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Synthesis of lipoic acid–peptide conjugates and their effect on collagen and melanogenesis

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ABSTRACT

We report new examples of lipoic acid (LA)–peptide conjugates, their potential as codrugs having anti-melanogenic and anti-aging properties was evaluated. These multifunctional molecules were prepared by linking lipophilic moiety (LA) to the pentapeptide KTTKS. The inhibitory effect of LA–peptide conjugates on melanin synthesis and tyrosinase activity is stronger than that of LA or the pentapeptide alone. Importantly, the conjugates display no cytotoxicity at a high concentration. LA–KTTKS and LA–PEG–KTTKS also inhibit UV-induced matrix metalloproteinase-1 expression up to 49.5% and 69.5% at 0.5 mM, respectively. LA–peptide conjugates stimulate collagen biosynthesis in fibroblasts more efficiently than their parent molecules do. These data suggest that LA–peptide conjugates may have cosmeceutical application as anti-melanogenic and anti-aging agents.

1. Introduction

The extracellular matrix (ECM) is the structural backbone of many tissues, especially the skin, and represents the main target for dermatology and cosmetic medicine applications. ECM proteins, including collagen and collagen fibers, play a pivotal role in cellular migration, proliferation, and gene regulation during wound healing. Collagen is the most abundant connective tissue in humans, and collagen fibers provide the tensile strength and resiliency that allows organs such as the skin to form organized structures [1,2]. The reduction of ECM components such as collagen loss or damage and excess accumulation of glycosaminoglycans (GAGs) leads to wrinkles that accompany aging [3]. These characteristics have led researchers to focus on the importance of collagen in the aging process and numerous products and chemical compounds specifically to stimulate ECM biosynthesis to improve the condition of the aging skin. In particular, some oligopeptides perform important functions in the human body and in the skin as bioactive messengers such as hormones, neurotransmitters, or neuromodulators, and some cosmeceutical peptides have been developed to improve skin physiology by acting as anti-aging or moisturizing agents in therapeutic and cosmetic medicine. For example, Val-Gly-Val-Ala-Pro-Gly (VGVAPG) hexapeptide stimulates human dermal skin fibroblast production while simultaneously downregulating elastin expression [4,5]. The Tyr-Tyr-Arg-Ala-Asp-Asp-Ala (YYRADDA) peptide sequence inhibits procollagen-C-proteinase, which cleaves C-propeptide from type I procollagen, thus leading to decreased collagen breakdown [6]. In addition, Lys-Thr-Thr-Ly-Ser (KTTKS), Glu-Glu-Met-Glu-Arg-Arg (EEMQRR), and the tripeptide Gly-His-Lys (GHK) were designed on the basis of the amine-acid sequences present in the proform of type-I collagen, the major form of collagen in human skin. These peptides stimulate new collagen production by fibroblasts [7–9].

The skin provides a physical and chemical barrier against the harmful effects of the external environment; typical peptides have relatively low *in vivo* bioavailability due to enzymatic digestion and other endogenous factors, including a circulating half-life measured in hours or more likely minutes. Therefore, numerous vehicles and penetration enhancers have been synthesized to increase safety, stability, and transdermal delivery of peptides. These include substituting D-amino acids for L-amino acids to reduce susceptibility to certain proteases, fusing them to albumin to improve half-life, and covalent modification by polymer conjugation to reduce enzymatic digestion, kidney clearance, and immunogenicity [10–

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12]. The most widely promoted peptide for topical cosmeceutical application is KTTKS which is comprised of five amino acid residues (hence 'pentapeptide'), that possesses the ability to enhance dermal remodeling by triggering cellular processes, such as increasing ECM production [3]. However, there are several issues to consider when formulating KTTKS as topical ingredients, particularly their passive permeation and penetration potential across the skin. One of the strategies available to improve topical delivery is to use a chemical penetration enhancer such as fatty acids [13,14], which has been applied to KTTKS as a means of improving its delivery across the epidermis, involving the attachment of a lipophilic group (palmitic acid) to lysine through the formation of an amide bond to form *N*-palmitoyl-KTTKS, a major ingredient of Matrixyl [15]. Recently, a novel compound based on KTTKS and ascorbic acid was developed which is known as stabilized ascorbyl pentapeptide; it is better able to stimulate *in vitro* human fibroblast cells to produce more collagen than its parent compounds alone [16]. Chemical modification with polyethylene glycol (PEG) can also improve drug performance through chemical and biological stability, as well as drug penetration in hygroscopically manipulated skin [17].

