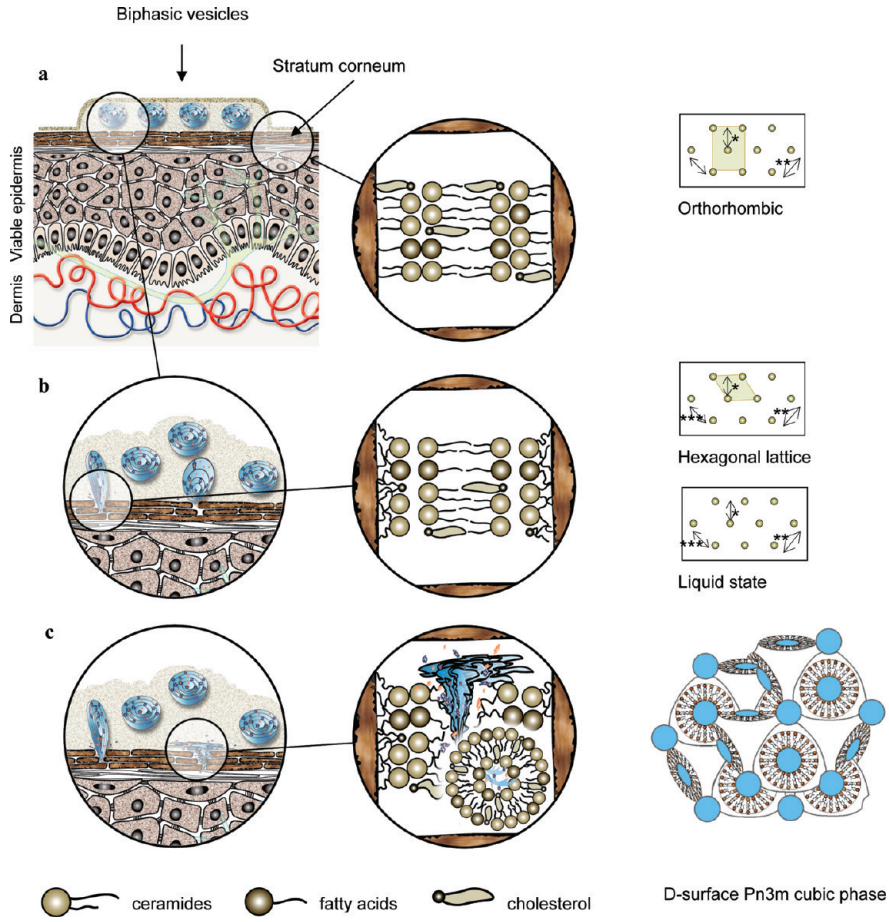

Figure 4. Proposed mechanism of interaction of biphasic vesicles with the SC. (a) Application of biphasic vesicles in a cream formulation on skin under an occlusive patch (left illustration). In normal SC intercellular lipids are organized in bilayers with a regular repeat pattern; i.e., a periodicity of about 13 nm, and lipid packing in an orthorhombic configuration, providing a strong barrier to permeation of substances[32] (center illustration). Orthorhombic lateral packing of lipids shows spacings *3.7, **4.1 Å (WAXS data and ref 78) (right illustration). (b) Biphasic vesicles initiate interaction with the skin (left illustration). Biphasic vesicles interact with intercellular lipids and create an initial disorder, which is more apparent among the lipids most closely surrounding the corneocytes (deduced from DSC data); intercellular lipid packing within the SC is also affected (WAXS data), indicating appearance of a hexagonal and a liquid state associated with high permeability (center illustration). Hexagonal lateral packing of lipids show spacings *4.1, **4.1, ***4.1 Å and liquid phase shows spacings *4.6, **4.6, ***4.1 Å (WAXS data and ref 78) (right illustration). (c) Biphasic vesicles induce increased disorder of SC lipid channels and possibly enlarge the lipid spacings through which vesicles/vesicle fragments and encapsulated drug are delivered into the skin (left illustration). Biphasic vesicles induce a polymorphic change within the SC (supported by SAXS data). A Miller plot analysis of the position of the reflections as a function of the Miller indices ($\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$, $\sqrt{8}$, $\sqrt{9}$, and $\sqrt{10}$ reflections) indicates the presence of a *Pn*3*m* bicontinuous cubic phase (center illustration; 3-D representation of D surface *Pn*3*m* cubic phase (adapted from ref 79) (right illustration).

the cubic phase, can be modulated by charged lipids and surfactants.[58−60]

