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Skin permeation of retinol in Tween 20-based deformable liposomes: in-vitro evaluation in human skin and keratinocyte models

Yu-Kyoung Oh, Mi Young Kim, Jee-Young Shin, Tae Woon Kim, Mi-Ok Yun, Sung Joon Yang, Sang Sook Choi, Woon-Won Jung, Jung Ae Kim and Han-Gon Choi

Abstract

To develop a more effective transdermal delivery method for lipophilic functional cosmetic compounds such as retinol, we formulated various deformable liposomes and compared their transdermal delivery efficiency with those of neutral or negatively-charged conventional liposomes. We tested the deformability of liposomes containing edge activators such as bile salts, polyoxyethylene esters and polyoxyethylene ethers. As indicators of deformability, we used the passed volume and phospholipid ratios during extrusion, as well as the deformability index. We found that the type of edge activator significantly affected the extent of deformability, and that Tween 20 provided the highest level of deformability. Accordingly, we used Tween 20 to formulate deformable liposomes containing retinol in the membrane bilayers, and conducted a skin permeation study in Franz diffusion cells, using dermatomed human skin and three-dimensional human keratinocyte layers. As compared with the use of conventional neutral or negatively-charged liposomes, the use of Tween 20-based deformable liposomes significantly increased the skin permeation of retinol. These results suggested that deformable liposomes might be of potential use for the formulation of retinol and other lipophilic functional cosmetic compounds.

School of Life Sciences and Biotechnology, Korea University, Anam-dong, Seungbuk-gu, Seoul 136-701, South Korea

Yu-Kyoung Oh, Mi Young Kim, Jee-Young Shin

Hans Biomed Corporation, Daejeon, South Korea

Tae Woon Kim

Korea Food and Drug Administration, Seoul, South Korea

Mi-Ok Yun, Sung Joon Yang, Sang Sook Choi

MyGene Bioscience Institute, Seoul, South Korea

Woon-Won Jung

School of Pharmacy, Yeungnam University, Kyongsan, South Korea

Jung Ae Kim, Han-Gon Choi

Correspondence: Y.-K. Oh, School of Life Sciences and Biotechnology, Korea University, Anam-dong, Seungbuk-gu, Seoul 136-701, South Korea. E-mail: ohyk@korea.ac.kr

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Introduction

Cosmeceuticals have emerged as an important new field requiring the delivery of functionally active compounds via topical routes. Though some cosmeceuticals (e.g. photoprotectants) function at the skin surface, many lipophilic cosmeceuticals exert their physiological benefits on cells in the epidermis or dermis layers (Kligman 2000). However, the physiological effects of these lipophilic cosmeceuticals have been limited to date, due to their inefficient penetration through natural skin barriers. One such lipophilic functional cosmetic, retinol, is widely used as a major component in functional cosmetics, but does not penetrate the skin well (Abdulmajed et al 2004).

Thus, a strong need exists for new delivery vehicles capable of enhancing the skin permeation of lipophilic cosmeceuticals such as retinol. Liposomes have been studied as vehicles for increasing the skin permeation of retinol. Cosmeceutical liposomes typically consist of phospholipids and cholesterols, which form concentric hydrated bilayers capable of entrapping and delivering functional cosmetics to the skin. Previous studies have shown that liposomes accumulate in the stratum corneum or other upper skin layers (Dayan & Touitou 2000), and only rarely penetrate into viable skin (Touitou et al 1994). To overcome this limitation, other vehicles have been examined, including deformable liposomes, which are specially designed vesicular particles containing at least one inner aqueous compartment surrounded by a lipid bilayer. Although deformable liposomes are similar to conventional liposomes in their morphology and construction, they differ in terms of elasticity. In addition, deformable liposomes contain membrane-softening molecules called edge activators, which most commonly consist of bile salts (Paul et al 1998; Kim et al 2004; Trotta et al 2004).

Deformable liposomes have been shown to improve the transdermal delivery of anti-inflammatory agents, antitumour agents and plasmid DNA as compared with conventional liposomes lacking edge activators (Cevc & Blume 2004; Trotta et al 2004; Lee et al 2005). In addition, deformable liposomes have been shown to be effective for transcutaneous immunization with antigenic proteins (Paul et al 1998; Hofer et al 2000).

