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Articles

Skin permeation of 5-aminolevulinic acid encapsulated in liposomes

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I. Introduction

5-Aminolevulinic acid (ALA) is the first compound of the biosynthetic pathway of heme. It has been used in photodynamic therapy for skin diseases such as cutaneous carcinoma and intractable acne [1–4]. Recently, some cosmetics including ALA have been launched to the market.

Skin has an outermost thin layer, stratum corneum, and underlying viable epidermis and dermis. Since the stratum corneum is highly hydrophobic, dry, and a percutaneous barrier, hydrophilic compounds are inferior to hydrophobic compounds in permeating through the stratum corneum [5]. When ALA is topically applied to skin, it is hard for ALA to permeate through the stratum corneum, because ALA is hydrophilic. Thus, ALA skin permeation needs to be improved.

Liposomes are defined as a structure composed of lipid bilayer membranes with an inner core of water phase. They can incorporate water-insoluble compounds in the lipid bilayers and water-soluble compounds in the inner core [6]. Liposomes are well known as a drug carrier to enhance skin permeation [7]. In the present study, we prepared ALA-loaded liposomes and determined *in vitro* ALA permeation through Yucatan micropig (YMP) full-thickness skin using a Franz-type diffusion cell, demonstrating their ability to enhance ALA skin permeation.

II. Experimental

1. Materials

L-α-dipalmitoylphosphatidylcholine (DPPC, 99.7%), cholesterol (99%), and 5-aminolevulinic acid hydrochloride (ALA, 98%) were purchased from NOF. Co., SIGMA, and Cosmo Bio Co., Ltd., respectively. They were used without further purification. Yucatan micropig (YMP) full-thickness skin from CHARLES RIVER LABORATORIES JAPAN, INC. was used for *in vitro* skin permeation measurements. Distilled water was used for all the measurements.

2. Preparation of ALA-loaded liposomes

ALA-loaded liposomes were prepared using Bangham's method [8]. A 1.83 x 10^{-2} g of DPPC and 3.8 x 10^{-3} g of cholesterol were dissolved in 5 mL of chloroform in a test tube. The solvent was

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evaporated under N_2 gas, and a thin lipid film was formed on the wall of the test tube. To remove the residual solvent completely, the test tube was allowed to stand in a desiccator under reduced pressure. After a 5 mL of 0.28 M aqueous ALA solution was added into the test tube, the test tube was kept for 5 min at 60 °C, then agitated on a vortex mixer for 5 min to give liposomes encapsulating ALA. The liposome dispersion was extruded through Millipore filters (Whatman Co.) having 100 nm pore diameter with an extruder (Lipex Biomembranes Inc.) to give homogeneously sized liposomes. Finally, the liposome dispersion was dialyzed against 0.9 wt% aqueous sodium chloride solution for a few hours, with the solution changed every hour. After the dialysis, the concentration of ALA in the aqueous sodium chloride solution was measured to confirm no presence of free ALA in the solution.

3. Measurement of ALA concentration

The concentration of ALA was measured by the fluorometric method reported by Okayama [9]. A 50 μL of ALA solution was treated with a mixture of 3.5 mL of acethyl acetone, ethanol, and water (15:10:75 in volume ratio) containing 4 g of sodium chloride per liter, and then with 450 μL of 85 ml/L aqueous formaldehyde solution. The treated solution was heated in boiling water for 30 min and was cooled under iced water for 5 min. These reactions eventually gave us the fluorescent ALA derivative, 2-methyldeneamin-3,5-diacetyl-4,6-dimethylphenylpropionic acid. The fluorescence intensity of the ALA derivative was measured with a fluorescence spectrophotometer (FP-6500, JASCO Co.). The excitation and emission wavelengths were 363 and 465 nm, respectively.

4. Trapping efficiency of liposomes for ALA

After the ALA-loaded liposome dispersion was dialyzed, the liposome was broken by addition of ethanol (the volume ratio between the liposome dispersion and ethanol was 1:4), and the concentration of ALA encapsulated in the liposome was detected. The trapping efficiency of the liposome for ALA was estimated by the *Eq*. (1). Here, the concentration of ALA in the solution used for preparing the liposome dispersion was 0.28 M, as mentioned above.

