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Recombinant Human Epidermal Growth Factor (rhEGF)-loaded Solid Lipid Nanoparticles: Fabrication and Their Skin Accumulation Properties for Topical rhEGF Delivery

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For the present study, rhEGF was encapsulated into solid lipid nanoparticles (SLNs). The SLNs were prepared by the $W_1/O/W_2$ double emulsification method combined with the high pressure homogenization method and the physical properties such as particle size, zeta-potential and encapsulation efficiency were measured. The overall particle morphology of SLNs was investigated using a transmission electron microscopy (TEM). The percutaneous skin permeation and accumulation property of rhEGF was evaluated using Franz diffusion cell system along with confocal laser scanning microscopy (CLSM). The mean particle size of rhEGF-loaded SLNs was 104.00 ± 3.99 nm and the zeta-potential value was in the range of −36.99 ± 0.54 mV, providing a good colloidal stability. The TEM image revealed a spherical shape of SLNs about 100 nm and the encapsulation efficiency was 18.47 ± 0.22%. The skin accumulation of rhEGF was enhanced by SLNs. CLSM image analysis provided that the rhEGF rat skin accumulation is facilitated by an entry of SLNs through the pores of skin.

Key Words : Solid lipid nanoparticle (SLN), Recombinant human epidermal growth factor (rhEGF), $W_1/O/W_2$ double emulsification, Skin accumulation, Topical drug delivery

Introduction

Epidermal growth factor (EGF) is a mitogenic polypeptide composed of 53 amino acids containing 3 intramolecular disulphide bonds (Mw: *ca.* 6,045).[1] EGF was for the first time discovered from the submaxillary glands of a mouse by Stanley Cohen in 1960.[2-5] It is a strong mitogen and is found in diverse body fluids such as tears, blood plasma, salivary, urine, glands, semen and so on. It stimulates the proliferation of skin epidermis, promotes blood vessels formation and enhances wound healing by stimulating the regeneration of epithelial tissues *e.g.* intestinal mucosa, cornea epithelial tissues and liver.[6] In addition EGF stimulates synthesis of extracellular molecules by binding to an EGF receptor known as tyrosine kinase.[7]

With the advances of biotechnology, the mass production of recombinant human epidermal growth factor (rhEGF) was possible and many formulations have been developed for chronic wounds, burns and diabetic foot ulcers healing.[7-9] Recently, rhEGF has been used even for cosmetic purposes also.[10-12]

Among all delivery routes, skin has been considered as an attractive route for delivering therapeutic agents for by-passing of first-pass liver metabolism and biological activity loss by exposure to gastrointestinal enzymes along with an enhancement of patient compliance.[13,14] Although rhEGF is beneficial for aesthetic purposes, a topical application of rhEGF is limited because of its large molecular size, hydrophilicity and the barrier effect of the stratum corneum.[15,16] Many strategies have been proposed to improve the skin permeability of drugs. In general, chemical enhancers[17-19] are common strategies for skin permeation but physical methods such as iontophoresis, electroporation and sonophoresis and the combination of both methods are advantageous for the drug delivery through skin.[20-22] In addition, the delivery systems like emulsions, liposomes, microemulsions and solid lipid nanoparticles (SLNs) have been studied for delivery of macromolecules through skin.[23,24] Among them, SLNs have been studied as a representative topical drug delivery system due to their biodegradability, non-toxic property, and biocompatibility with human skin.[25,26] The main reason for a topical application of SLNs is their ability to facilitate the skin accumulation of various drugs within viable skin layers and their high skin occlusive effect.[27]

In the present study, we have fabricated rhEGF-loaded SLNs systems using the $W_1/O/W_2$ double emulsion method. This is the first report regarding the topical rhEGF delivery using SLNs. We investigated the rhEGF-loaded SLNs using transmission electron microscopy (TEM) and measured their physicochemical properties, including particle size, polydispersity index (PI), encapsulation efficiency (EE, %) and zeta (ζ) potential. Effects of the SLNs on topical delivery of rhEGF were evaluated using Franz diffusion cells and visually investigated by confocal laser scanning microscopy

(CLSM).