However, no previous work has sought to deliver lipophilic cosmeceuticals using deformable nanoparticles. Moreover, although the edge activators play an important role in the deformability of liposomes, few studies have systemically compared the effect of edge activators on deformability. Here, we have compared the effects of various edge activators on the deformability of nanoparticles, and have examined the use of optimized deformable liposomes for enhancing human skin permeation of retinol, as compared with delivery with conventional neutral or negatively-charged liposomes.

Materials and Methods

Materials

Phosphatidycholine from egg yolk (PC), phosphatidylglycerol (PG), and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Tween 20 and Triton X-100 were from USB (Cleveland, OH, USA). Sodium cholate, sodium deoxycholate, sodium taurocholate, Tween 80, Brij 35, Brij 58, Myrj 45, Myrj 53, and phosphorus standard solution were from Sigma Chemical Co. (St Louis, MO, USA). The Nuclepore polycarbonate membranes (pore size: 0.05, 0.1, 0.2 μm) were from Whatman (Clifton, NJ, USA). All the materials used in this study were of reagent grade with the purity of 99% and used without further purification.

Preparation of liposomes with or without edge activators

To serve as control liposomes without an edge activator, neutral liposomes were prepared according to Oh & Straubinger (1997). In brief, 15 μmol lipids (PC:Chol, molar ratio, 10:5) were dissolved in 300 μL chloroform, the chloroform was removed on a rotary evaporator under reduced pressure, and nitrogen gas was purged on the resulting dried lipid film to remove trace organic solvents. The lipid film was suspended in 0.5 mL NTE buffer (pH 7.0, 145 mM NaCl, 10 mM TES (N-Tris(hydroxymethyl)-2-aminoethane sulfonic acid), 0.1 mM EDTA) with vigorous vortexing, and the resulting liposomes were extruded three times through polycarbonate membrane filters. The deformable liposomes with edge activators were prepared as described by Hofer et al (2000) with slight modification. In short, deformable nanoparticles were formed by mixing 15 μmol PC with each edge activator in NTE buffer (6:1 w/w) and extruding the resulting particle suspension three times as above.

Measurement of particle size

The mean particle sizes of the liposomes were determined by the dynamic light scattering method using a Submicron Particle Sizer (Nicomp 370, Particle Sizing Systems, Inc., Santa Barbara, CA, USA). Liposomes were diluted in distilled water for intensity adjustment before size measurement, as recommended by the manufacturer. A colloidal solution of anhydrous caffeine was used to validate the particle size analyser.

Measurement of passed volume ratios

For evaluating the deformability of the various edge activator-based liposomes, permeation across an artificial membrane was surveyed using an extruder (Lipex Thermobarrel Extruder, Northern Lipids Inc., Canada) under the indicated pressure. Liposomes were diluted with NTE buffer to yield a final lipid proportion of 1.5%. External pressure (1.5 MPa) was used to drive the diluted liposomes through three sheets of 50-nm polycarbonate membrane for 10 min, and the extruded volume was measured using a syringe.

Quantitative determination of phospholipids

The concentration of phospholipids before and after extrusion of the liposomes was determined using the inorganic phosphate assay method (Torchilin & Weissig 2003). In brief, liposome suspensions were digested with 400 mL 5 M H_2SO_4 at 170 °C, the samples were cooled, and 100 mL 30% H_2O_2 was added. The mixtures were heated at 170°C until the H_2O_2 was completely removed. The removal of H_2O_2 was confirmed using H_2O_2 detection strips. To the cooled sample, 4.6 mL 0.2% ammonium molybdate and 100 mL 15% fresh ascorbic acid were sequentially added with vortexing. The resulting mixtures were then boiled for 10 min at 100°C, cooled to room temperature, and the absorbance read at 830 nm. Standards were prepared with phosphorus standard solution (Sigma cat. no. P3869). The passed phospholipid ratio was calculated by dividing the total amount of phospholipids in the pass-through sample by that in the initial suspension.

Calculation of the deformability index

The deformability index was defined as a multiplication value of the passed volume ratio, the passed phospholipid ratio, and the factor related to the particle size: $(r_v/r_p)^2$. In this equation, r_v is the radius of a phospholipid nanoparticle and r_p is the radius of the membrane micropores (Cevc et al 1998).

