Liposomes Encapsulating *Aloe vera* Leaf Gel Extract Significantly Enhance Proliferation and Collagen Synthesis in Human Skin Cell Lines

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Abstract: *Aloe vera* leaf gel extract (AGE) are widely used as cosmetic and pharmaceutical ingredients because of its versatile skin care properties. In order to enhance the bioavailability of AGE, liposomes encapsulating AGE were prepared and examined for their interfacial and biochemical properties. The liposomes prepared from a soybean lecithin (SLP-WHITE, 1.0 wt%) by the Bangham method gave relatively a good trapping efficiency up to the AGE concentration of 0.5 wt%. The stable liposomes were then prepared from 1.0 wt% of SLP-WHITE and different concentrations of AGE by the mechanochemical method using a homogenizer and microfluidizer. The liposomes obtained from 0.25 wt% of AGE were confirmed to be small unilamellar vesicles with a diameter of less than 200 nm, and remained well dispersed for at least two weeks. The obtained liposomes encapsulating AGE were further examined for the effects on proliferation and type I collagen synthesis in normal human neonatal skin fibroblasts, NB1RGb cells. Liposomal AGE clearly showed higher proliferation rate than that of AGE alone. In addition, compared to the control, liposomal AGE significantly increased the collagen synthesis by 23%, while AGE alone showed a small effect. Liposomal AGE was also assayed for the effect on proliferation in normal human epidermal keratinocytes, NHEK(F) cells. Interestingly, liposomal AGE fractions containing 4 and 20 μg/mL of the extract considerably increased the proliferation rate by 77% and 101%, respectively. In contrast, AGE alone fractions containing 4 and 20 μg/mL of the extract increased the rate by 41% and 60%, respectively. Accordingly, the bioavailability and skin care properties of AGE will be significantly enhanced by liposome encapsulation, and the present liposomal AGE should have a great potential as an effective skin care formulation.

Key words: *Aloe vera*, liposome, bioavailability, skin care, fibroblast proliferation, collagen synthesis, keratinocyte proliferation

1 INTRODUCTION

*Aloe barbadensis* M. (*Aloe vera*) is a perennial succulent, which is widely used for different therapeutic purposes¹,². Especially, a clear gel in *Aloe vera* leaf has been used as food supplements, cosmetic and pharmaceutical materials. Several hydrophilic components of the gel are considered to be highly functional and responsible for its versatile skin care properties³,⁴.

For example, glycoproteins in the gel are reported to enhance epidermal turnover through kerationcyte proliferation⁵,⁷. Aloeisin⁸ is also useful component, and induces proliferation and type I collagen synthesis in human fibroblasts⁹. Type I collagen is a major structural protein and is found in large quantities in various parts of the body, especially in the skin. A decrease of type I collagen induces wrinkle formation, which is a common phenomenon of aged skin¹⁰. Therefore, *Aloe vera* gel has a great potential as a highly effective skin care material, and various products using *Aloe vera* leaf gel extract (AGE) have been manufactured and marketed.

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Accepted September 5, 2009 (received for review August 3, 2009)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/

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However, the externally use of the gel often minimize the skin care activities, because the penetration of such hydrophilic components into the skin are highly suppressed by a water impermeable barrier of the stratum corneum, which is composed of protein-rich nonviable cells and intercellular lipid domains. Although the penetration may be improved by the high-dose administration of these components, this would cause skin inflammation.

To solve these problems, various derivatives of the gel components have been synthesized to improve the penetration into the skin. However, the possibility of using these derivatives is far from straightforward due to the high cost and decrease of the skin care properties. Accordingly, it is of great interest to develop new formulations using AGE, which enhance the skin penetration as well as the skin care properties.

We thus focused our attention on the feasible use of liposomes to attain the breakthrough. Liposomes have been receiving increasing attention as a drug carrier, because they are able to retain hydrophilic substances in the inner aqueous phase and hydrophobic substances in the bilayer wall. In addition, they also have proved to be effective vehicles for various drugs to enhance the permeation into the skin.