α-Lipoic acid (LA) is a sulfur-containing fatty acid, exerts several biological effects. It is not only restricted to its antioxidant properties and even dopaquinone trapping [18–20], but also extends to the modulation of melanogenic enzyme expression [21]. Therefore, it is a possible candidate for adjuvant melanoma therapy. The design and synthesis of hybrid molecules encompassing two pharmacophores on the same scaffold is a well-established approach to the synthesis of more potent drugs with dual activity. Using this approach, LA has been widely used for the synthesis of various conjugates, possessing multifunctional activity [22–24].

In this study, we proposed the synthesis of novel LA–peptide conjugates (**1**–**2**) in which pentapeptide is linked to a lipophilic moiety (LA). We have described the synthetic progress of LA–peptide conjugates (**1**–**2**) and have compared its biological activity with that of the original activity of the pentapeptide. We showed that LA–peptide conjugates significantly inhibit melanin synthesis better than parent molecule. Furthermore, we investigated its biological activity in human dermal fibroblasts (HDFs), including inhibitory effect on matrix metalloproteinase-1 expression and stimulating effect in collagen biosynthesis. Our results indicate that LA–peptide conjugates may be strong candidates for use as anti-melanogenic and anti-aging agents.

2. Results and discussion

2.1. Chemistry

The general strategy for synthesis of the LA–peptide conjugates (**1**–**2**) is shown in Scheme 1 and 2. There are three parts of synthesis in conjugates **1** (LA–PEG–KTTKS). The first part is the synthesis of water-soluble PEGylated LA ester derivative **4** (LA–PEG). The second part is peptide synthesis in solid phase chemistry. And third part is the conjugation of LA–PEG and pentapeptide. In the case of synthesis of conjugates **2** (LA–KTTKS), the first part is pentapeptide synthesis, and second part is the conjugation of LA and pentapeptide.

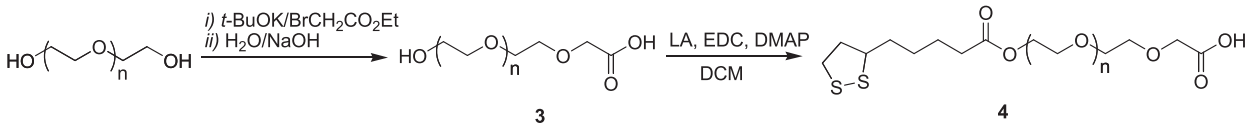
The synthesis of LA–PEG was accomplished using previously developed methodology (Scheme 1) [25]. The heterobifunctional α-hydroxyl-ω-carboxyl PEG (HO–PEG–COOH) was obtained by a simple procedure that starts from commercially available homobifunctional PEG, and involves one chemical and an ion exchange chromatography step. The heterobifunctional PEG has one terminal hydroxyl group available for conjugation with LA, thereby suppressing the formation of byproducts and to provide a carboxyl functional group for conjugation to the pentapeptide moiety.

The linear pentapeptide KTTKS was synthesized by solid-phase peptide synthesis (SPPS) using the 2-chlorotrityl chloride resin preloaded with fluorenylmethyloxycarbonyl-serine (Fmoc-serine). Fmoc was used to temporarily protect the N-terminal amino groups, and *N*-*tert*-butyloxycarbonyl (Boc) and *tert*-butyl (*t*-Bu) were used for side-chain protection. Couplings of protected amino acids were carried out with a solution of HBTU/HOBt reagents, and 20% piperidine/dimethylformamide (DMF) solution was used to remove the Fmoc group. Then, the resin-bound peptide was washed extensively with dichloromethane (DCM) and DMF, and coupled to LA or LA–PEG using *N*,*N*′-diisopropylcarbodiimide (DIC) and HOBt. The resulting LA–peptide conjugates were incubated in a standard cleavage cocktail to remove the peptide side-chain protection groups and induce their cleavage from the resin (Scheme 2).

