Journal of Industrial and Engineering Chemistry 98 (2021) 289–297

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

Asterias pectinifera derived collagen peptide-encapsulating elastic nanoliposomes for the cosmetic application

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ARTICLE INFO

Article history:
Received 26 January 2021
Received in revised form 8 March 2021
Accepted 23 March 2021
Available online 31 March 2021

Keywords:
Asterias pectinifera
Starfish collagen
Elastic nanoliposome
Cosmetic application
Anti-aging
Marine biotechnology

ABSTRACT

Asterias pectinifera, a starfish that has been known to ruin the aquaculture industry owing to its voracious appetite, has recently been identified as an eco-friendly source of non-toxic and highly water-soluble low-molecular weight collagen peptides, which promotes wound healing, bone regeneration, and skin protection. Although they have potential applications in biomedical applications, including pharmaceuticals and cosmetic products, it remains unclear how to improve the *in vivo* absorption of collagen peptides. Here, we present a novel method to enhance the absorption rate of collagen peptides using a lipid-based nanocarrier. We prepared an elastic nanoliposome by controlling the composition ratio of phospholipids and low-molecular weight collagen peptides. Our results indicate that low-molecular weight collagen peptides extracted from *Asterias pectinifera* have higher encapsulation efficiency than the collagen peptides extracted from pork and fish, which have traditionally been considered as a conventional source of collagen. Moreover, we demonstrate that the elastic nanoliposome containing the collagen peptide of *Asterias pectinifera* can reduce MMP-1 expression caused by ultraviolet radiation-induced photoaging. Therefore, the combination of *Asterias pectinifera*-derived low-molecular-weight collagen peptides and elastic nanoliposomes may be a promising formulation as an eco-friendly source of materials for anti-aging cosmetics.

Introduction

Asterina pectinifera, a marine species of starfish found mainly in Korea, eastern Russia, Japan, and China [1], is considered a critical marine hazard because it destroys marine aquacultures such as oysters, abalone, and clam farms, and impedes to fishing activities, resulting in economic loss [2]. The body wall of starfish is composed of magnesium calcite ossicles connected by 10% collagenous tissues and muscle proteins, of which 60% is composed of collagen [3]. Thus, collagen-rich starfish is expected to be a useful raw material for pharmaceutical and cosmetic products, particularly in applications that prohibit the use of collagen originating from pork, which is conventionally considered as a source of collagen [4].

Collagen is the major structural protein constituting the extracellular matrix of connective tissues and widely used in bioindustries such as tissue regeneration [5], pharmaceuticals, food, and cosmetics, because its highly biocompatible and biodegradable characteristics enable superior cell accommodation, while its immunogenicity is relatively low compared to other types of biomaterials [6]. To further utilize collagen in biotechnology, it is hydrolyzed to collagen peptides by proteolysis [7,8]. Collagen peptide have low molecular weight and excellent solubility in water, which enables diverse functions such as wound healing [9], bone regeneration [10], and anti-aging of skin [11]. Hydrolyzed collagens, small-molecular-weight peptides, are easy to penetrate deep layers of the skin compared to high-molecular-weight native collagen. They play an important role in fibroblast growth [12], collagen expression [13], and strong collagen fiber formation in epidermis [14]. Moreover, they are critical molecular components to improve the functionality of the skin layer by increasing the water content of the stratum corneum [15]. Therefore, it is a widely used in the cosmetic applications to maintain skin homeostasis and to restore disrupted skin layer [16]. While collagens are

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https://doi.org/10.1016/j.jiec.2021.03.039

primarily derived from terrestrial animals such as pigs and cattle, collagen peptides extracted from marine organisms such as marine sponges and starfish have a lower molecular weight than collagen peptides derived from terrestrial animals [17].

Liposomes composed of phospholipid bilayers are non-toxic, biodegradable, and biocompatible drug-encapsulating vesicles. Spherical-shaped liposomes have been utilized to deliver various components such as DNA, vaccines, and drugs into the body in pharmaceutical and cosmetics applications [18–22]. However, conventional liposomes are not well-soluble in water and their low stability in water reduces structural integrity by promoting oxidation and hydrolysis of phospholipids, resulting in low permeability through the skin layer [22,23]. Recently, to overcome these issues, modified liposomes featuring enhanced deformable properties, e.g., ethosome [24] and elastic nanoliposome [25] have been developed. In particular, elastic nanoliposomes consisting of phospholipids and surfactants induce a high radial curvature of the vesicles because the surfactant acts as an edge activator between the lipid bilayers [25]. This structure makes the liposomes more elastic and flexible than conventional liposomes, which ultimately enables more effective penetration of the skin layer without disassembly of the liposome architecture [23]. Elastic liposome differs depending on the composition of lipid and surfactant but has the elasticity to pass through a very narrow space, about 0.2 times the diameter [26]. Furthermore, liposome membrane structure with edge activators enhances the drug encapsulation efficiency by increasing the solubilization of hydrophobic drugs [27].

While liposomes have been applied as carriers to increase the absorption rate of collagen peptides originating from pork, elastic nanoliposomes encapsulating collagen peptides originating from starfish have not yet been developed [28]. In this study, therefore, we extracted collagen from *Asterina pectinifera* to produce low-molecular weight collagen peptides and embedded them into elastic nanoliposomes to maximize their skin permeation. We aimed to design *Asterina pectinifera*-derived collagen peptide-encapsulated elastic nanoliposomes and demonstrated their superior anti-aging potential compared to conventional collagen peptides originating from pork and fish.