We have previously reported a new method for the large-scale preparation of liposomes using a homogenizer and microfluidizer. This mechanochemical method allowed us to efficiently prepare liposomes encapsulating functional food materials such as Ukon extract. We thus tried to prepare liposomes containing AGE by the method, and to characterize the skin care properties.

In this study, we prepared liposomes from a soybean lecithin and AGE by the Bangham method, and examined the trapping efficiency. We then prepared stable liposomes by the mechanochemical method, and investigated the particle size and dispersibility. We also evaluated the effects of liposomal AGE on proliferation and collagen synthesis in human skin cell lines. This is the first report on the interfacial and biochemical characterization of liposomes encapsulating Aloe vera leaf gel extract.

2 EXPERIMENTAL

2.1 Materials

A soybean lecithin (SLP-WHITE) was purchased from Tsuji Oil Mill Co., Ltd (Mie, Japan), and used without further purification. The composition of SLP-WHITE was as follows: phosphatidylcholine (20 wt%), phosphatidylethanolamine (26 wt%), phosphatidylserine (16 wt%), phosphatidic acid (16 wt%), others (22 wt%). A hydrophilic fluorescence probe, 3'-Bis[N,N-bis(carboxymethyl)-aminomethyl]fluorescein (calcein) (purity 98.0%) was purchased from DOJINDO Lab (Kumamoto, Japan), and used for evaluating the trapping efficiency of liposomes.

Alpha modified eagle minimum essential medium (α-MEM) and fetal bovine serum (FBS) for fibroblast cultivation were purchased from Invitrogen Co. (Tokyo, Japan). Serum-free keratinocyte growth medium (HuMedia-KG2) and growth additive sets [bovine pituitary extract (BPE), human recombinant epidermal growth factor (hEGF), bovine insulin, hydrocortisone, gentamicin and amphotericin] for keratinocyte cultivation were purchased from Kurabo Co. (Tokyo, Japan).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was purchased from Promega Co. (Tokyo, Japan), and used for cell proliferation assay. L-Ascorbic acid was purchased from Kanto Chemical Co. (Tokyo, Japan). All other chemicals and reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan).

2.2 Preparation of Aloe vera leaf gel extract (AGE)

Aloe vera leaf was kindly supplied by Coralvegetable Co. Ltd. (Okinawa, Japan). The flesh, colorless gel fillet (2.46 kg) was separated from the green rind, then homogenized in a blender at 80°C for 30 min. The gel was frozen at -25°C, and then left at room temperature to thaw completely. The resulting gel was centrifuged at 8,000 rpm for 30 min, and then 2.30 kg of the clear yellow supernatant was obtained. The supernatant was diluted to an appropriate concentration and used as Aloe vera leaf gel extract (AGE) in the following experiments.

2.3 Measurement of the trapping efficiency of liposomes

Liposomes encapsulating AGE were prepared from SLP-WHITE according to the Bangham method as follows. The lecithin was dissolved with chloroform (2 mL) in a test tube. The solvent was then removed under the stream of nitrogen gas, and the residual solvent was further dried overnight under vacuum. The dried lipid films were hydrated with 10 mM Tris-HCl buffer (pH 7.3) containing calcein (0.1 mM) and AGE, and warmed (55-60°C) above its phase transition temperature for 10 min. The test tube was then shaken on a vortex mixer for 5 min. The total lecithin concentration was fixed at 1 wt% in all experiments, while AGE concentration was changed from 0.25 to 1 wt%.

The trapping efficiency for calcein was determined by a fluorescence method with a spectrophotometer (FP-6500, JASCO Co., Japan) using excitation and emission wavelengths at 490 and 520 nm, respectively. The liposomal solution was diluted 50 times by the Tris-HCl buffer, and the total fluorescence intensity was measured (I_{total}). The calcein presented in the outer aqueous phase was then quenched by complexation with Co^{2+} (CoCl_2, 10 mM), and the fluorescence intensity was measured again (I_{Q}). Finally, the liposomes were destroyed by adding Triton X-100, and the fluorescence intensity was measured again.
Characterization of Liposomes Encapsulating Aloe vera Leaf Gel Extract

1. Introduction

In this study, we aimed to characterize liposomes encapsulating Aloe vera gel extract (AGE) and evaluate their efficacy in skin regeneration. Liposomes were prepared using an mechanochemical method as described previously. The trapping efficiency was calculated according to the Equation 1:

\[
\text{Trapping efficiency (\%) = } \frac{(I_{\text{in}} - I_{\text{TX}} \cdot r)}{(I_{\text{final}} - I_{\text{TX}} \cdot r)} \times 100
\]  

where \(r\) is the volume correction factor. The efficiency was expressed in terms of the average of the values measured at least three times independently.