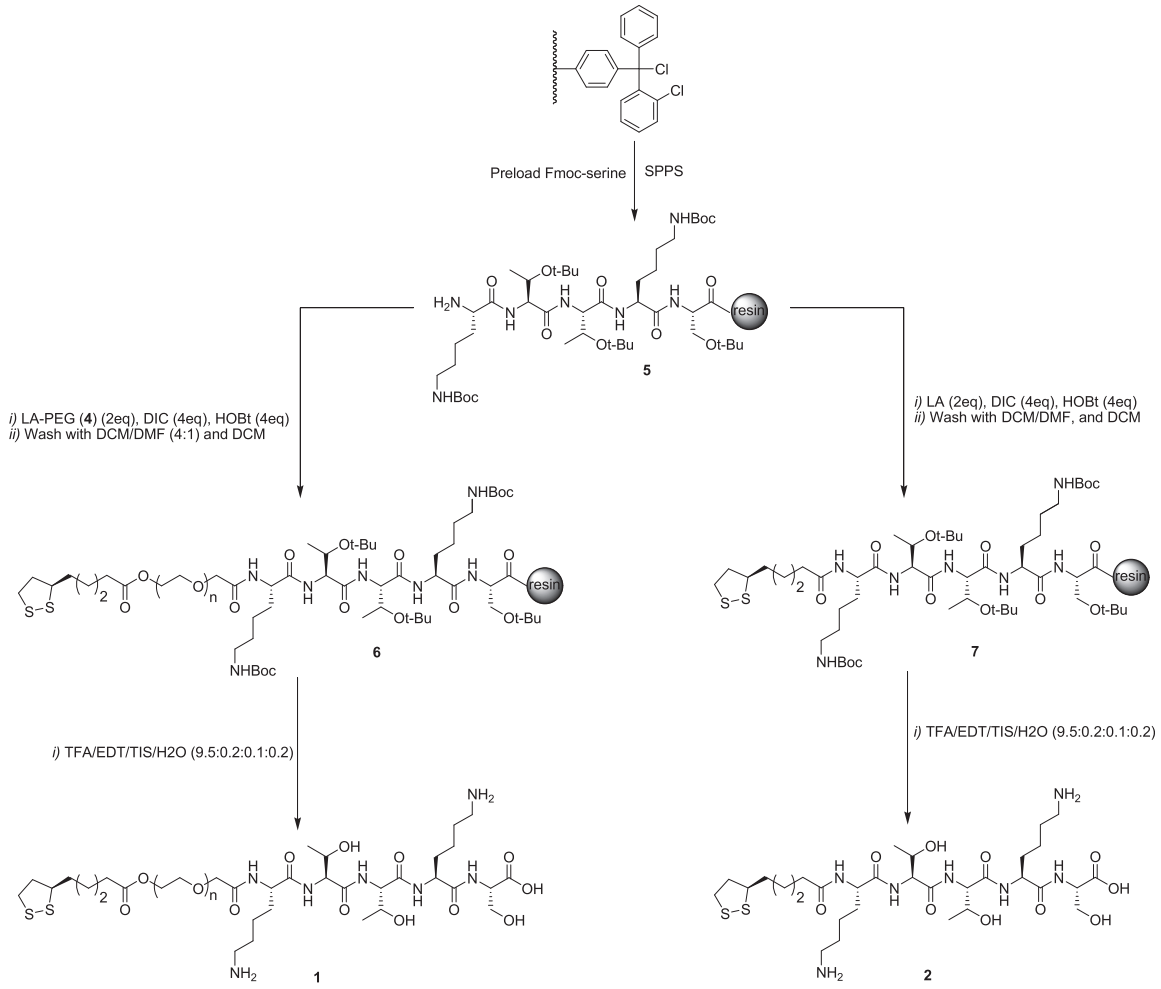
Characterization of the compounds was accomplished by NMR spectroscopic technique. The NMR data of LA–PEG (**4**) indicate that when appending LA to PEG, the resulting spectrum is essentially a composite of the two starting materials. The main contribution from the PEG moiety appears as a large broad multiplet at 3.5–3.7 ppm with an additional two-proton triplet at 4.2 ppm and LA moiety appears at 1.4–3.2 ppm. Since the LA–PEG derivative was thoroughly washed with ether for several times, the NMR result suggests that LA has successfully been appended to the PEG through the esterification reaction. The ^{1}H NMR spectrum of conjugate **1** was similar to that of conjugate **2** except the large broad multiplet at 3.5–3.7 ppm integrated for PEG backbone as discussed previously for LA–PEG (**4**). It is these characteristic resonances that lead us to unambiguously identify the compounds prepared in this investigation. The purities of LA–peptide conjugates (**1**–**2**) were shown in Fig. S1–S2 of the Supplementary data.