Experimental

Materials. Recombinant human epidermal growth factor (rhEGF) was kindly donated by Daewoong Pharmaceuticals Co, Ltd. (Seoul, Korea). Glyceryl monostearate was purchased from Azing (Korea), Poloxamer® 188 was purchased from BASF (Ludwigshafen, Germany) and ELISA kit (DuoSet ELISA Development System, Cat. No. Dy236) and TMB microwell peroxidase substrate (KPL, Cat. No. 52-00-00) for ELISA analysis were purchased from R&D system (Seoul, Korea). The wash buffer was made with phosphate buffer solution (pH 7.4) containing 0.05% Tween 20®. Other chemicals and solvents were of reagent grade and used as received.

Preparation of SLNs using $W_1/O/W_2$ Method. The SLNs was prepared by $W_1/O/W_2$ double emulsification method combined with high pressure homogenizer. Briefly, the lipid phase of melted glyceryl monostearate was pre-heated to 70 °C and 40 °C of prepared rhEGF water phase was added to glyceryl monostearate oil phase, which was mixed and homogenized by homo-mixer with 8,000 rpm for 1 minute at 70 °C. This hot primary emulsion (W_1/O) was added into hot Poloxamer® 188 solution and passed through high pressure homogenizer for three cycles at 500 bar. The obtained SLNs solution was cooled down to room temperature. All processes were promptly performed to prevent a degradation of rhEGF.

Measurement of Particle Size and Zeta-Potential. The mean particle size and PI value of the SLNs were determined using dynamic light scattering (BI-90 plus, Brookhaven Instruments Corp., Holtsville, NY, USA). The SLNs formulation was diluted to 1:1,000 with distilled water and its light scattering was measured in triplicate. The zeta-potential values of the formulation were determined by dynamic light scattering (OTSUKA Electronics, ELS-8000, Japan). The SLNs formulation was 1,000-fold diluted with distilled water to measure the zeta-potential. The experiment was performed in triplicate and averaged data were presented.

Observation of Transmission Electron Microscopy (TEM). The morphological characteristics of rhEGF-loaded SLNs were investigated by TEM. Aliquots of SLNs dispersion diluted as 1:10 with distilled water were placed on a carbon-coated copper grid for the observation of TEM image and negatively stained with uranyl acetate before the imaging analysis. Then the excessive uranyl acetate was wiped out by filter paper. The TEM image was obtained using JEM 1200 EX II (JEOL, Tokyo, Japan).

Measurement of Encapsulation Efficiency. For the measurement of encapsulated rhEGF in SLNs, rhEGF-loaded SLNs were dissolved with 6 mL of warm methanol and cooled to room temperature. The dissolved solution was centrifuged (Optima L-100XP, Beckman, USA) with 130,000 × g at 4 °C for 15 minutes and the supernatant was collected. rhEGF were analyzed by enzyme-linked immunosorbent assay (ELISA) kit to determine the encapsulation efficiency. The encapsulation efficiency of rhEGF-loaded SLNs was calculated (%) with following equation:

$$EE(\%) = \frac{Q_t - Q_s}{Q_t} \times 100$$

where Q_t is the total amount of rhEGF in SLNs dispersion and Q_s is the amount of untrapped rhEGF.