2. Preparation of liposomes by the mechanochemical method using a homogenizer and microfluidizer

Liposomes encapsulating AGE were also prepared by the mechanochemical method as reported previously. Equal volume of the lecithin aqueous solution (500 mL) and AGE (500 mL) were mixed with a homogenizer (TK HOMO MIXER MARK II, Primix Co. Ltd., Japan) at 35°C with 4,000 rpm for 15 min. The obtained suspensions were then processed with a microfluidizer (M110-E/H, Mizuho Industrial Co., Ltd., Japan) under an inlet pressure of 100 MPa at 1 pass for 5 min, unless otherwise indicated.

The total lecithin concentration was fixed at 1 wt% in all experiments, while AGE concentration was set at 0.25 or 0.5 wt%. Unless otherwise indicated, the liposomes were prepared from 1 wt% lecithin and 0.25 wt% AGE were used in the following experiments.

2.5 Measurement of particle size of liposomes

The particle size of liposomes encapsulating AGE was estimated by dynamic light scattering at 24°C using a FPAR-1000 (Otsuka Electronics Co., Ltd., Japan). The light source was a diode-pumped solid state laser with a wavelength of 632 nm as a light source. The scattering angle was 90°. The diffusivity of liposomal suspension (D) was obtained with above measurement, and the average liposome diameter (\(d_{\text{hy}}\)) was calculated according to Equation 2:

\[
d_{\text{hy}} = \frac{kT}{3\pi\eta D}
\]  

where \(k\) is the Boltzman constant, \(T\) is the absolute temperature, \(\pi\) is the circular constant, and \(\eta\) is the viscosity.

2.6 Freeze-fracture electron microscopy (FFEM)

FFEM was used to determine the structure of liposomes encapsulating AGE. The sample was frozen with liquid nitrogen at -189°C. The fracture process was performed with a JFD-9010 (JOEL, Japan) at -130°C, and the fractured surface was then replicated by evaporating platinum at an angle of 60°, followed by carbon at an angle of 90° to strengthen the replica. The replica was placed on a 400 mesh copper grid after being washed with water, methanol, and chloroform. It was then examined and photographed using a JEM-1011 (JOEL, Japan) transmission electron microscope.

2.7 Fibroblast culture

Normal human neonatal skin fibroblasts (NB1RGB cells) were purchased from Riken Cell Bank (Ibaraki, Japan). NB1RGB cells were cultivated in α-MEM supplemented with 10% FBS under a humidified atmosphere at 37°C, 5% CO₂. Then, the cells were assayed for proliferation and type I collagen synthesis, as described below.

2.8 Keratinocyte culture

Normal human epidermal keratinocytes of neonatal foreskin (NHEK(F) cells) were purchased from Kurabo Co. (Osaka, Japan). NHEK(F) cells were cultivated in HuMedia KG2 supplemented with 0.4% BPE, 0.2 ng/mL hEGF, 10 µg/mL bovine insulin, 0.18 µg/mL hydrocortisone, 50 µg/mL gentamicin, and 50 ng/mL amphotericine under a humidified atmosphere at 37°C, 5% CO₂.