Experimental

Starfish collagen extraction and collagen peptide production

To produce collagen peptide derived from starfish, we used *Asterina pectinifera* collected from the western sea of South Korea. *Asterina pectinifera* was treated overnight with a 5% NaOH solution (Samchun) to remove non-collagen substances and was placed in a distilled water and 0.25% tartaric acid (Duksan) solution to precipitate the starfish fragments so that only collagen attached to the bone fragments remained. It was then treated ultrasonically at 38 kHz for 1 h using an ultrasonic processor (Sonics & Materials, VCX-500). The collagen extraction process was performed in ice water to prevent collagen degeneration. Subsequently, the extracted collagen was hydrolyzed into collagen peptide by treatment with subtilisin (0.1% by weight). After dividing the calcium tartrate and collagen peptide solution using a centrifuge (Hanil, MF80) at 2000 rpm for 3 min, the solution was frozen in a deep freezer and freeze-dried to prepare a starfish-derived collagen peptide powder.

Amino acid composition analysis

The amino acid composition of the collagen peptide was analyzed using high-performance liquid chromatography (HPLC). The collagen peptide extracted from *Asterina pectinifera* was hydrolyzed with HCl at 110 °C for 24 h. Through the derivatization process, the hydrolyzed samples were labeled with fluorescein isothiocyanate (FITC). After drying, the samples were dissolved in a solution composed of 40 mM sodium acetate trihydrate, 0.15% triethylamine, 0.03% ethylenediaminetetraacetic acid (EDTA), and 6% CH_3CN. The supernatant was extracted using centrifugation and analyzed on an HPLC system (Agilent, Agilent 1260 series).

Fabrication of elastic liposome

Elastic liposomes were produced using thin-film hydration [29]. The ratios of the constituents constituting the elastic liposome were determined, as shown in Table 1. In a 50 ml round bottom flask, L-α-phosphatidylcholine (sigma), TEGO® Care CG 90 surfactant (EVONIK), and collagen peptide were added according to the composition ratio given in Table 1, and then dissolved with 20 ml ethyl alcohol (Merck). After the ethanol was completely evaporated using a rotary evaporator, a lipid membrane was formed on the flask wall, which was then hydrated in 20 ml of distilled water to produce an elastic nanoliposome. Mechanical vibration was applied to the solution for 15 min at 20 kHz, 30% amplitude using an ultrasonic processor to manufacture an elastic nanoliposome of constant size. The elastic nanoliposome solution thus prepared was stored at 4 °C after removing the unloaded collagen using a 0.45 μm syringe filter (Advantec).

Particle size and zeta potential measurement

To confirm the stability of the elastic nanoliposome particle size and liposome structure, we used a particle size and zeta potential analyzer (Ostuka Electronics, ELSZ-1000). An argon laser was used for light scattering, and the measurement was carried out at 25 °C. The average particle size distribution was measured based on the intensity, and was analyzed using the CONTIN algorithm [30]. The zeta potential was also measured using the same equipment.

Morphological observation

Transmission electron microscopy (TEM) was used to confirm the morphology of the elastic nanoliposome [29]. After dropping 5 μl of elastic nanoliposome on the mesh structure carbon-coated copper grid, the solvent was absorbed for 2 min and allowed to be removed completely using paper tissue. The sample on the grid was stained with 0.5% uranyl acetate solution for 1 min. The prepared elastic nanoliposome sample was imaged using a TEM system (Hitachi H-7100) that was operated at an accelerating voltage of 80 kV.

Encapsulation efficiency of collagen in elastic nanoliposome

To measure the amount of collagen loaded in the elastic nanoliposome, the same collagen concentration used in the liposome preparation process was dissolved in distilled water. Using a 0.45 μm syringe filter, 0.5 ml of the elastic nanoliposome solution from which the unloaded collagen was removed was added to 0.5 ml of distilled water, and the collagen and lipid layers

Table 1
Molecular weight of collagen peptide measured by GPC. Mw and Mn represent the weight-average molecular weight and the number-average molecular weight, respectively. Mw/Mn indicates the width of the molecular weight distribution.

Source	Mw	Mn	Mw/Mn
Pork	2406	1703	1.41
Fish	1901	1306	1.46
Starfish (*Asterina pectinifera*)	1587	1541	1.03

were separated through ultracentrifugation (1 h, 10,000 rcf at 4 °C). The amount of collagen loaded in the liposome in the supernatant was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, 23225). The absorbance in the assay was measured at 555 nm using a microplate reader (Biotek, Epoch). The encapsulation efficiency of the elastic nanoliposome is calculated as follows:

$$\text{Encapsulation efficiency}(\%) = \frac{Collagen_{total} - Collagen_{super}}{Collagen_{total}} \times 100$$

where $Collagen_{total}$ represents the total amount of collagen dissolved in the distilled water and $Collagen_{super}$ indicates the amount of unloaded collagen peptide remaining in the supernatant.

Cell viability test (Live/Dead assay & MTT assay)

To evaluate the toxicity of the collagen-loaded elastic nanoliposome, we performed cell viability test with CCD-986sk human dermal fibroblast cell line, which was obtained from the Korea Cell Line Bank. After seeding 3×10^4 cells/well on a 48-well cell culture plate, the cells were cultured for 24 h in Dulbecco's modified eagle's medium (DMEM) cell culture media (Corning) composed of 10% fetal bovine serum (FBS) (Merck) and 1% penicillin-streptomycin (Thermo Scientific). The elastic nanoliposomes were then mixed with the culture media at a concentration of 0.8 mg/ml and the cells were cultured for 24 h before performing the cell viability tests. For the live/dead assay (Invitrogen, L3224), we used calcein-AM and ethidium homodimer-1 that indicated whether cells were alive in response to intracellular esterase activity and whether cells were dead due to impaired integrity of plasma membrane, respectively. Calcein-AM and ethidium homodimer-1 were dissolved in phosphate-buffered saline (PBS) at a concentration of 0.4 μl/ml and 1 μl/ml, respectively. Cells cultured in elastic nanoliposomes containing culture media for 24 h were treated with these solutions for 30 min. and then imaged using a Nikon A1R confocal microscope equipped with a Plan 20× lens. In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the culture media containing the elastic nanoliposomes was replaced with the cell culture media containing 5% MTT solution. After an additional incubation for 4 h, the media was removed, and 100 μl of dimethyl sulfoxide (DMSO) (Merck) was added to dissolve the insoluble formazan crystal in living cells. The absorbance was measured at 560 nm using a microplate reader (Biotek, Epoch).