Encapsulation of retinol in conventional or deformable liposomes

Retinol was encapsulated in neutral or negatively-charged liposomes, and in deformable liposomes. To encapsulate retinol in the lipid bilayers of neutral liposomes, 15 μmol lipids (PC:Chol, molar ratio, 10:5) and 2 μmol retinol were dissolved in 300 μL chloroform. To encapsulate retinol in negatively-charged liposomes, 15 μmol lipids (PG:PC:Chol, molar ratio, 3:7:5) and 2 μmol retinol were dissolved in 300 μL chloroform. These lipid mixtures were then placed in a rotary evaporator for removal of the organic solvent, and the dried lipid film was hydrated with 0.5 mL isotonic NTE buffer (pH 7.0). After vigorous vortex mixing, the liposomes were extruded three times with 0.2-μm polycarbonate membrane filters, and three times with 0.1-μm membrane filters. To load retinol in deformable liposomes containing Tween 20

as the edge activator, 15 μmol PC and 2 μmol retinol were dissolved in 300 μL chloroform and placed in a rotary evaporator for removal of the organic solvent. The dried lipid film was hydrated for 15 min with 0.5 mL isotonic NTE buffer containing 0.25% Tween 20, and vortexed. The resulting lipid particles were extruded three times with 0.2 μm polycarbonate membrane filters, and three times with 0.1 μm membrane filters.

Permeation through human skin and epidermis skin models

The human skin study was approved by the Bundang CHA ethics committee and conducted according to the declaration of Helsinki. Human cadaver skin was obtained from the thigh area of a female donor. Dermatomed human cadaver skin samples (Hans Biomed Co., Daejeon, South Korea) or KeraSkin™ (round pieces, 2.1 cm diameter, Modern Tissue Technologies, Inc., Seoul, South Korea) were mounted in Franz diffusion cells with a diffusional area of 1.77 cm^2. The Franz cells have the average receiver volumes of 12 mL. Dermatomed human cadaver skin (300-μm thick) was set in place with the dermis facing the receptor compartment. KeraSkin™, used as an epidermis skin model, consists of normal human epidermal keratinocytes cultured to form a multilayered, highly differentiated human epidermis (Kang et al 2004). The receptor compartment of the Franz diffusion cell was filled with phosphate-buffered solution (pH 7.0) and maintained at 37 ± 0.5°C. Retinol-containing liposome, or free retinol was given as infinite dose in non-occluded condition. After application of 300 μL retinol-containing liposomes, or free retinol onto the human skin or KeraSkin™, 100-μL samples were collected from the receptor side of the diffusion cell at various time points and replaced with the same volume of phosphate-buffered solution. The cumulative amounts of retinol penetrating across the skin were measured using a Kontron SFM 25 spectrofluorometer (Kontron, Zurich, Switzerland) with an excitation wavelength of 395 nm and an emission wavelength of 485 nm. The 100-μL samples taken at various time points were added to 2.5 mL 1% Triton X-100 in phosphate buffered saline for fluorescence measurement. The concentrations were determined by comparison with known standards of retinol dissolved in 1% Triton X-100 in phosphate buffered saline.

Statistical analysis

The data are expressed as mean±s.d. (n=4). Statistical differences were evaluated using the unpaired Student's *t*-test or analysis of variance, with $P<0.05$ considered statistically significant. Duncan's multiple range test was used as a post-hoc test.

Results

Effect of edge activators on the deformability of liposomes

Various edge activators were tested for their ability to affect the deformability of liposomes, as measured by the passed volume ratios, the passed phospholipid ratios, and the deformability index values. First, we measured the passed volumes ratios of liposomes with edge activators before and after extrusion (Table 1). We tested three bile salts, four polyoxyethylene esters, and three polyoxyethylene ethers. Of the tested bile salts, sodium deoxycholate showed the highest passed volume ratios followed by sodium cholate, while sodium taurocholate showed no detectable volume after extrusion, similar to the control liposomes composed of PC and Chol (Table 1). In the case of polyoxyethylene ester-type edge activators, Tween 20 had the highest passed volume ratio, followed by Tween 80, while Myrj 45 and 53 could not pass through the polycarbonate membranes during extrusion under reduced nitrogen pressure (Table 1). Interestingly, the polyoxyethylene ethers tested in this study (Brij 35 and 58, and Triton X-100) all yielded positive passed volume ratios higher than 0.4 (Table 1).