2.9 Assay of fibroblast proliferation

Four culture groups were prepared in this study. NB1RGB cells were seeded at a concentration of 1 × 10⁵ cells/dish (60 mm φ × 15 mm high) and cultivated as above. After 24 h, the medium was changed to α-MEM containing 10% FBS supplemented with L-ascorbic acid (4 µg/mL), AGE or liposomal AGE, respectively, and cells were cultivated for another 72 h. In this assay, the final concentration of the gel extract was fixed at 4 µg/mL. The control cells were cultivated in α-MEM containing 10% FBS without the above supplements. After cultivation, cells were harvested by digestion with 0.05% trypsin and 0.53% ethylenediamine tetraacetic acid solution. The viability of harvested cells was over 95% by the trypan blue dye exclusion test. Only the live cell number was measured using a blood corpuscle counting chamber. Data were expressed in terms of cell proliferation rate (%) compared to the control. The rate (%) was calculated according to the Equation 3:

\[
\text{Cell proliferation rate (\%) = } \frac{(C_s)}{(C_c)} \times 100
\]  

where \(C_s\) is the live cell number in the supplemented medium, \(C_c\) is the live cell number in the control medium.

2.10 Assay of type I collagen synthesis

NB1RGB cells of four groups were prepared by the same conditions as above. After cultivation with the supplemented or control medium, the medium was removed, and the cells were scraped without digestion of trypsin. The amount of type I collagen in the scraped cells was then measured by the enzyme-linked immunoassay (ELISA) using a commercially available kit (Human collagen type I ELISA KIT, Cosmo Bio Co., Ltd., Tokyo, Japan) following the manufacturers' instructions. Briefly, the scraped cells were treated with 2N NaOH overnight, and the resulting collagen was then measured using a colorimetric assay.
were incubated with 0.01 wt% of pepsin (5mL) for 18 h at 4 °C. After incubation, the supernatants were collected by centrifugation at 10,000 g for 10 min at room temperature, and mixed with 200 mM Tris-HCl buffer (pH 7.2, 150 mM NaCl) to stop pepsin digestion. The treated supernatants contained atelo-collagen, which were derived from type I collagen of the extracellular matrix. The amount of atelo-collagen was then monitored by microplate reader (Model 450, Bio-Rad, CA, U.S.A). Data were expressed in terms of collagen production rate (%) compared to the control. The production rate (%) was calculated according to the Equation 4:

\[
\text{Collagen production rate} \, (\%) = \left( \frac{A_S}{A_C} \right) \times 100
\]

where \(A_S\) is the amount of atelo-collagen with the supplemented medium, \(A_C\) is the amount of atelo-collagen with the control medium.

2.11 Assay of keratinocyte proliferation
Five culture groups were prepared in this study. NHEK(F) cells were seeded on 24-well microplates (1 × 10⁴ cells/well) and cultivated as above. After 24 h, the medium was changed to HuMedia-KG2 (1 mL) supplemented with AGE or liposomal AGE instead of hEGF and BPE, and the cells were cultivated for another 72 h. In this assay, final concentration of the gel extract was fixed at 4 or 20 µg/mL. The control cells were cultivated in HuMedia-KG2 (1 mL) including no hEGF and BPE.

After cultivation, cell proliferation was determined by MTS assay. 200 µl of MTS solution (1.9 mg/mL) was added to each well, and the cells were incubated at 37°C, 5% CO₂, for 60 min. After incubation, the absorbance at 490 nm of the cells was determined by a microplate reader (Model 450, Bio-Rad, CA, U.S.A).

Data were expressed in terms of cell proliferation rate (%), which was calculated according to Equation 5:

\[
\text{Cell proliferation rate} \, (\%) = \left( \frac{A_{490}^S}{A_{490}^C} \right) \times \left( \frac{A_{490}^C}{A_{490}^{b490}} \right) \times 100
\]

where \(A_{490}^S\) is the absorbance with the supplemented medium, \(A_{490}^C\) is the absorbance with the control medium, and \(A_{490}^{b490}\) is the absorbance of the medium itself (without cells).

2.12 Statistical analysis
All data were expressed as the mean ± S.D. Data were analyzed by one-way ANOVA, and differences among the means of groups were analyzed by the Bonferroni procedure as a post hoc test. Differences were considered significant at \(P < 0.05\).