Permeation of elastic nanoliposome

We performed the skin parallel artificial membrane permeability assay (PAMPA) (Pion, 120663) to measure the absorption rate of the collagen peptides encapsulated by elastic nanoliposomes. A skin PAMPA (a permeation assay using artificial skin) was carried out as follows [31]. Briefly, the skin layer of the acceptor plate coated with artificial skin was completely hydrated with a hydration solution, and buffers and samples were added to the deep-well plate and sufficiently mixed using a shaker. After moving the samples to each well on the donor plate, the acceptor plate (filled with the buffer) was carefully placed on the donor plate so that the artificial skin layer touched the sample solution and allowed the sample to penetrate the artificial membrane. Subsequently, the absorbance of each plate at 275 nm was analyzed with a microplate reader (Biotek, Epoch) to measure skin transmission.

UV-induced MMP-1 inhibition assay

CCD-986sk human dermal fibroblasts were seeded in 48-well plates (3×10^4 cells/well) and cultured at 37 °C in 5% CO_2. After 24 h of incubation, cell culture media were aspirated, and cells were washed with PBS three times. The cells were irradiated with an ultraviolet B (UVB) lamp (Sankyo, maximum wavelength of 306 nm) for 20 min in PBS. After UVB irradiation, the PBS was aspirated, and the cell culture media containing the elastic nanoliposome were replaced. The cells were cultured for 24 h. The amount of MMP-1 expression was measured in the supernatant of the cells using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad software, USA). For multiple comparisons, one-way analysis of variance (ANOVA) was applied. The statistical methods and results are summarized in each figure caption.

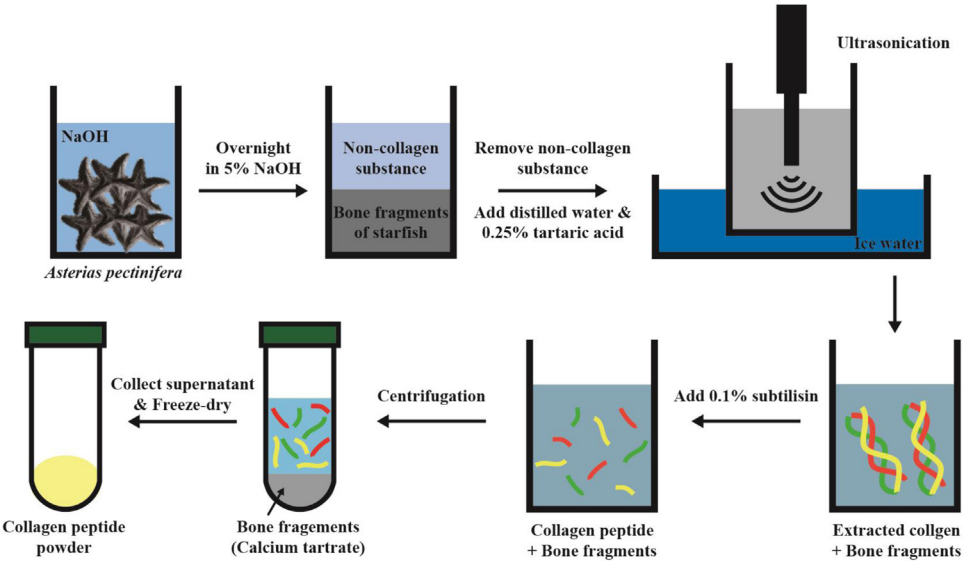

Fig. 1. Preparation of collagen peptide from *Asterina pectinifera*.

Results

Extraction of collagen peptide from Asterina pectinifera

To produce an elastic nanoliposome with high collagen loading efficiency, we extracted collagen peptide from *Asterina pectinifera*. First, we treated *Asterina pectinifera* with 5% NaOH overnight. After removing the supernatant, we added tartaric acid and distilled water to the remaining bone fragments of *Asterina pectinifera* to achieve collagen extraction, which was followed by ultrasonication at 38 kHz for 1 h in ice water. Next, we added 0.1% of the protein-digesting enzyme subtilisin (w/w%) to create a collagen peptide. After centrifugation at 2000 rpm for 3 min, the supernatant segregated from calcium tartrate was freeze-dried to collect collagen peptide powder, where the extraction efficiency of the collagen peptide was 3.8% by weight (Fig. 1).

The collagen peptide produced from *Asterina pectinifera* in this manner has a lower molecular weight than conventionally utilized collagen peptides extracted from other animals such as pork and fish (Table 1). Gel permeation chromatography (GPC) indicated that the molecular weight of the collagen peptide (1587) extracted from starfish was 34% and 17% lower than that of pork (2406) and fish (1901), respectively (Table 1). Moreover, the dispersity index that defines the ratio of weight-average molecular weight to number-average molecular weight indicates that the collagen peptide from *Asterina pectinifera* has a more uniform molecular weight compared to other types of collagen peptides (Table 1).

We further analyzed the amino acid composition of the collagen peptides from *Asterina pectinifera*. HPLC indicated that the main components of amino acids in the collagen peptides extracted from *Asterina pectinifera* were glycine, proline, alanine, and hydroxyproline (Table 2), which also constitute the major population of collagens obtained from other species [32–35]. Moreover, the proportion of hydrophilic amino acids such as glutamic acid, arginine, aspartic acid, serine, lysine, threonine, histidine, and tyrosine is 5% and 3% higher than that of collagens obtained from pork (25%) and fish (27%), respectively [33,34]. These results indicate that the collagen from *Asterina pectinifera* has greater potential as a viable substance inside the liposome.