Table 1 Passed volume ratio, relative phospholipid ratio, and deformability index values of various liposomes with edge activators

Edge activators	Passed volume ratio	Relative phospholipid ratio	Deformability index
Sodium cholate	0.22 ± 0.08	0.13 ± 0.06	1.62 ± 0.39
Sodium deoxycholate	0.60 ± 0.02	0.59 ± 0.04	6.39 ± 0.18
Sodium taurocholate	0.00	0.00	0.00
Tween 20	0.64 ± 0.01	0.58 ± 0.02	8.45 ± 0.27
Tween 80	0.49 ± 0.08	0.42 ± 0.08	6.01 ± 0.46
Myrj 45	0.00	0.00	0.00
Myrj 53	0.00	0.00	0.00
Brij 35	0.42 ± 0.09	0.34 ± 0.07	4.40 ± 0.36
Brij 58	0.52 ± 0.10	0.44 ± 0.08	5.53 ± 0.42
Triton X-100	0.74 ± 0.06	0.72 ± 0.07	8.30 ± 0.91
Control	0.00	0.00	0.00

Liposomes composed of PC and Chol without an edge activator were used as a control. Data are expressed as mean ± s.d. (n = 4).

To confirm that the extruded suspensions actually contained the phospholipid vesicles rather than buffer alone, we further tested the amounts of phospholipids before and after extrusion. As shown in Table 1, the ratios of passed phospholipids paralleled the patterns of passed volume ratios regardless of the edge activator utilized. Consistent with the above results, sodium deoxycholate showed the highest passed phospholipid ratio of liposomes among bile salt edge activators, and Tween 20 revealed the highest passed phospholipid ratio of the polyoxyethylene ester-type edge activators. These results indicated that the liposomes indeed passed through the membrane during the extrusion.

The deformability index was used as another measure of liposome elasticity. Of all the edge activators tested in this study, Tween 20 and Triton X-100 showed the highest deformability index values. Although sodium deoxycholate and Tween 20 exhibited similar passed volume ratio and relative phospholipid ratio, Tween 20-based liposomes showed particle sizes higher than sodium deoxycholate-based liposomes, resulting in the higher deformability index. Among the edge activators showing positive passed volume ratios, sodium cholate revealed the lowest deformability index (Table 1).

Formulation and deformability of retinol-loaded liposomes

We formulated retinol-loaded liposomes using Tween 20 as an edge activator, and compared their deformability with those of conventional neutral (containing PC and Chol) and negatively-charged (containing PG, PC and Chol) liposomes. Although Tween 20 and Triton X-100 showed higher deformability than the others, we decided to use Tween 20 for retinol formulation owing to the previous toxicity reports on Triton X-100 (Dayeh et al 2004). Due to the high lipophilicity, retinol was mixed with phospholipids to form a thin lipid film during liposome preparation. NTE buffers or the edge activator buffer solution was then added to the thin film before extrusion. The light scattering data indicated that the mean sizes of conventional neutral (PC:Chol), negative (PG:PC:Chol), and deformable (PC:Tween 20) liposomes did not significantly differ after extrusion at a high nitrogen pressure of 4 MPa. We found that Tween 20-based liposomes were deformable, but that the neutral and negatively-charged liposomes were not. Tween 20-based liposomes passed though the 50-nm pore sized membrane under reduced nitrogen pressure (1.5 MPa), whereas the retinol-containing neutral and negatively-charged liposomes did not show any passed volumes after extrusion under the same nitrogen pressure.

Effect of deformable liposomes on human skin permeation of retinol

We tested whether topically-applied deformable liposomes could enhance the permeation of retinol through human skin as compared with the use of conventional liposomes. In-vitro permeation studies were performed using a Franz diffusion cell apparatus with dermatomed human skin and KeraSkin™, which mimics the keratinocyte layers of human skin. Deformable liposomes showed higher human skin permeation of retinol than did neutral or negatively-charged liposomes (Figure 1A). Similar to human skin, the KeraSkin™ model system showed enhanced penetration of retinol delivered in deformable liposomes vs conventional neutral and negatively-charged liposomes (Figure 1B). As a negative control we tested the penetration of retinol powder suspended in NTE buffer through the human skin and keratinocyte model systems, and observed no skin permeation of unencapsulated retinol.