Based on considerations and equations described by Briggs et al.[61] and Kraineva et al.[62] for monoolein cubic phase systems under limited hydration conditions, we used the measured lattice parameters from SAXS data to estimate the water channel radius in the cubic phase formed after biphasic vesicle treatment. The water channel radius (r_w) is calculated using the following equation:

$$r_w = (-A_0/2\pi\chi)^{1/2}\alpha - l \qquad (1)$$

A_0 is a constant (its value for *Pn*3*m* phase is −2), and χ is the Euler−Poincare characteristic (a space group dependent characteristic, having a value of 1.919 for the *Pn*3*m* phase).[63−65] Using these values for A_0 and χ,

$$D_w = 2r_w = 0.391\alpha - l \qquad (2)$$

l can be calculated from the lattice parameter, α from SAXS data and the sample composition such as the volume fraction of the lipid (ϕ_l),

$$\phi_l = 2A_0(l/\alpha) + 4\pi\chi/3(l/\alpha)^3 \qquad (3)$$

ϕ_w is volume fraction of water, and ϕ_l is volume fraction of lipid (0.4595, based on the partial specific volume of lipid v_l and water v_w, which are 1.062 cm^3/g and 0.903 cm^3/g at

20 °C). These two parameters are assumed to be independent of composition and minimally dependent on temperature and pressure.[62] The calculated lattice constant for the induced cubic phase by biphasic vesicles (without IFN α) is 101 Å and by biphasic vesicles encapsulating IFN α is 106 Å. The resulting estimates for the diameter of the aqueous channels for each of these two phases are 44.98 and 48.89 Å, respectively, when using 17 Å for lipid length.[61] The hydrodynamic radius (R_H) of IFN α with a molecular weight (MW) of 19.4 kDa, calculated from the equation $MW = (1.6800R_H)^{2.3398}$, is 2.11 nm (diameter = 4.22 nm). The radius of gyration (R_g) for IFN α, which would describe its effective size, is not known in the absence of crystallography data; however, based on estimates of similarly sized proteins it may be around 18 Å.[66] Considering the aqueous channel and IFN α molecular dimensions as calculated above, the transport of IFN α appears feasible. This is further supported by results of Clogston et al.,[66] who showed that the diffusion characteristics of a protein depend on the size of the aqueous channels. However, as the cubic phase is quite flexible, it can accommodate somewhat larger sized molecules.

The transformation between lamellar (Lα) and cubic phases was described previously.[67,68] Chung and Caffrey[69] as well as other research groups[66,70] have demonstrated that relatively minor changes in molecular compositions within small regions of lipids and resulting changes in curvature energies will contribute to polymorphic phase changes. The lipids in mitochondria, for example, are arranged in a cubic array system during inactivity,[71] and bilayer-to-cubic or cubic-to-bilayer organizational switching may be a form of cellular functioning such as an on−off mechanism of membrane continuity or intracellular trafficking and transportation.[72] According to Anderson et al.[63] the transformation of a lamellar phase into a cubic phase is more likely than to an inverted hexagonal phase because of the geometrical considerations related to κ_m (bending rigidity constant of a lipid monolayer) and l_0 (length of the lipid molecule).

The ability of SC lipids to rearrange from a bilayer configuration to other polymorphic phases has been demonstrated previously. The two prominent models explaining the formation and molecular organization of SC lipids indicate two possibilities: a lamellar-to-lamellar transition and a cubic-to-lamellar transition between the as-produced lipids by granulocytes and the intercellular lipid barrier layer in the SC. The Landmann model[73] postulates that lamellar granules produced by granular cells in the upper viable epidermis fuse with the cell membranes and extrude into the extracellular spaces to form uninterrupted bilayer sheets. Hence, the Landmann model represents a lamellar-to-lamellar phase transition. Research on liposomal topical drug delivery systems supports the possibility that lamellar-to-lamellar phase interaction could take place between liposomes and SC lipids and could create some disorder, which results in