Determination of the optimal formulation of elastic nanoliposomes

To maximize the skin penetration of *Asterina pectinifera* collagen peptides, we used elastic nanoliposomes as the carriers for collagen peptides because they are more elastic and flexible than conventional liposomes [25]. Thus, we evaluated various composition ratios among the phosphatidylcholine, surfactant (cetearyl glucoside) by controlling the amount of phosphatidylcholine-surfactant (1, 3, 5, 10% w/w), and collagen amount (0.1%, 0.5%, 1.0% w/w) relative to the total solution (Table 3), where the ratio of phosphatidylcholine and surfactant in each elastic nanoliposome was maintained at 9:1, because this ratio has been reported to have the highest encapsulation efficiency [29].

Because the size of the liposomes as well as the lipid composition and surface charge govern the delivery of liposomes into the skin [36], we first measured the particle size of the prepared elastic nanoliposomes using dynamic light scattering (DLS) (Fig. 2). Measurements indicated that the particle size of the elastic nanoliposome increased as the amount of phosphatidylcholine-surfactant (PS) increased by the same amount as the collagen peptide (distinguished by different colors, Fig. 2). For the 0.1% collagen peptide, the particle size of the elastic nanoliposome containing 1% PS was 134.8 nm, which was 2.46 times less than that of the 10% PS sample, which was 331.8 nm. Even at higher collagen concentrations (0.5%, 1.0%), the particle size with 1% PS was observed to increase by 1.3 times. These results indicate that the particle size increases as the fraction of PS in the elastic nanoliposomes increases.

Surface charge of liposomes plays a critical role in preventing particle aggregation by acting as a repulsive force between the elastic nanoliposomes. Thus, we assessed the surface charge of elastic nanoliposomes after loading the collagen peptide using a zeta potential analyzer because it can indicate the dispersion stability, that is, against sedimentation and/or aggregation of *Asterina pectinifera* collagen peptide-embedded elastic nanoliposomes. The elastic liposome containing 0.1% of the collagen peptide exhibited a zeta potential of −12.44 to −18.48 mV (Table 4), which corresponds to a stable liposome [37]. On the other hand, elastic nanoliposomes loaded with a high concentration of collagen peptide (0.5% and 1.0%) did not exhibit constant surface charge

Table 2
Amino acid composition of the collagen peptides derived from pork, fish, and *Asterina pectinifera*. Bold indicates hydrophilic amino acids.

Amino acid	Pork [33]	Fish [34]	Starfish *(Asterina pectinifera)*
	(residues/1000 total amino acid residues)		
Glycine	341.0	333.0	285.9
Proline	123.0	119.0	102.2
Alanine	115.0	124.0	91.7
Hydroxyproline	97.0	86.0	142.0
Glutamic acid	72.0	76.0	54.1
Arginine	48.0	51.0	23.2
Aspartic acid	44.0	46.0	29.3
Serine	33.0	35.0	40.1
Lysine	27.0	23.0	28.2
Valine	22.0	19.0	21.2
Leucine	22.0	23.0	17.4
Threonine	16.0	23.0	99.0
Phenylalanine	12.0	14.0	13.1
Isoleucine	10.0	10.0	7.6
Hydroxylysine	7.0	–	–
Methionine	6.0	2.0	23.6
Histidine	5.0	8.0	4.8
Tyrosine	1.0	3.0	16.6
Cysteine	–	3.0	–
Total	1000.0	1000.0	1000.0
Hydrophilic amino acids (%)	**25%**	**27%**	**30%**

Table 3
Formulation of various elastic nanoliposomes with collagen peptide derived from *Asterina pectinifera*. Formulation code denotes the ratio of lipid surfactant and collagen peptide, where the ratio of lipid and surfactant is constant at 9:1.

Formulation Code	Lipid content (w/w%)	Surfactant content (w/w%)	Collagen peptide (w/w%)
EL1/0.1	0.9	0.1	0.1
EL3/0.1	2.7	0.3	0.1
EL5/0.1	4.5	0.5	0.1
EL10/0.1	9	1	0.1
EL1/0.5	0.9	0.1	0.5
EL3/0.5	2.7	0.3	0.5
EL5/0.5	4.5	0.5	0.5
EL10/0.5	9	1	0.5
EL1/1	0.9	0.1	1
EL3/1	2.7	0.3	1
EL5/1	4.5	0.5	1
EL10/1	9	1	1

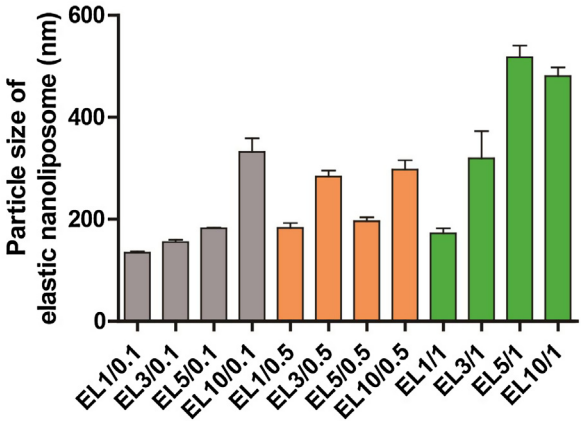

Fig. 2. Particle size of elastic nanoliposomes of formulated elastic nanoliposomes. Each bar graph shows the mean ± SD based on three independently conducted experiments per condition.

(Table 4). These results indicate that loading excessive amount of collagen peptides onto limited-size elastic nanoliposomes disrupts the construction of stable liposomes, as it attenuates the stability of dispersions, for example, sedimentation and/or aggregation of *Asterina pectinifera* collagen peptide-embedded elastic nanoliposomes.