2.2. Biological evaluation

The water solubility of LA–peptide conjugates (**1**–**2**) was more than 100 mg/mL. The cytotoxicity of LA–peptide conjugates was monitored by evaluating the effects on cell viability. The maximum non-toxic concentration for B16F10 melanoma cell viability, which was the highest dose tested that did not reduction in viability of 100% of B16F10 melanoma cells after 48 h of incubation at 37 °C, is reported in Table 1. The analysis of the values of the maximum non-toxic concentration for cell viability indicated that LA–peptide conjugates (**1**–**2**) were less toxic for B16F10 melanoma cells than LA. LA exhibited a significant cytotoxic effect on cell viability, down to 0.1 mM, whereas a great variability has been observed in LA–peptide conjugates (**1**–**2**). The dose-dependent effect of LA–peptide conjugates on the cell count of human dermal fibroblasts (HDFs) was investigated. The cells were incubated with 0.5, 2.5, and 5 mM of samples for 48 h. As shown in Fig. 1, LA–peptide conjugates stimulated the fibroblast proliferation activity, and the number of cells was increased maximally of 24.5% for LA–KTTKS and 13.3% for LA–PEG–KTTKS at 0.5 mM, respectively. The



Scheme 1. Synthesis of a novel water-soluble PEGylated lipoic acid ester.



Scheme 2. Reaction conditions for the preparation of the LA–peptide conjugates, followed by deprotection and cleavage from the solid support.

maximum proliferation effects were 12.9% for LA–PEG and 13.4% for KTTKS at 5 mM, respectively. LA did not affect cell proliferation at low concentration (0.1 mM) and to be toxic to cells in culture at high concentrations.

To assess the ability of LA–peptide conjugates to reduce melanin synthesis, we examined the inhibitory effects of LA–peptide conjugates versus parent molecules on the activity of tyrosinase in B16F10 melanoma cells. As shown in Fig. 2a, tyrosinase activity was strongly influenced by LA–peptide conjugates; all LA–peptide conjugates had stronger inhibitory effects on tyrosinase than KTTKS or LA alone did. LA–PEG, LA–KTTKS, and LA–PEG–KTTKS reduced tyrosinase activity up to 92.7%, 60.2%, and 78.4% at 2.5 mM, respectively. We also measured the levels of melanin in the B16F10 melanoma cells cultured with LA–peptide conjugates for 72 h, and compared the results with that of the parent molecule KTTKS.

Table 1
The maximum non-toxic concentration of LA, LA–PEG, KTTKS and LA–peptide conjugates (**1**–**2**).

Compound	Max. non-toxic conc. (mM)[a]
LA	0.1
LA–PEG	5
LA–PEG–KTTKS	2
LA–KTTKS	1
KTTKS	5

[a] The maximum non-toxic concentration was the highest dose tested that did not reduction in viability of 100% of B16F10 melanoma cells after 48 h of incubation at 37 °C.