3 RESULTS AND DISCUSSION
3.1 The trapping efficiency of liposomes encapsulating AGE
The present study aims to enhance the bioavailability of AGE as a skin care ingredient by liposome encapsulation. We previously demonstrated that a commercially available lecithin, SLP-WHITE, self-assembles to form liposomes, which can efficiently encapsulate functional food materials\(^23\). We thus prepared liposomes from SLP-WHITE (1 wt%) and AGE by the Bangham method, and checked the fundamental interfacial properties.

AGE comprises of different hydrophilic components as indicated above, and would thus be encapsulated in the inner aqueous phase of liposomes. Here, the trapping efficiency of liposomes was thus investigated using calcein as a hydrophilic fluorescence probe. Figure 1 shows the efficiency of the prepared liposomes at a concentration range from 0.25 to 1 wt% of AGE.

Little change was observed in the trapping efficiency at the concentrations up to 0.5 wt%. Further increase in the concentration caused significant decrease of the efficiency. Accordingly, SLP-WHITE is likely to form liposomes, which are able to retain calcein as well as AGE to some extent in the inner aqueous phase.

3.2 The dispersibility of liposomes encapsulating AGE
The dispersibility of liposomes is very important when liposomes are used in practice. The effect of AGE concentration on the dispersibility was thus examined by visual observation. Figure 2 shows the time-dependence of the visual observation on liposomal solutions prepared from SLP-WHITE (1 wt%) and 0.25 or 0.5 wt% of AGE, respectively.

One h after preparation, each liposomal solution was well dispersed (Fig. 2a). Interestingly, the solution prepared from 0.25 wt% of AGE remained well dispersed for at least 14 d, while the solution from 0.5 wt% of AGE developed phase separation and precipitation (Fig. 2b). AGE is reported to include different polysaccharides such as a polydispersed β-(1,4)-linked acetylated mannan.

![Fig. 1 Relationship between the Trapping Efficiency and AGE Concentration.](image)

Values were expressed as the means ± S.D. (n=3).
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(acemannan) which might have cross-linking affinity toward phosphatidylcholine. The liposomal solution prepared from 0.5 wt% of AGE might thus show less stability compared to that from 0.25 wt%.

3.3 Preparation of liposomes by the mechanochemical method

We previously reported that highly stable liposomes encapsulating the extract of Ukon are efficiently obtained by the mechanochemical method using a homogenizer and microfluidizer. In order to improve the dispersibility and stability of liposomes encapsulating AGE, the liposomes were thus prepared by the new method with SLP-WHITE of 1 wt% and 0.25 or 0.5 wt% of AGE.

**Table 1** Relationship between the Mean Particle Size of Liposomal AGE and Concentration of AGE after the Treatment by Homogenization with/without Microfluidization.

<table>
<thead>
<tr>
<th>Concentration of AGE (wt%)</th>
<th>Mean particle size (nm ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenization</td>
</tr>
<tr>
<td>0.25</td>
<td>684.0 ± 15.8</td>
</tr>
<tr>
<td>0.5</td>
<td>1167.3 ± 19.1</td>
</tr>
</tbody>
</table>

Fig. 2 Visual Observation of Liposomal AGE Solutions Prepared from Different Concentrations of SLP-WHITE.
(a) 1 h after preparation (b) 14 d after preparation

Fig. 3 Freeze Fracture Electron Micrograph of Liposomal AGE Prepared from SLP-WHITE by the Mechanochemical Method.

![Fig. 3](image-url)
than 200 nm (Fig. 3), which corresponds well to the mean particle size in Table 1.

Accordingly, highly stable liposomes encapsulating AGE were efficiently obtained with SLP-WHITE by the mechnochemical method. The obtained liposomes with a diameter of less than 200 nm were extensively used in the following experiments.

3.4 Effect of liposomal AGE on proliferation and type I collagen synthesis in normal human neonatal skin fibroblasts

The effect of the obtained liposomal AGE on proliferation and type I collagen synthesis in normal human neonatal skin fibroblasts (NB1RGB cells) was examined, aiming to tentatively evaluate the anti-aging properties.