Preparation of Rat Skin and *in vitro* Skin Permeation Study. Male Sprague-Dawley rats (7–8 weeks old, 220–240 g) were anesthetized with diethyl ether by inhalation, the hair was removed from their dorsums with electric clippers and the dorsal skin was excised using surgical scissors. Adipose tissues were carefully removed with a surgical scalpel from the skin and the skin was immediately used thereafter. The *in vitro* skin permeation properties of rhEGF-loaded SLNs were evaluated using vertical type Franz diffusion cells. The dorsal rat skin was mounted onto the diffusion chamber with the stratum corneum (SC) side facing the donor compartment. The effective diffusion area was 2.0 cm^2, each receptor compartment was filled with 12.5 mL of phosphate buffer solution (PBS, pH 7.4) and the receptor phase was stirred with a magnetic bar at 600 rpm. After equilibration of the rat skin with PBS (pH 7.4) at 37 °C; 1 mL of i) rhEGF solution; ii) mixture of rhEGF and SLNs vehicles; and iii) rhEGF-loaded SLNs were mounted onto each donor compartment. The rhEGF concentration for all three samples was 25 ug/mL. Aliquots (400 μL) of samples were withdrawn from the receptor compartment at predetermined time intervals of 2, 4, 8 and 12 h and replaced with the same volume of fresh buffer. Experiments were performed in triplicate and the permeated amount of rhEGF was analyzed with a human EGF ELISA kit. All protocols for these experiments were approved by the ethical committee for animal experimentation of the university.

Measurement of Accumulated Amounts of rhEGF within Skin Layer. At the end of the permeation study, the rat skins were separated from Franz diffusion cells and were clearly washed three times with PBS (pH 7.4). The 2 cm^2 of effective diffusion area was taken out from the skin samples. The tissues were weighed and kept under liquid nitrogen before homogenization of skin samples. Frozen skin tissues were mashed with hammer and homogenized in PBS (pH 7.4). The homogenate was centrifuged with 14,000 rpm at 4 °C for 30 minutes (Centrifuge 5417R, Eppendorf, Germany). The distributed amount of rhEGF within rat skin tissue was analyzed with human EGF ELISA kit and the potentials of interference effects were proved prior to the analysis.

Immunofluorescence labelling and CLSM study CLSM studies were performed using immunofluorescence (IF) technique to visualize the permeation profile of rhEGF-loaded SLNs. After the permeation study, each dorsal rat skin was separated from the diffusion cells and rinsed with PBS (pH 7.4) five times. They were dried with a paper towel and fixed within 4% paraformaldehyde in PBS (pH 7.4) for 4 h. After rinsing with phosphate buffer, skin samples were step by step dehydrated with 12%, 16% and 20% of sucrose solutions. Then, the samples were embedded into optimal

cutting temperature (OCT) compound in aluminum molds and were rapidly frozen by liquid nitrogen. The frozen blocks containing skin samples were perpendicularly sectioned with a cryostat microtome (Microtome CryoStat HM 505E, Microm International GmbH, Walldorf, Germany) under −20 °C condition. The slices of skin tissues on the slide glasses were labeled with fluorescein isothiocyanate (FITC) conjugated antibody. Briefly, the sectioned slices were incubated within 4% paraformaldehyde in PBS for 20 minutes at 4 °C and 5% bovine serum albumin (BSA) diluted with PBS for 20 minutes at room temperature. After rinsing three times with phosphate buffer for 5 minutes, the sectioned slices were incubated with goat polyclonal IgG (sc-1341), a primary antibody, diluted in the range of 1:50 and 1:500 for 90 minutes at room temperature. After complete washing with PBS three times for 10 minutes, sectioned skin slices were incubated with donkey anti-goat conjugate FITC (sc-2024), a secondary antibody, at room temperature for 45 minutes using dark conditions. After washing three times with PBS for 5 minutes, all samples were mounted onto mounting medium (Vectashield®). The specimens images were observed using a CLSM (LSM 510META, Carl Zeiss, Germany).[28]

Evaluation of Occlusive Effect of SLNs. An *in vitro* occlusion test was performed in order to evaluate the occlusive properties of SLNs. Briefly, 50 mL beakers with a diameter of 45 mm were filled with 30 g water and sealed with cellulose filter paper (Whatman number 6, cutoff size 3 mm, USA). The SLNs dispersion was homogeneously spread with a spatula on the filter surface (300 μL/15.90 cm^2) and stored for 48 h at 32 °C to mimic the temperature of skin surface. The weight of water remaining in the beaker was measured after 8, 12 and 24 h. The beaker covered with the filter paper where water was spread was used as a reference. All experiments were performed in triplicate. The occlusion factor (F) was obtained from following equation:

$$F(\%) = \frac{A-B}{A} \times 100$$

where A is the water loss without sample (reference) and B is the water loss with sample. F(%) of 100 implies maximum occlusive effect, while F(%) of 0 implies no occlusive effect.