Conjugates **1** and **2** at 0.25 mM for 72 h yielded 56.3% and 46.1% inhibition, respectively, whereas KTTKS decreased melanin formation by only 7.5% (Fig. 2b). LA–peptide conjugates suppress melanin biosynthesis better than the parent molecules does.

It is also important to identify the original activity of ECM production for the development of oligopeptide cosmetic

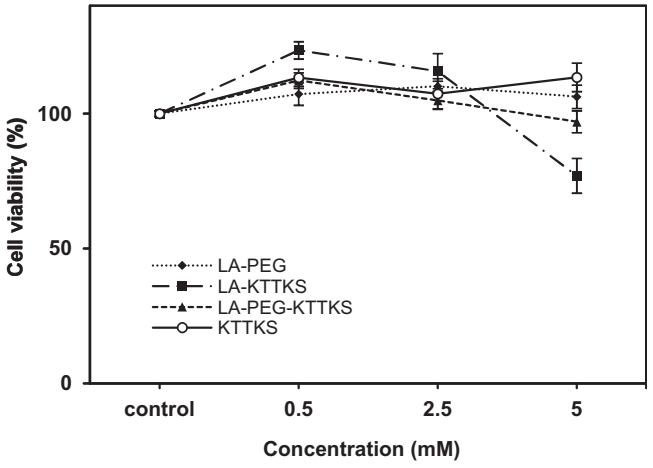


Fig. 1. Relative cell viability of LA–peptide conjugates on human dermal fibroblasts (HDFs) by MTT assay. Cells were treated with various concentrations of samples for 48 h. The results are the mean ± SD of the triplicate measurements.

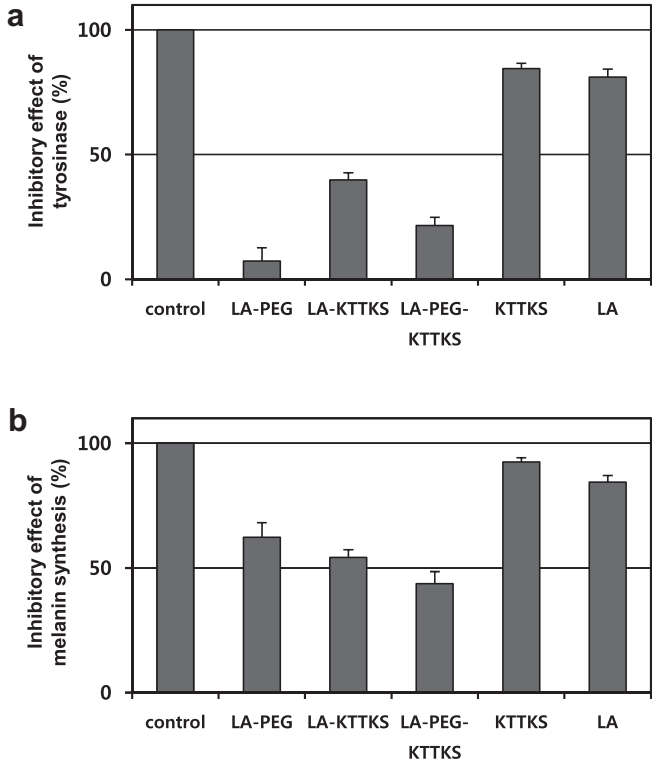


Fig. 2. Inhibitory effect of LA–peptide conjugates on mushroom tyrosinase activity. The values are represented as inhibition (%). (b) Effect of LA–peptide conjugates on melanin synthesis. Total melanin is represented as a percentage of the control. B16 melanoma cells were cultured with or without the samples (LA–PEG, LA–KTTKS, LA–PEG–KTTKS, KTTKS, LA, respectively; 0.2 mM). The values are expressed as means ± SD of the triplicate measurements.