Stability of deformable liposomes

Stability is an essential factor for functional cosmetic delivery vehicles. The stability of deformable liposomes during storage was studied in terms of size. The size of conventional and deformable liposomes was initially measured after preparation. We found that entrapment of retinol in the deformable liposomes did not significantly change the particle size. Moreover, the storage of deformable liposomes at 4°C had no significant effect on the particle size for up to three months (Table 2).

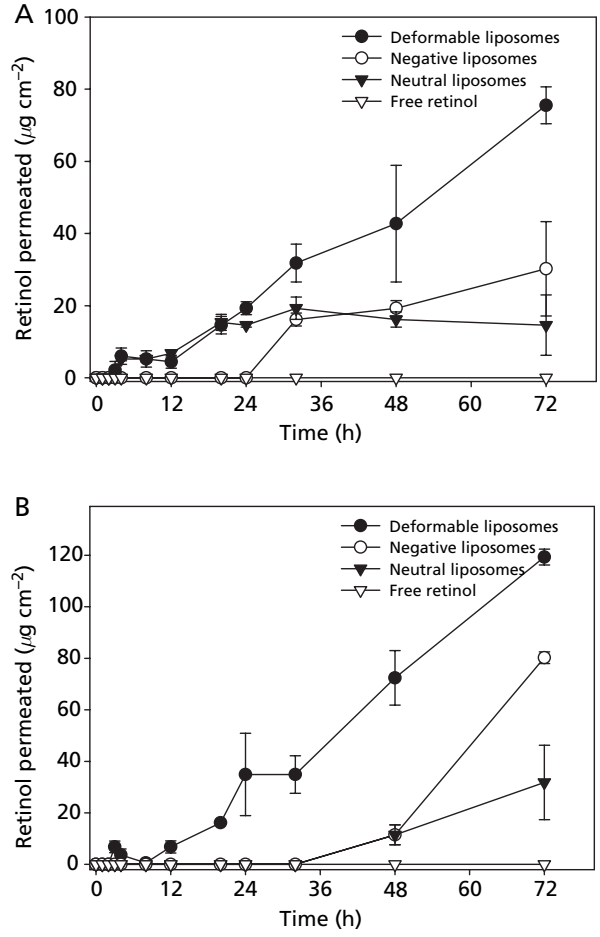

Figure 1 Permeation of retinol across human skin or human keratinocyte layers. Permeation of retinol was studied using dermatomed human skin samples (A) or KeraSkin samples (B) mounted in Franz diffusion cells. The cumulative amounts of retinol permeated across the skin were measured by a spectrofluorometer.

Table 2 Physical stability of deformable liposomes as a function of time

Time	Size of empty liposomes (nm)	Size of retinol-loaded liposomes (nm)
One day	85.30 ± 14.10	97.50 ± 36.50
One week	90.80 ± 49.00	81.50 ± 20.51
One month	133.50 ± 45.40	78.60 ± 35.72
Two months	103.20 ± 33.00	95.00 ± 36.21
Three months	104.00 ± 51.00	128.00 ± 43.10

The average particle sizes of deformable liposomes with or without retinol were measured at various days. Deformable liposomes were stored at 4°C. Data are expressed as mean ± s.d. (n = 4).

Discussion

Our results indicated that Tween 20-based deformable liposomes might have potential for the effective delivery of lipophilic functional cosmetics such as retinol. Previous studies focused primarily on using bile salt-based deformable lipid vesicles as carriers of pharmaceutical drugs, proteins and genes (Paul et al 1998; Kim et al 2004; Trotta et al 2004). We previously reported that edge activators such as sodium cholate, sodium deoxycholate and Tween 80 provided

deformability to lipid vesicles (Lee et al 2005), but herein showed that Tween 20 might be a more effective edge activator.

Interestingly, Triton X-100 provided a deformability index value only slightly lower than that of Tween 20. Trinton X-100 appeared to be better than the previously examined edge activators in terms of this value. However, the usefulness of this edge activator may be limited, as Triton X-100 was reported to exert cytotoxicity on various mammalian cell lines such as H4IIE, Caco2, and HepG-2 (Dayeh et al 2004). In contrast, Tween 20 has been long used as a component of various cosmetic formulations, and sorbitan fatty acid esters such as Tween 20 have been shown to confer minimal to mild skin irritation in animal studies, and to be relatively nontoxic via ingestion in acute and long-term studies (Lanigan & Yamarik 2002). Further work is required to examine the mechanism by which Tween 20 enhanced deformability, but it was possible that the molecule was capable of close physicochemical interaction with PC in the lipid membrane bilayer.