These results highly suggest that the ratio of 1:0.1 (denoted by EL1/0.1) between the vehicle and the collagen peptide could maximize the structural stability of elastic nanoliposome loading the *Asterina pectinifera* collagen peptide. TEM evaluation of this condition further confirmed the uniform size (~130 nm) and spherical shape of 0.1% collagen peptide-embedded elastic nanoliposomes (Fig. 3), consistent with the DLS-based particle size analysis (Table 4).

Encapsulation of collagen peptide into elastic nanoliposome

Next, we quantified the encapsulation efficiency of collagen peptide in the prepared elastic nanoliposomes using a BCA assay to double check if the previously determined formulation could efficiently load the collagen peptides. Collagen encapsulation was maximized using a 0.1% collagen peptide concentration (62–88%, gray bars in Fig. 4) compared to collagen encapsulations of 0.5% and 1% collagen peptide-loaded elastic nanoliposomes corresponding to 10–50% (orange bars in Fig. 4) and <10% (green bars in Fig. 4), respectively. We further noted that these results were maintained for various composition ratios of PS, where elastic nanoliposomes composed of 1% PS exhibited the maximum encapsulation efficiency (>88%), strongly suggesting that *Asterina pectinifera* collagen peptides were densely packed into minimized elastic liposomes (Compare Figs. 2 and 4).

Finally, we tested if our elastic nanoliposomes could load the collagen peptides originating from fish and pork that are widely utilized as cosmetic ingredients. Surprisingly, we found that collagen peptides derived from pork and fish were hardly loaded in the elastic nanoliposomes (Fig. 4), which could be attributed to the relatively high molecular weight and low hydrophilicity of these collagen peptides compared to the starfish-extracted collagen peptides. This notion was supported by the previous data showing that the molecular weights of collagen peptides originating from pork, fish, and starfish were 2406, 1901, and 1587, respectively (Table 1), and the composition of hydrophilic amino acids in the pork, fish, and starfish derived collagen peptides were 25%, 27%, and 30%, respectively (Table 2).

Together, these results demonstrate that the nanoliposomes consisting of 1% PS and 0.1% collagen peptide denoted by EL1/0.1 represent the optimal formulation between lipid membrane structure and encapsulated collagen peptide derived from *Asterina pectinifera*.

Table 4
Physical characteristics of elastic nanoliposomes with collagen peptide derived from *Asterina pectinifera*.

Formulation code	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
EL1/0.1	134.8 ± 1.9	0.303 ± 0.013	−16.2 ± 0.11
EL3/0.1	155 ± 4.7	0.319 ± 0.009	−15.1 ± 1.07
EL5/0.1	182.3 ± 1.3	0.337 ± 0.006	−18.5 ± 0.3
EL10/0.1	331.8 ± 27	0.362 ± 0.005	−12.4 ± 1.04
EL1/0.5	183.5 ± 9.1	0.289 ± 0.02	2.4 ± 2.62
EL3/0.5	283.6 ± 12.3	0.227 ± 0.013	−3.2 ± 0.09
EL5/0.5	196.3 ± 7.3	0.335 ± 0.004	−8.9 ± 0.67
EL10/0.5	297.2 ± 18.5	0.312 ± 0.026	−5.9 ± 0.52
EL1/1	172.5 ± 9.6	0.303 ± 0.016	0.2 ± 0.59
EL3/1	319.3 ± 53.6	0.292 ± 0.05	1.5 ± 0.49
EL5/1	517.9 ± 23.1	0.336 ± 0.013	−2.9 ± 0.2
EL10/1	480.9 ± 16.9	0.305 ± 0.008	−2.6 ± 2.01

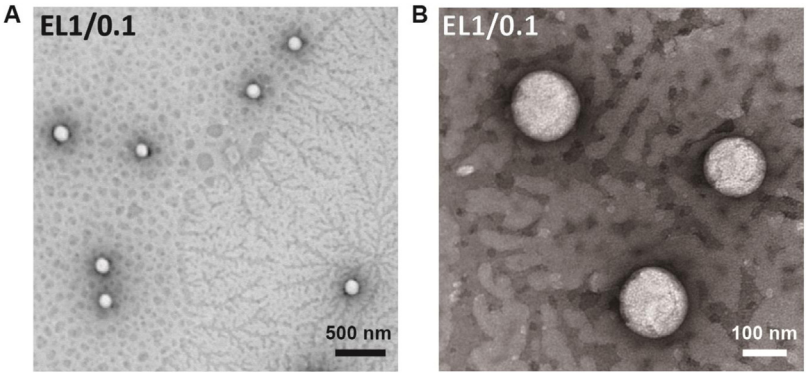

Fig. 3. Transmission electron microscopy (TEM) image of collagen peptide-embedded elastic nanoliposomes formulated in EL1/0.1. A. low magnification, B. high magnification.

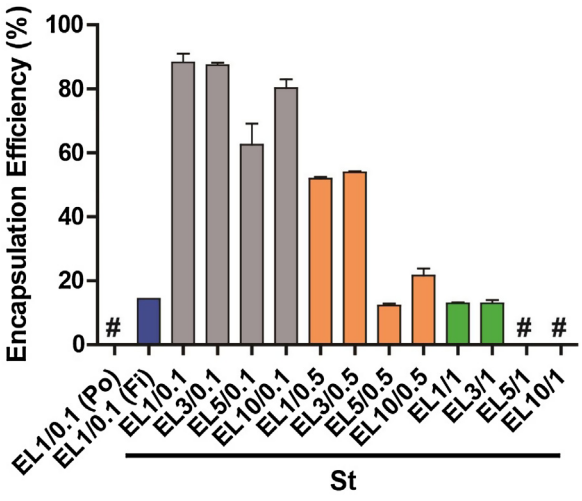

Fig. 4. Encapsulation efficiency of collagen peptide in elastic nanoliposomes. # indicates no encapsulation detected. Data are presented as the mean ± SD (three experiments performed per condition).