L-ascorbic acid is well known to act as an inducer of cell proliferation and type I collagen synthesis in fibroblasts, and thus the acid was used as a positive control. Figure 4 shows cell proliferation rate of L-ascorbic acid, AGE and liposomal AGE, respectively, in comparison with the control cells. L-ascorbic acid, AGE and liposomal AGE showed positive effects on the cell proliferation. Interestingly, the proliferation rate with liposomal AGE was significantly higher than that with AGE alone.

In addition, compared to the control, liposomal AGE significantly increased the collagen synthesis by 23%, while L-ascorbic acid and AGE alone increased the synthesis by only 8 and 4%, respectively (Fig. 5). These results may indicate that the skin care activities and bioavailability of AGE are significantly enhanced by liposome encapsulation.

The particle size of liposomes plays a dominant role in the absorption rate into cells. Especially, the liposomes of around 200 nm promote membrane fusion with target cells and efficiently deliver components into the cells. Liposomal AGE as nano-size particles of less than 200 nm would thus be efficiently incorporated into the skin fibroblasts, as a result of the increased affinity toward the cell surface. Nevertheless, further physiochemical and biochemical studies on the present liposomes should be done to shed light on the detailed proliferation mechanism.

3.5 Effect of liposomal AGE on proliferation in normal human epidermal keratinocytes

A recent study demonstrated that hydrophilic components of Aloe vera leaf have a keratinocyte proliferation activity in vitro. Keratinocytes are often used for evaluating a regeneration of skin. We then evaluated the effect of liposomal AGE on proliferation in normal human epidermal keratinocytes of neonatal foreskin (NHEK(F) cells), aiming to tentatively evaluate the skin whitening properties.

Here, the control cells were cultivated in HuMedia-KG2 without both BPE and hEGF, which are known to be effective growth factors of keratinocytes. Compared to the control, two kinds of AGE fractions, which include 4 and 20 µg/mL of the gel extract instead of BPE and hEGF, increased the proliferation rate by 41 and 60%, respectively. These results revealed that AGE possesses a positive effect on the keratinocyte growth (Fig. 6).

Fig. 5 Effect of AGE and Liposomal AGE on Type I Collagen Synthesis in Normal Human Neonatal Skin Fibroblasts.

NB1RGB cells were cultivated for 72 h in α-MEM containing 10% FBS, which supplemented with L-ascorbic acid, AGE or liposomal AGE. L-ascorbic acid was used as a positive control. Final concentration of the gel extract was fixed at 4 µg/mL. Values were expressed as the means ± S.D. (n=3). Different letters are significantly different (P<0.05).
bioavailability of AGE are highly improved by liposome encapsulation. This implies that the administration of the present liposomal AGE should accelerate a regeneration of skin, which leads to wound healing in the epidermis.

4 CONCLUSION

Liposomes including *Aloe vera* leaf gel extract (AGE) were prepared from a commercial soybean lecithin (SLP-WHITE) by the Bangham method, and were examined for the fundamental interfacial properties. The liposomes prepared from SLP-WHITE of 1 wt% and AGE of 0.25 wt% showed relatively a good trapping efficiency and dispersibility. Highly stable liposomes were then prepared by the mechanochemical method using a homogenizer and microfluidizer. With the combination of homogenization and microfluidization, SUVs with a diameter of less than 200 nm were efficiently prepared from SLP-WHITE of 1 wt% and AGE of 0.25 wt%.

We also investigated the upgraded effects of liposomal AGE on proliferation and type I collagen synthesis in human fibroblasts, and on proliferation in human keratinocytes, from the view points of the skin care properties. Interestingly, the present liposomal AGE significantly enhanced the cell proliferation and collagen synthesis, compared to the case of AGE alone. Therefore, these findings indicate that the skin care properties and bioavailability of AGE are highly improved by liposome encapsulation. These upgraded effects of liposomal AGE should be attributed from the increased transdermal activity, although further studies should be performed to clarify the skin permeation mechanism. The present liposomal AGE should have a great potential as an effective skin care formulation, especially for anti-aging and/or regeneration of skin.

ACKNOWLEDGEMENT

This work was supported in part by Grant-in-Aid for Consortium R&D Projects for Regional Revitalization from Ministry of Economy, Trade and Industry.

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