Notably, Brij and Myrj provided totally different deformability to the lipid vesicles. Previously, polyoxyethylene lauryl ether (Brij 30) was used to prepare tretinoin-loaded niosomes (Manconi et al 2003), while Myrj was used for the formulation of lipid emulsions containing gandolinium (Miyamoto et al 1999). We found that Brij, but not Myrj, conferred substantial liposome deformability. The different activity of these edge activators might be explained in part by the chemical structural differences between polyoxyethylene esters and ethers. Our observation seemed to indicate that Brij might be a better edge activator than Myrj, but that Tween 20 was better than both.

Currently, the mechanism of how Tween 20 might have interacted with PC in the lipid membrane layer remains to be elucidated. The possibility exists that Tween 20 might have interacted with PC with strong affinity but in reversible mode. The strong affinity between Tween 20 and PC might have allowed the fast reconstruction of liposome spheres after extrusion. The reversible binding mode might have provided the deformability upon physical stress. The use of the fluorescence resonance energy transfer technique may be useful to test the actual interaction between Tween 20 and PC in the future.

We tested the permeation of encapsulated retinol in human skin and human keratinocyte layers, the latter of which has been used as an epidermis model in studies assessing the skin irritation properties of cosmetics (Faller et al 2002). Previously, rat keratinocyte culture model was shown to be useful in screening transepidermal drug permeability together with possessing potential for research on dermal formulations, irritation and toxicity (Marjukka et al 2003). It was unlikely that the keratinocyte culture contained substantial intercellular lipid layers. However, given the three dimensional construction characteristics of keratinocyte culture, it was possible that the liposomes might have traversed via intercellular spaces among the cells in the culture layers.

In the skin permeation plot, we observed a step-like profile at some time points. Currently, the reasons for this profile remain unclear. It might have been due to experimental errors. Alternatively, the profile might have been related to our dosing pattern. In the cases of the keratinocyte culture experiment, the finite dose study was actually difficult to establish. Accordingly, we chose the infinite dose model rather than finite dose, which might be partially associated with the step-like profile of the permeation plot.

We found that Tween 20-based liposomes conferred significantly higher skin permeation of retinol. This might have been at least partially due to the increased deformability of these liposomes. In this study, we tested the deformability and the skin permeation of retinol encapsulated in Tween 20-based lipid vesicles or in neutral and negatively-charged liposomes. Neutral liposomes, which are widely used for the formulation of cosmeceuticals, did not show any deformability, nor did negatively-charged liposomes. In contrast, retinol-containing deformable nanoparticles showed significant passed volume ratios. Thus, our results indicated that the highly deformable Tween 20-based liposomes conferred higher skin permeation of retinol. Regarding the mechanisms by which deformable liposomes enhanced the delivery of retinol, Cevc (1996) suggested that deformable liposomes might be driven across the skin by the naturally occurring, concentration-insensitive, and probably hydration based, transepidermal gradient. Our observation of retinol-loaded deformable liposomes agreed with that of Cevc (1996) that deformable liposomes might be efficient for delivering various lipophilic or hydrophilic agents into the body. Moreover, El Maghraby et al (1999) studied possible mechanisms of deformable liposome-mediated drug delivery through human skin. Although they found a possible penetration-enhancing effect for pure PC vesicles, they reported that there might be several operating mechanisms.

Lastly, we observed that retinol-containing deformable liposomes were physically stable for at least three months. Kristl et al (2003) suggested that the location of the sensitive group of the drug-molecule in a colloidal carrier system such as liposomes could be crucial for its stability. As previous studies have shown that retinol is prone to chemical oxidation (Byrn et al 2001), it might be desirable to co-entrap an antioxidizing agent such as ascorbic acid in the aqueous phase of the lipid nanoparticles in future studies.

Conclusions

Our results collectively indicated that Tween 20 might be a highly effective edge activator for use in deformable liposomes. Moreover, the substantial human skin permeation of retinol following topical delivery with Tween 20-based deformable liposomes indicated that deformable liposomes could be of potential use for delivery of lipophilic functional cosmetics and other bioactive substances.

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