Biological compatibility of collagen peptide encapsulated elastic nanoliposomes

To test if collagen peptide-encapsulated elastic nanoliposomes are biologically safe, we performed a cell viability test using CCD-986sk, a human dermal fibroblast cell line. Cells were exposed to cell culture media for 24 h, where elastic nanoliposomes loaded with pork, fish, and *Asterina pectinifera*-derived collagen peptides, denoted by EL-Po, EL-Fi, and EL-St, respectively, were diluted to 0.8 mg/ml. Because the Live/Dead assay can distinguish living cells that exhibit green fluorescence due to esterase activation from dead cells that exhibit red fluorescence due to the loss of plasma membrane integrity [38], we first performed fluorescence confocal microscopy to estimate the population of dead cells (Fig. 5A and Supplementary Fig. S1). 0.77% of cells were dead in *Asterina pectinifera*-derived collagen peptide embedded elastic nanoliposomes-treated cells. This result was similar in pork (0.94%) and fish (2.38%) collagen peptides embedded elastic nanoliposomes treated cells. While some dead cells were detected in each condition, a majority of the cells were alive in all three conditions (Fig. 5A and Supplementary Fig. S1).

To further quantitatively assess cell viability, we performed the MTT assay based on cellular metabolic activity. All samples exhibited >90% cell viability and no significant difference in cell survival rate was detected (Fig. 5B), consistent with the result of Live/Dead assay.

These results confirm that the collagen peptide-embedded elastic nanoliposomes formulated in EL1/0.1 are biologically safe.

Skin permeation of starfish collagen peptide-encapsulated elastic nanoliposomes

Because the transport of particles through the skin is critical for cosmetic formulations of skin care products, we assessed the permeation capability of collagen peptide-loaded elastic nanoliposomes. To this end, we performed *in vitro* skin permeation assays using a skin PAMPA to directly evaluate the skin absorption of the collagen peptide encapsulated by elastic nanoliposomes. The skin PAMPA assay measures the amount of permeated solution

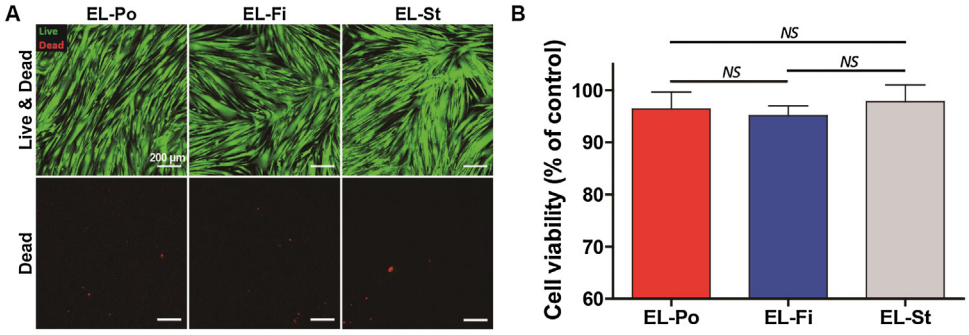

Fig. 5. Effects of elastic nanoliposomes on cell viability. A. Live/dead cell viability assay of cells. Cells were incubated for 24 h in DMEM containing 10% FBS with collagen peptide-embedded elastic nanoliposomes at a concentration of 0.8 mg/ml. Encapsulated-collagen peptides are derived from pork, fish, and starfish, respectively. B. MTT assay. Cell viability was determined by sample absorbance at 570 nm. Bar graphs show the mean ± SD. 1-way ANOVA using Tukey's test was applied for the statistical comparison (*NS*: $p > 0.01$).

through an artificial lipid mixture-based membrane that mimics human skin [31]. Comparing the penetration ratio of collagen peptides from diverse origins with/without the nanocarrier, we noted that collagen peptides that were not loaded onto the nanoliposomes did not penetrate the skin during the PAMPA (Fig. 6A). However, regardless of the type of collagen peptide, elastic nanoliposome-loaded collagen peptides were able to permeate the membrane, and more importantly, *Asterina pectinifera* collagen peptide embedded into elastic nanoliposomes penetrated the membrane 6 times and 2 times more than the pork and fish collagen peptide-loaded elastic nanoliposomes, respectively (Fig. 6A). These results indicate that the elastic nanoliposome facilitates collagen peptide absorbance into the skin, and the elastic nanoliposome with collagen peptide from *Asterina pectinifera* displayed a higher protein absorption rate than other types of collagen peptides.

Furthermore, a control liposome that lacks surfactant was prepared to confirm the effect of elastic nanocarriers on the absorption rate. The absorption rate of collagen peptide encapsulated by the basic liposome was compared with the elastic nanoliposomes. The non-surfactant liposome (NL-St) was composed of 1% phosphatidylcholine and 0.1% collagen peptide derived from *Asterina pectinifera*. In skin PAMPA assay, the collagen peptides loaded on elastic nanoliposomes were detected more than the collagen peptide loaded on the non-surfactant liposomes (Supplementary Fig. S2). This result showed that the elastic nanocarriers help the collagen peptide to penetrate the skin layer efficiently.

To further confirm skin permeability of collagen peptide loaded on the elastic nanoliposomes, we visualized the *in vitro* distribution of fluorescence-labeled collagen peptides using pork skin [29]. We conjugated collagen peptides to fluorescein-isothiocyanate (FITC), a lipid-soluble fluorescent compound with excitation and emission peaks at 491 nm and 519 nm [39], respectively, prior to loading into the elastic nanoliposome. Using laser confocal microscopy, we detected the distribution of FITC-binding collagen peptides in the dermal layer (Fig. 6B).