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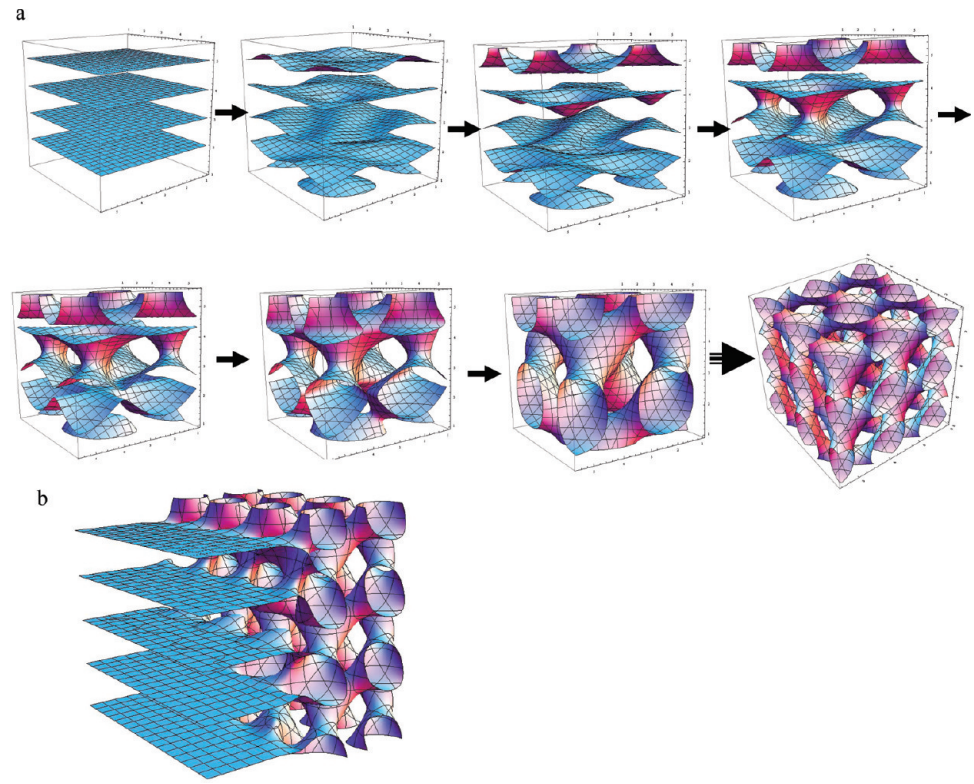

Figure 5. Simulation of cubic phase formation in SC lipids: hypothetical models. (a) Induction model: The lamellar lipid phase in the SC undergoes a series of molecular reorganization steps (shown here as a seven-step process) including possible fusogenic processes as a result of biphasic vesicle interaction and reform as a 3-dimensional cubic phase with aqueous channels (modeled using Mathematica version 6). The diamond surface (D-surface) *Pn*3*m* (space group no. 227) is described by the following equation: $\cos(2x)\cos(2y) + \cos(2y)\cos(2z) + \cos(2z)\cos(2x) + \sin(2x)\sin(2y)\sin(2z) = 0$. (b) Merging model: Hypothetical merging of lamellar phases between the biphasic vesicle lamellar structures and SC lamellar structures to form a cubic phase. $L(x,y,z)$ represent the equation for the lamellar phase, and $Q(x,y,z)$ represent the equation for the *Pn*3*m* cubic phase. The equation describing the merging of the two phases as shown is given by $H(-3 - z)L(x,y,z) + H((z + 3)(5 - z))[T(z,-3,5,4)L(x,y,z) + (1 - T(z,-3,5,4))Q(x,y,z)] + H(z - 5)Q(x,y,z) = 0$.

enhanced drug delivery. More recent studies indicate that the lipids extruded by granular cells during lipid barrier formation may be in other polymorphic states such as a cubic phase, which merges into the lamellar intercellular space.[74] The membrane folding model by Norlen[74] implies the flexibility of lipid structural arrangement and suggests that intercellular lipid formation via cubic-lamellar phase transition is more energetically favorable than is lamellar granule fusion,[75] which further supports that intercellular lipids can rearrange into a cubic phase under specific circumstances.

Mathematical modeling of the bilayer-to-cubic phase transformation that may take place upon biphasic vesicle application to the SC is shown in Figure 5. The formation of cubic phase configuration could occur in two ways: (1) by regional fusion of the lamellar lipid layers in the SC, resulting in a series of molecular reorganization steps (shown here as a seven-step process) and re-forming as a 3-dimensional cubic phase with aqueous channels (induction model; Figure 5a), or (2) by the merging of lamellar phases between the biphasic vesicle lamellar structures and SC lamellar structures to form a cubic phase (merging model; Figure 5b).

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Conclusion

The interaction of biphasic vesicles with SC lipids results in the formation of a three-dimensional cubic *Pn*3*m* polymorphic phase by the molecular rearrangement of intercellular lipids. This is the first instance demonstrating the induction of a cubic phase in the SC by an external formulation. This observation is significant because most previous literature data that describe intercellular permeation of compounds attribute the enhanced permeation to the disordering, disturbance, fluidization or extraction of lipids in the stratum corneum. The results reported here suggest that enhanced dermal and transdermal delivery of proteins may not depend on a disordered state; rather, it may depend on a different order of the lipids. This novel nanopathway through the SC induced by biphasic vesicles could make delivery of large proteins into the skin possible.

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