5. Measurement of *in Vitro* skin permeation of ALA

In vitro skin permeation of ALA in the liposome through YMP full-thickness skin was determined with a Franz-type diffusion cell having donor and receptor compartments. The available diffusion area of the skin was 1.77 cm^2, and the volume of the receptor compartment was 12 mL. The skin was mounted in the Franz-type diffusion cell. The ALA-loaded liposome dispersion prepared was added in the donor compartment, and phosphate buffered saline (pH: 7.4) was poured into the receptor compartment. After the cell stood for a certain time at 37 °C with stirring of the buffered saline with a magnetic stirrer, 200 μL of buffered saline in the receptor compartment was taken out. The concentration of ALA permeated was then measured, and the cumulative amount of permeated ALA per unit skin area was calculated.

III. Results and discussion

It is well known that Bangam's method produces multilamellar vesicle types of liposomes and that the polarization microscopic observation of the liposome provides us a cross Nicol texture

[*Equation (1)*]

$$\text{Trapping efficiency} = \frac{\text{Concentration of ALA encapsulated in liposome}}{\text{Concentration of ALA in the solution for preparing liposome dispersion}} \times 100\ (\%) \qquad (1)$$

coming from the multilamellar structure of the lipid membrane [10]. When observing the prepared liposome dispersion with a polarization microscopy, we could recognize a cross Nicol texture, as expected. Additionally, the concentration of ALA encapsulated in the liposome was 2.0 mM; the trapping efficiency of the liposome for ALA was estimated at 0.71 %. These findings indicate that ALA-loaded liposomes can be constructed obviously by Bangam's method in this study.

Figure 1 represents the change in cumulative amount of permeated ALA per unit skin area with the permeation time. Here, we employed three types of ALA systems for the measurement: ALA-loaded liposome dispersion, aqueous ALA solution including empty liposomes, and aqueous ALA solution (ALA alone). The concentration of ALA was 2 mM in all of the systems. The aqueous ALA solution including empty liposomes was prepared by the same method as the ALA-loaded liposome dispersion using 2 mM ALA without the dialysis. In consideration of the fact that the trapping efficiency of ALA in the ALA-loaded liposome was 0.71 %, as mentioned above, we guess that a large number of ALA are in the outer water phase, but not in the inner water phase.

As can be seen from ***Fig. 1***, the cumulative amount of permeated ALA increases in increasing permeation time. In comparison at the same permeation time, the cumulative amount of permeated ALA in the ALA-loaded liposome dispersion is the largest among the systems; the cumulative amount of ALA in the aqueous ALA solution including empty liposomes is almost equal to that in the aqueous ALA solution.

We evaluated permeation parameters estimated from the permeation profile shown in ***Fig. 1*** [10]. The flux, J, of ALA permeated through YMP skin was calculated from the slope of the steady-state portion of the permeation profile between the cumulative amount of permeated ALA per unit skin area and the permeation time. Besides, the

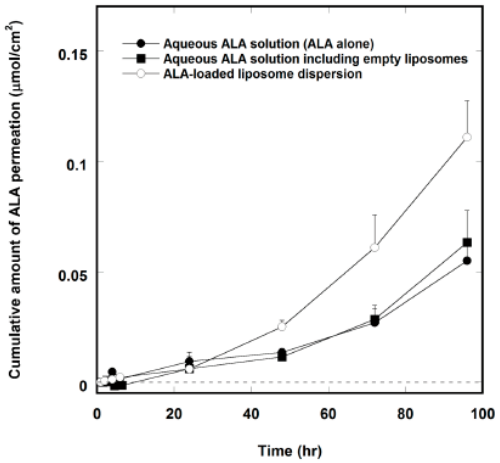

Fig. 1 Change in cumulative amount of permeated ALA per unit skin area with permeation time. Data show mean±SD (n=3).

lag time, T_{lag} was estimated from the linear extrapolation of the steady-state portion to the X-axis of the permeation profile. We then estimated the J and T_{lag} from the linear relationship using three plots at the permeation time more than 48 hr in ***Fig. 1***. Furthermore, we calculated the permeability coefficient of ALA in the stratum corneum, K_p, diffusion coefficient of ALA in the stratum corneum, D, and partition coefficient of ALA between the stratum corneum and the solution in the donor compartment, K, using *Eqs*. (2) to (4) below:

$$K_p = \frac{J}{C_D} \qquad (2)$$

$$K_p = \frac{DK}{L} \qquad (3)$$

$$T_{lag} = \frac{L^2}{6D} \qquad (4)$$

where C_D is the initial concentration of ALA in the donor compartment, L the diffusion path length, that is, the thickness of YMP skin. According to the previous paper, we assigned 0.2 cm to L in this study [11].