These results confirm that elastic nanoliposomes are necessary to transport collagen peptides into the skin layer. Furthermore, *Asterina pectinifera* collagen peptides displayed superior permeation efficiency compared to conventional pork or fish collagen peptides.

Anti-aging effect of starfish collagen peptide-encapsulated elastic nanoliposomes

To estimate their potential for functional cosmetic applications, we assessed the anti-aging effects of *Asterina pectinifera* derived collagen peptide-loaded elastic nanoliposomes. UV rays are known to induce biophysical and biochemical damage in skin tissue through the formation of excessive free radicals that lead to DNA damage and abnormal activation of various enzymes and proteins [40]. It has been further reported that the production of reactive oxygen species by UV irradiation promotes the production of inflammatory cytokines, resulting in inflammatory responses [41] and degradation of the collagen matrix in the skin by overexpression of matrix metalloproteinase (MMP) [42,43]. On the other hand, collagen peptide is known to reduce photoaging-induced MMP-1 production by promoting the expression of the TIMP metalloproteinase-1 (TIMP-1) inhibitor that suppresses MMP-1 expression [44,45].

Therefore, we tested whether starfish collagen peptide-loaded elastic nanoliposomes could reveal anti-aging effect by reducing MMP-1 expression. To this end, we first irradiated the human dermal fibroblasts with UVB (wavelength: ~306 nm) to induce MMP-1 expression, which corresponds to the process of photoaging. Compared to MMP-1 expression of the collagen peptide unloaded control condition (indicating 100%, Fig. 7A), the expression level of MMP-1 was reduced in cells treated with collagen peptide-loaded elastic nanoliposomes (less than 100%, Fig. 7A). More importantly, elastic nanoliposomes containing collagen peptides extracted from *Asterina pectinifera* exhibited a higher reduction of MMP-1 than pork and fish extracted collagen peptide-loaded elastic nanoliposomes.

We further noted that this effect varied in a dose-dependent manner, and these changes were most pronounced in elastic nanoliposomes containing collagen peptides from *Asterina pectinifera* (compare black vs. red and blue curves in Fig. 7A). The elastic nanoliposomes containing collagen peptide extracted from *Asterina pectinifera* at a concentration of 0.8 mg/ml induced a 40% reduction in MMP-1 expression compared to the control, while elastic nanoliposomes loaded with other collagen peptides decreased by ~10% (Fig. 7B). These results indicate that elastic nanoliposomes encapsulated with *Asterina pectinifera* collagen peptides have superb anti-aging potential.

Discussion

Since the configuration of liposomes mainly depends on the material properties of the substance encapsulated by the liposomes, the hydrophilic interior of a liposome easily accommodates DNA, RNA, and hydrophilic drugs [22]. We demonstrated that elastic nanoliposomes loaded with low-molecular weight collagen peptides extracted from *Asterina pectinifera* starfish appears to be a promising biomaterial for cosmetic applications owing to its low toxicity, enhanced skin permeability, and anti-aging efficacy. Our results indicate that starfish collagen peptides have lower molecular weight (Table 1) and more hydrophilic amino acid residues (Table 2) than collagen peptides extracted from pork and fish, which are conventional sources for collagenous materials. Thus, the combination of low molecular weight and high hydrophilicity of collagen peptides extracted from starfish enables efficient encapsulation into the elastic nanoliposomes displaying high loading efficiency (Fig. 4). Our elastic membrane structure has

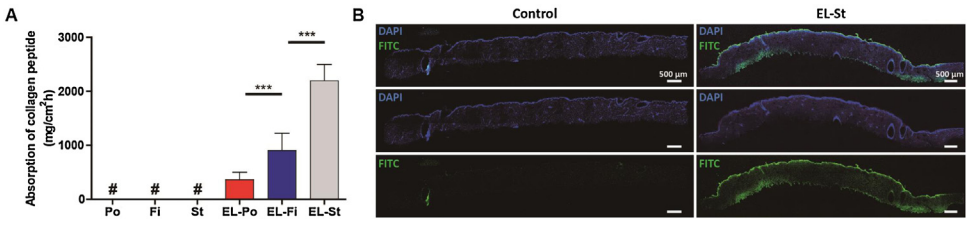

Fig. 6. Transdermal absorption of collagen peptide encapsulated by elastic nanoliposomes. A. *In vitro* penetration of collagen peptide embedded into elastic nanoliposomes through skin-PAMPA, a permeation assay using an artificial skin membrane. # represents no detection of permeated collagen peptide. Data are presented as the mean ± SD and 1-way ANOVA using Tukey's test was applied (***: $p < 0.001$). B. Visualization of *in vitro* dermal penetration using a pork skin. FITC-conjugated collagen peptide-embedded elastic nanoliposomes formulated in EL1/0.1 was applied to the pork skin to confirm the penetration of collagen peptide loaded by elastic nanoliposome through the animal skin. In the control condition, no treatment was performed. Samples were stained with DAPI to mark nuclei of cells in the skin tissue.

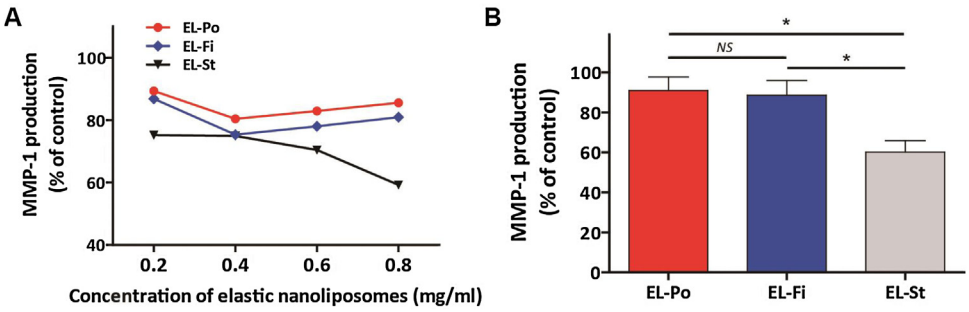

Fig. 7. Alleviation of UVB-induced photoaging. A. Quantification of MMP-1 expression in human dermal fibroblast cells in response to changing amount of collagen peptide-embedded elastic nanoliposome formulated in EL1/0.1. B. Detection of the maximum anti-aging effect. UV irradiation-induced photoaging is minimized in the condition of using 0.8 mg/ml of elastic nanoliposomes. Data were normalized based on the control values in each condition, Error bars indicate SEM. 1-way ANOVA using Tukey's test was applied for the statistical comparison (*: $p < 0.01$, NS: $p > 0.01$).