Data Analysis. All results are indicated as mean ± standard deviation. Statistically significant differences were determined using the Student's t-test with $P < 0.05$ as a minimal level of significance.

Results

Physicochemical Properties of the SLNs Formulation. In this study, rhEGF-loaded SLNs were successfully prepared by the $W_1/O/W_2$ double emulsification method combined with the high pressure homogenization. Samples of this formulation were diluted with distilled water and measurements were made of particle size, PI and ζ potential. As shown in Table 1, the mean particle size was 104.00 ± 3.99 nm, the PI was 0.20 ± 0.01 and the zeta-potential of rhEGF-loaded SLNs was −36.99 ± 0.54 mV. The mean size and zeta-potential value of rhEGF-loaded SLNs were similar with SLNs vehicle and the SLNs seemed to show a good colloidal stability. The calculated encapsulation efficiency was 18.47 ± 0.22%. Generally the entrapment efficiency of peptide drug in SLNs is very low because of the hydrophilic property of peptides. However, the $W_1/O/W_2$ double emulsification method enabled a relatively high rhEGF entrapment efficiency.

Table 1. Physicochemical characteristics of rhEGF-loaded SLNs and rhEGF-free SLNs vehicles

Parameters	SLNs vehicles	rhEGF-loaded SLNs
Particle size (nm)	107.03 ± 7.93	104.00 ± 3.99
Polydispersity index	0.21 ± 0.06	0.20 ± 0.01
Zeta-potential (mV)	−30.29 ± 0.47	−36.99 ± 0.54

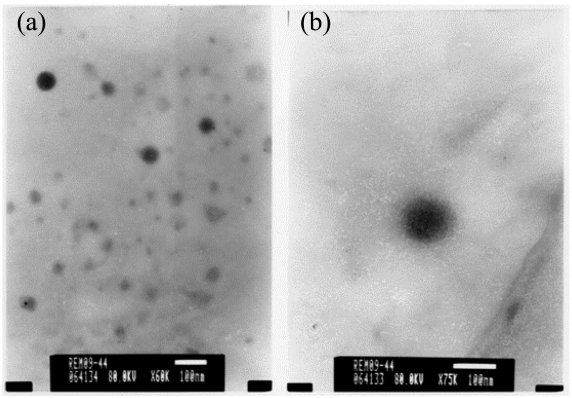

Figure 1. TEM images of the rhEGF-loaded SLNs. The resolution is 60,000 × (a) and 75,000 × (b).

TEM (Transmission Electron Microscopy) Imaging. In order to investigate the morphology of rhEGF-loaded SLNs, the SLNs formulations were observed with TEM. Figure 1 shows the TEM images of rhEGF-loaded SLNs. The SLNs were spherical and the mean particle size of rhEGF-loaded SLNs from TEM images was about 100 nm. The particle size observed by TEM correlated well with the result of the particle size analysis. Also, rhEGF-loaded SLNs showed narrow size distribution through the TEM image.

***In vitro* Skin Permeation and Accumulation of rhEGF.** In order to evaluate the permeation profile of rhEGF, we performed a skin permeation study using Franz diffusion cells. The permeated and accumulated amounts of rhEGF were evaluated with a human EGF ELISA kit. First we investigated the possibility of interference effects induced by rat skin. We added 100 pg/mL of rhEGF solution to rat skin homogenates and identified the matrix effect of skin tissues. The recovery (%) of rhEGF from diluted skin homogenates was 99–115.27% and the accuracy was *ca.* 80–120%. Also the calibration curve showed good linearity ($y = 0.647x - 1.396$, $R^2 = 0.995$, data not shown). rhEGF permeated through the rat skin tissue was not detected. The accumulated rhEGF within rat skin is presented in Figure 2. The accumulated amount of rhEGF in rat skin was increased in order of the