materials. Ultraviolet (UV) irradiation from sun exposure is widely held to be the primary factor in premature skin aging, or photoaging. MMP-1/interstitial collagenase is over-expressed in human fibroblasts within hours of exposure to UV radiation [26,27]. MMP-1 is considered to be a key regulator, and it plays a prominent role in the breakdown of dermal extracellular matrix, namely of collagen types I and III, during the photoaging process, which finally results in collagen deficiency and leads to wrinkling. Inhibitors of the major collagen-degrading enzymes like MMP-1 could be useful anti-aging agents. To examine the effect of LA–peptide conjugates on expression of MMP-1 in HDFs, cultured fibroblasts were exposed to UVA (6.3 J/cm^2) using UV light which did not influence cell viability. After 72 h UV irradiation, MMP-1 protein levels in the culture media were determined by performing the enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 3, treatment with LA–peptide conjugates inhibited UVA-induced MMP-1 expression in a dose-dependent manner, and LA–PEG–KTTKS had the most potent and distinct inhibitory activity against collagenase, inhibiting at least 69.5% MMP-1 expression induced by UVA irradiation at 0.5 mM. LA–PEG, LA–KTTKS, and LA–PEG–KTTKS inhibit UV-induced MMP-1 expression with IC_{50} values of 0.82, 0.5, and 0.16 mM, respectively. The IC_{50} value was the dose of compound inhibiting UV-induced MMP-1 expression by 50%. To evaluate anti-aging activities of LA–peptide conjugates, we also examined the stimulate effect on collagen synthesis of HDFs. Cells treated with LA–peptide conjugates increased collagen production (Fig. 4) than the cells treated with KTTKS did. The results show the ability of conjugates to stimulate collagen synthesis in fibroblasts.

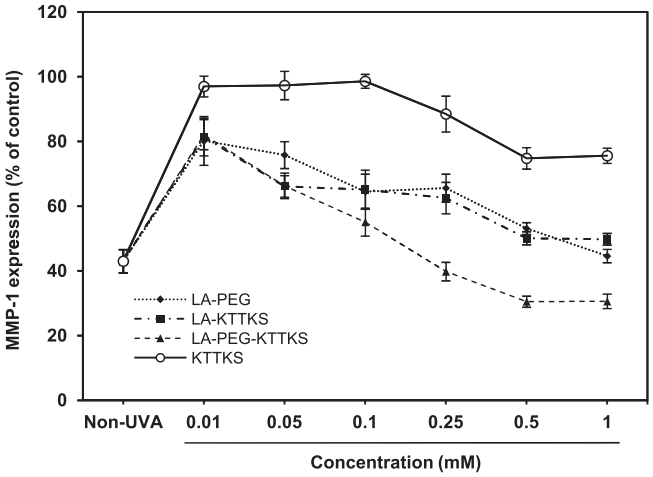


Fig. 3. Inhibitory effect of LA–peptide conjugates on UVA-induced MMP-1 expression. Values are expressed as means ± SD of the triplicate measurements.

3. Conclusions

We designed and synthesized two LA–peptide conjugates with anti-melanogenic and collagen biosynthesis-stimulating effects. Conjugates were non-cytotoxic and inhibited melanin synthesis and tyrosinase activity in B16F10 melanoma cells. Conjugates **1** and **2** inhibited UVA-induced collagenase expression; to pentapeptide did not show such an activity. These results represent possible cosmetic and pharmaceutical applications of the conjugates as effective skin-whitening and anti-aging agents.

4. Experimental protocols

4.1. Chemistry

All Fmoc-protected amino acids, HBTU, HOBt and 2-chlorotrityl chloride resin (0.82 mmol/g) were purchased from GL Biochem of China, whereas all the other chemicals were purchased from Sigma–Aldrich and used without further purification. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ or CD_3OD on a JEOL FT/NMR spectrometer at 500 MHz using tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in ppm (δ). Reactions were routinely monitored by performing thin-layer chromatography (TLC) on silica-gel F254 Merck plates, with detection by iodine vapor or UV lamp.