surfactants as an edge activator between lipid bilayers, making the liposomes more deformable and permeable than conventional liposomes. Furthermore, relatively low molecular weight of collagen peptides from starfish might help compact encapsulation into the elastic nanoliposomes. Thus, more starfish-derived collagen peptides could be detected in the skin layer by using a small amount of elastic nanoliposome as a carrying agent (Fig. 6A). This high-performance nanocarrier containing the high content of starfish collagen peptide improved the permeability of collagen peptides (Fig. 6B) and also suppressed the expression of MMP-1 resulting in photo-aging of skin layer (Fig. 7).

Collagen peptide is known as excellent antioxidants [11]. Highly reactive chemical species with non-covalent unpaired electrons, for example, free radicals, are attributed as causes of aging because intracellular oxidation induces damages to dermal collagenous extracellular matrix that weakens structural integrity of skin tissue [46]. While collagen peptides react with free radicals to create a stable form that functions as an electron donor [47], their distinct properties can be further modulated by the substances added during the extraction process. For instance, collagen peptides extracted using ascorbic acid provide higher antioxidant activity than ordinary collagen peptides, which enables collagen peptides to be utilized in various industries such as cosmetics, foods, pharmaceuticals, and regenerative biomaterials [48–50].

Nevertheless, delivery of collagen peptides deep into the skin layers has been considered as a technical hurdle that prevents multiple applications, which caused the development of diverse nanocarriers e.g., liposomes [51]. In this study, we used elastic nanoliposomes to overcome the weakness of the conventional liposome e.g., low encapsulation efficiency and the structural instability of lipid membrane [52]. Liposomes are suitable for peptide encapsulation because of their amphiphilic structure with various properties such as biocompatibility, biodegradable and high drug-loading ability [22]. However, the loading efficiency of hydrophilic substances in the conventional liposomes is poor and the unstable structure of the lipid membrane weakens their structural stability [26]. In contrast to conventional liposomes, elastic nanoliposomes overcome these shortcomings by the edge activators, which improves the loading efficiency and also structural flexibility to penetrate into tiny spaces like skin layer. In constituting the elastic nanoliposomes, the ionic characteristics of the edge activator are important. e.g., Anionic surfactants have higher skin penetration than cationic and nonionic surfactants and non-ionic surfactants have higher tolerance and lower toxicity than charged surfactants [53].

We fabricated elastic nanoliposomes which have lower toxicity (Fig. 5A and B) using Tego care, non-ionic surfactant. Elastic nanoliposomes made by this membrane structure displayed the smallest size (Fig. 2) and the highest encapsulation efficiency (Fig. 3) in EL1/0.1 of formulation code. Therefore, while elastic nanoliposomes still have several disadvantages such as low stability to chemical oxidative decomposition, low purity of phospholipid, and high manufacturing cost, they are considered one of the best drug delivery carriers [26].

Because collagen peptides commercially utilized in various industries have been mainly extracted from terrestrial animals such as cattle and pigs, they are susceptible to biosafety issues induced by animal diseases such as swine flu [54] and/or bovine spongiform encephalopathy [55]. As an alternative source, marine life such as marine sponges, fish, jellyfish, and starfish have been tested [56]. Although starfish provide ecological benefits as a primary scavenger of the carcasses of various animals on the seabed and other organisms [1], they can cause significant economic losses to the marine industry by destroying aquacultures, such as oyster, abalone, and shellfish farms. As predators of marine ecosystems in particular, they aggressively expand their population and invade the surrounding marine ecosystem [57]. Therefore, our elastic nanoliposomes loaded with starfish collagen peptides may not only be a new type of anti-aging cosmetic agent, but could also convert an invasive marine species into an eco-friendly biomaterial that is expected to create high value.

Conclusions

We prepared collagen peptide-loading nanoliposomes exhibiting high biological safety, skin absorption rate, and excellent anti-aging effects. Instead of previously utilized collagen peptides derived from pork and fish, collagen peptides from *Asterina pectinifera* starfish with a lower molecular weight were encapsulated into elastic liposomes with high efficiency to overcome the low absorption of collagen peptides into the skin. Ultimately, we demonstrated their superior anti-aging efficacy by measuring the reduction of MMP expression in photoaged cells. We expect that starfish collagen peptide-loaded elastic nanoliposomes could be widely used as a practical cosmetic ingredient.

Conflict of interest

The authors declare that there is no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

This work was performed by the collaboration with Starstech Co. partly supported by the Technology Development Program

(S2788590) funded by the Ministry of SMEs and Startups (MSS, Korea). The authors thank members of the Applied Mechanobiology Group (AMG) at Korea University and Stars' Tech for constructive discussion of marine biotechnology. D.K. was supported by National Research Foundation of Korea (NRF-2019R1A2C2004437) and the MSIT (Ministry of Science and ICT), Korea, under the ICT Creative Consilience program (IITP-2020-0-01819) supervised by the IITP (Institute for Information & communications Technology Planning & Evaluation). D.K. and S.H. appreciate the financial support from KU-KIST Graduate School of Converging Science and Technology Program.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiec.2021.03.039.

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