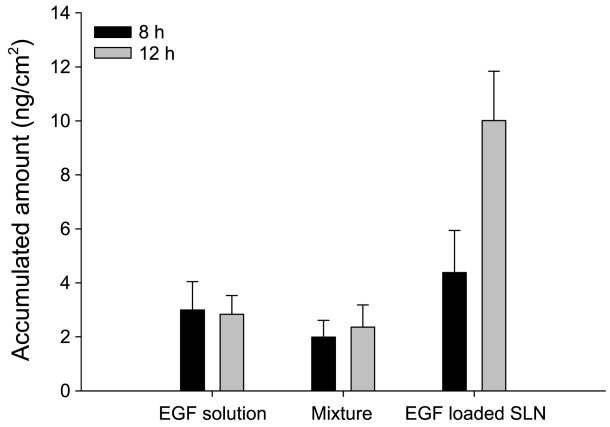

Figure 2. Skin accumulation profile of rhEGF solution, mixture of rhEGF and SLNs vehicles, rhEGF-loaded SLNs in terms of total accumulated amount of rhEGF (ng/cm^2) within skin layer after 8 and 12 h.

rhEGF mixtures with SLNs vehicle ≈ free rhEGF solution < rhEGF-loaded SLNs. With such result we can confirm that the topically applied SLNs can improve the skin accumulation of rhEGF in skin layer.

Confocal Laser Scanning Microscopy (CLSM) Imaging Analysis. The permeation of rhEGF-loaded SLNs was visualized using CLSM. We performed the observation of the auto-fluorescence of rat dorsal skin tissue prior to the observation of samples and confirmed that the auto-fluorescence of control rat skin tissue did not disturb the CLSM result (data now shown). The CLSM images were obtained after 12 h of *in vitro* skin permeation test. The fluorescence intensity of rhEGF-loaded SLNs was higher than that of the free rhEGF solution (Figure 3). Also, we observed that most of the present rhEGF was permeated through the pores of skin. The high fluorescence intensity associated with pores of skin suggests that the enhanced delivery of rhEGF by the SLNs occurred *via* pilosebaceous units like hair follicles, hair shafts and sebaceous glands.

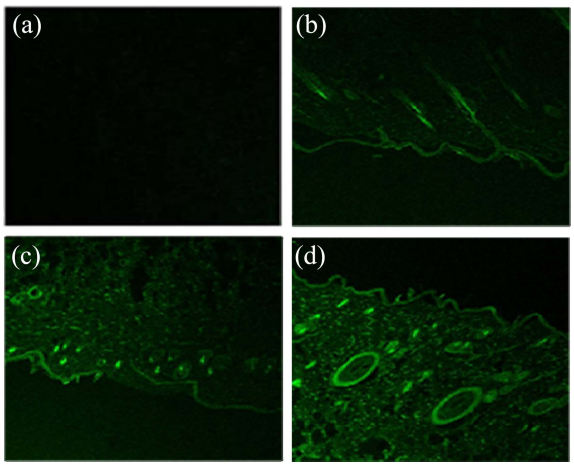

Figure 3. CLSM images of the rat skin treated with rhEGF formulations for 12 h. (a) Control PBS, (b) rhEGF solution, (c) Mixture of rhEGF and SLNs, (d) rhEGF-loaded SLNs.

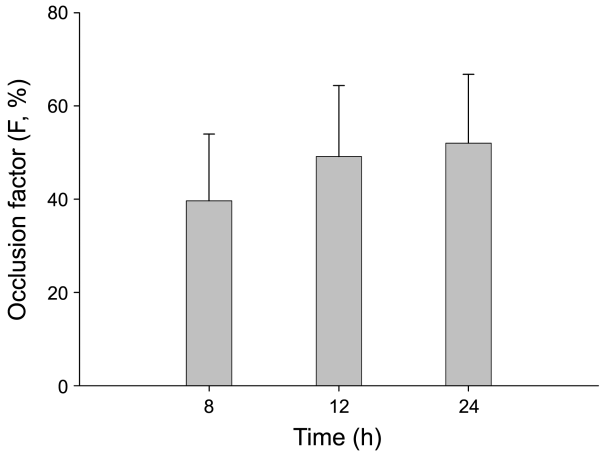

Figure 4. Occlusion factor of rhEGF loaded SLNs.