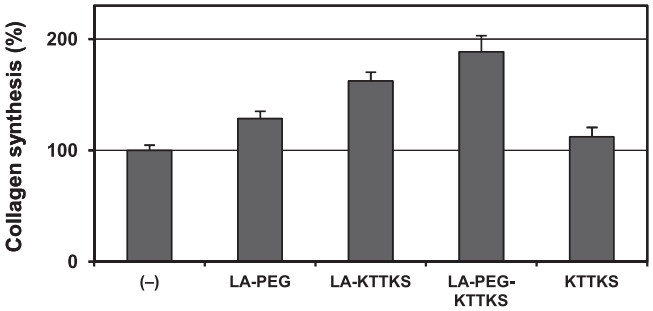


Fig. 4. Effect of LA–peptide conjugates on collagen synthesis. We measured the total collagen content in the cultured HDFs treated with the samples (0.5 mM) by using the enzyme immunoassay kit. The values represent mean ± SD of the triplicate measurements.

4.2. Synthesis of LA–peptide conjugates

4.2.1. Synthesis of PEGylated LA ester derivative (LA–PEG) (**4**)

LA (0.74 g, 3.6 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.69 g, 3.6 mmol), 4-(dimethylamino)pyridine (DMAP) (0.032 g, 0.2 mmol), and dichloromethane (15 mL) were placed in a flask and degassed with a stream of N_2 for 20 min. The reaction mixture was cooled to 0 °C in an ice bath, and a solution of α-hydroxyl-ω-carboxyl PEG (6.2 g, 3 mmol) in dichloromethane (15 mL) was added. The reaction mixture was stirred at 0 °C for 1 h before it was warmed to room temperature and stirred for 24 h. The resulting mixture was washed three times with the same volume of water to remove the side products. The organic phase was dried over anhydrous $MgSO_4$, filtered, precipitated by cold ether and dried *in vacuo* to give compound **4** (LA–PEG) (96%) as a white solid with a slight yellow color. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.21 (t, 2H, J = 5.0 Hz), 4.19 (t, 2H, J = 4.5 Hz), 3.63 (m, ~180H, PEG backbone), 3.08–3.22 (2H, m), 2.72 (br s, 1H), 2.39–2.47 (m, 1H), 2.33 (t, 2H, J = 7.5 Hz), 1.83–1.90 (m, 1H), 1.57–1.70 (m, 4H), 1.36–1.50 (m, 2H).

4.2.2. Synthesis of KTTKS-Resin (**5**)

The linear pentapeptide KTTKS was synthesized on 2-chlorotrityl chloride resin by standard Fmoc methodology. Each coupling was achieved by Fmoc protected amino acid (4 equivalents), HBTU (8 equivalents) and HOBt (8 equivalents) in DMF. Each coupling and deprotection step was monitored by the Kaiser ninhydrin test. After the peptide synthesis, a small portion of the peptide resin was placed in a solid phase reaction vessel and cleaved by a mixture of trifluoroacetic (TFA)/1,2-ethanedithiol (EDT)/triisopropylsilane (TIS)/H_2O (9.5:0.2:0.1:0.2 (v/v)) at room temperature for 3 h to remove the peptide-protection groups and resin. The resin was removed by filtration, and the reaction mixture was precipitated with cold ether, collected by centrifugation, washed with ether, and dried *in vacuo* to give pentapeptide KTTKS.