The permeation parameters calculated are summarized in ***Table 1***. As a result, the K_p of ALA in the ALA-loaded liposome dispersion is about 2 times larger than those in any other system ($p<0.05$ by Welch's t-Test vs. aqueous ALA

Table 1 Skin permeation parameters estimated from permeation profile.

	K_p (x 10^{-4} cm/hr)	T_{lag} (hr)	D (x 10^{-4} cm^2/hr)	K (x 10^{-1})
ALA-loaded liposome dispersion	8.95±1.57*	35.2±3.50	1.91±0.19	9.50±2.32
Aqueous ALA solution including empty liposomes	5.40±1.18	40.3±1.56	1.66±0.07	6.51±1.39
Aqueous ALA solution (ALA alone)	4.33±1.09	34.8±3.51	1.93±0.19	4.58±1.65

*: $p<0.05$ by Welch's t-Test vs. aqueous ALA solution

solution). Moreover, the K_p in the aqueous ALA solution including empty liposomes is almost the same as that in the aqueous ALA solution. K_p is the most important parameter for skin permeation of drugs. It can evaluate the skin permeation without depending on the initial concentration of the drug in the donor compartment; the larger the K_p of the drug is, the greater its skin permeation is. Therefore, the ALA-loaded liposome dispersion is superior in permeating ALA through the YMP skin.

The T_{lag} is the time at the beginning of the steady-state portion of the permeation profile between the cumulative amount of permeated ALA per unit skin area and the permeation time. ***Table 1*** shows that T_{lag} is almost constant in any system.

Equation (3) indicates that the K_p is closely related to K and D. Since the D in any permeation system is almost constant, shown in ***Table 1***, the change in K_p is attributed to K alone. ***Table 1*** depicts that the K of the ALA-loaded liposome dispersion is the largest among the systems. Therefore, the enhancement in skin permeation of ALA by the ALA-loaded liposome dispersion is caused by the enlargement of partition of ALA into the stratum corneum.

It is well known that the structures of stratum corneum are changed by phospholipids, leading to enhancement of drug penetration [12]. But there is little difference in K_p of the aqueous ALA solution with and without empty liposomes in this study. We consider that the enhancement of ALA permeation by the ALA-loaded liposome is independent of the permeation enhancement effect of phospholipids.

Intact skin penetration of liposomes carrying drugs was suggested in the first report on liposomes as skin drug delivery systems [7]. Assuming that the ALA-loaded liposome penetrates and passes through the skin, we expect that the D of ALA is different from that in the aqueous ALA solution. As can be seen from ***Table 1***, however, the D of ALA in the ALA-loaded liposome dispersion is almost equal to that in the aqueous ALA solution. It is then unlikely that the skin permeation of ALA-loaded liposomes enlarges the partition of ALA into the stratum corneum and promotes skin permeation of ALA.

It was reported that liposomes adhere onto the skin surface with fusing and/or mixing with the lipid matrix of stratum corneum, releasing drugs into stratum corneum [13]. This mechanism could reasonably explain the results in this study. That is, the release of ALA from liposomes on the skin surface elevates the concentration of ALA around the skin and promotes the partition of ALA into the stratum corneum, resulting in the acceleration of ALA penetration through the stratum corneum. Moreover, since ALA independently permeates through the skin in this way, we suggest that the D of ALA in the ALA-loaded liposome dispersion corresponds to that in the aqueous ALA solution.

In consequence, the ALA-loaded liposome dispersion promotes the partition of ALA into the skin and is effective to enhance the skin permeation of ALA. The interest in mechanism of drug permeation with liposomes warrants further investigation.

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