Occlusion Factor (F). Figure 4 shows the calculated occlusion factor (F) of rhEGF-loaded SLNs. The occlusion factor (F) of rhEGF-loaded SLNs after 8, 12 and 24 h were 39.64 ± 14.31%, 49.14 ± 15.25% and 52.02 ± 14.77%, respectively. With this result, we could conclude that the SLNs formulation was able to prevent water evaporation from the filter paper and rhEGF-loaded SLNs may induce skin hydration and helpful for rhEGF skin accumulation.

Discussion

In this study we developed a novel solid lipid nanoparticle system of rhEGF for topical application. Based on previous studies,[29,30] it is possible to consider the size of present rhEGF loaded-SLNs formulation is suitable to deliver rhEGF into rat skin layer. In particular, the system showed uniform particle size distribution and good physical colloidal stability as nanoparticle.

In the skin permeation study, rhEGF was not detected in the receptor solution. Although rhEGF-loaded SLNs did not penetrate skin, rhEGF was detected in the homogenized skin samples with the accumulated rhEGF as the greatest of all for the rhEGF-loaded SLNs formulations. This result could also be supported by a CLSM image analysis (Figure 3). The rhEGF-loaded SLNs formulation resulted in an accumulated amount of rhEGF which was 0.83-fold higher than that obtained with the free rhEGF solution and 3.53-fold higher than that obtained with the rhEGF-SLNs vehicle mixture.

We observed meager fluorescence in skin samples treated with a free rhEGF solution, though the intensity was weak. This suggests that free rhEGF molecules slightly permeated into the viable epidermal layers of skin. Considering the pores of skin as main delivery route (Figure 3), the rhEGF and SLNs seemed to be delivered *via* hair follicles and they were retained within rat skin. Hair follicles are known to be an effective topical drug delivery route for hydrophilic drug and particles, despite the surface area of skin occupied by hair follicles is only about 0.1% of the total skin surface area.[31,32] Recently, follicular penetration of nano- and microparticle has been investigated and showed hair follicles as a

primary penetration route for the particle-based deliver system containing macromolecules, chemical drug, vaccine.[33-35] The penetration depth depends on the size of the particles and the result isn't very similar because of difference in lipid type, combination of lipid, preparation methods.

SLNs have been developed as alternative drug delivery systems for emulsion, liposome and polymeric nanoparticles. SLNs have advantages of stability and controlled release of active ingredient and no toxicity.[36] Besides the above mentioned advantages, the skin retain property and skin hydration characteristics are other main reasons that SLNs have been used in topical drug delivery. SLNs have a skin occlusive effect and they can be used in order to increase the water content of the skin.[27] High occlusion factors over 50 can be obtained when the SLNs were applied for 12 h. Referring to the report of Wissing *et al*., the collusive effect is related with the particle size of SLNs and they reported high occlusion factors of 50–60 can be obtained with a particle size below 400 nm. Additionally they obtained as solid results for SLNs-containing cream that it was significantly more efficient in skin hydration.[37,38] With the results of occlusive test we could conclude that the present SLNs meet the size for a good occlusive value and this will be effective for skin hydration in *in vivo* studies.

Conclusion

In this study, we fabricated rhEGF-loaded SLNs by $W_1/O/W_2$ double emulsion method. We confirmed a good colloid system of the prepared SLNs and with circa 18% high encapsulation efficiency. The skin accumulation of rhEGF was enhanced by the use of SLNs, the results were greater than those for the rhEGF solution as well as for the mixture of rhEGF and SLNs vehicle. These results were supported by CLSM image analysis and which shown that rhEGF-loaded SLNs were penetrated through hair follicle. In conclusion, the results of the present study suggested rhEGF-loaded SLNs as good candidates for the topical delivery of rhEGF.

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