4.2.3. Synthesis of LA–peptide conjugate **1** (LA–PEG–KTTKS)

Resin-bound peptide **5** (0.8 mmol) was suspended in DCM/DMF (4:1) and LA–PEG **4** (1.6 mmol), DIC (3.2 mmol) and HOBt (3.2 mmol) in DCM/DMF was added to a resin. The mixture was stirred until a negative Kaiser test (about 24 h) at room temperature was observed. The resin was collected by filtration, washed with DMF, DCM, and dried in *vacuo* to yield **6**. The dried product **6** (0.8 mmol) was incubated in a mixture of TFA/EDT/TIS/H_2O (9.5:0.2:0.1:0.2) at room temperature for 3 h to remove the peptide-protection groups and resin. The resin was removed by filtration, and the reaction mixture was precipitated with cold ether, collected by centrifugation, washed with ether, and dried *in vacuo* to give conjugate **1** (880 mg, 49%). ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 4.46–4.43 (m, 4H), 4.35 (d, 2H, J = 3.5 Hz), 4.29–4.25 (m, 2H), 3.93–3.88 (m, 2H), 3.64 (m, ~180H, PEG backbone), 3.30 (m, 4H), 2.97–2.92 (m, 2H), 1.90–1.72 (m, 4H), 1.52–1.21 (m, 8H).

4.2.4. Synthesis of LA–peptide conjugate **2** (LA–KTTKS)

Conjugate **2** was prepared from LA and resin-bound peptide by following the procedure used for conjugate **1** (340 mg, 58%). ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 4.45–4.43 (m, 4H), 4.35–4.24 (m, 2H), 3.94–3.82 (m, 2H), 3.28–3.31 (m, 4H), 2.96–2.43 (m, 2H), 1.96–1.84 (m, 4H), 1.49–1.30 (m, 8H).

4.3. Cell culture

Human dermal fibroblasts (HDFs) or mouse melanoma B16F10 cells (Korean Cell Line Bank) were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 1% penicillin–streptomycin (Gibco BRL, NY, USA), and 0.2 μM α-melanocyte stimulating hormone (α-MSH, Sigma) at 37 °C in a humidified atmosphere containing 5% CO_2.

4.4. Cell viability and proliferation assay

The viability and proliferation of cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were pretreated with samples at various concentrations. After incubation for 48 h, MTT solution (final concentration: 5 μg/mL) was added and cells were incubated at 37 °C for 3 h. Finally, absorbance was measured on a microplate reader at 570 nm to obtain the percentage of viable cells.

4.5. Tyrosinase inhibition assay

Tyrosinase activity with L-DOPA substrate was assayed spectrophotometrically by the method described previously, with slight modification [28]. The reaction mixture consisting of 0.1 mL mushroom tyrosinase solution (625 U mL^{-1}), 0.9 mL 1/15 mM phosphate-buffered saline (PBS, 2.0 mM), and 0.1 mL of sample solution, was mixed and pre-incubated at 25 °C for 10–15 min. Then, a reaction was carried out by adding 0.03% L-DOPA solution. After incubation, the absorbance was monitored at 475 nm in a microplate reader.

4.6. Measurement of melanin content

B16F10 cells were treated with test samples for 72 h. Cells were counted and washed with PBS and collected by centrifugation (3000 rpm, 5 min). After washing the cell pellet with PBS, cells were solubilized with 0.2 mL of 1 N NaOH and l mL of homogenization buffer solution (50 mM sodium phosphate, pH 6.8; 1% Triton X-100; and 2 mM PMSF). Melanin absorbance was monitored at 405 nm, and the quantity of melanin was measured using a standard curve created using synthetic melanin (Sigma).

4.7. Enzyme-linked immunosorbent assay

The expression of MMP-1 was assayed by performing ELISA. HDFs were exposed to UVA irradiation (6.3 J/cm^2) in a UV chamber. Prior to UVA irradiation, the cells were washed twice with PBS. Anti-MMP-1 monoclonal antibody was added at 37 °C and incubated for 60 min. Secondary antibody (anti-mouse IgG conjugated with alkaline phosphatase) in PBS was added to each well and further incubated for 30 min. After washing, the optical density was measured at 405 nm after 30 min.

4.8. Collagen synthesis assay

HDFs were cultured in 96-well plates (5×10^4 cells) with DMEM containing 10% fetal bovine serum for 24 h, which was then replaced with serum-free medium containing the test samples. After incubation for 24 h, the supernatant was collected from each well and type I procollagen was measured by using an enzyme immunoassay (EIA) kit (MK101, Takara, Japan).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.011.

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