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Biological Activity of Multifunctional Oligopeptide Derivatives

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Abstract

The peptide sequences, GHK(Gly-His-Lys) and KTTKS(Lys-Thr-Thr-Lys-Ser), using a collagen stimulator recently were manipulated at N-terminal as a multifunctional peptide derivative with PEG(polyethyleneglycol) linker connected to gallic acid which presents anti-inflammatory activity. The multifunctional peptide derivatives were obtained in a normal peptide preparation method through SPPS(solid phase peptide synthesis) using Fmoc chemistry and a carboxyl group insertion reaction of PEG-3,4,5-triacetoxy benzoate by using potassium *tert*-butoxide and ethyl bromoacetate, which was separated by Sephadex DEAE. It gave a good compromise to a cosmetic application for cell cytotoxicity, anti-wrinkle, and anti-inflammation.

Keywords: Oligopeptide Derivative, SPPS, PEGylation, Cosmetics

1. Introduction

The dictionary meaning of aging is not due to illness or accident but phenomena of biological decay over time,h,[1] but anti-aging business has focused on pursuit of youthful and healthy management according to social changes of an improved life quality and a long lifespan. The main areas of interest in the cosmetic industry are a development of functional components that generate new skin cells or protect skin aging from toxic environment such as UV and hazardous materials, and measurement methods to help the efficacy of skin safety. As effective cosmetic ingredients for anti-aging, vitamins, ceramide, glucan, enzymes, cytokine modulator, peptides, various animal and plant extracts have been investigated in many studies[2-5].

It is a barrier sometimes to use the biological active peptides in pharmaceuticals and cosmetics because of their physicochemical properties depending on solubility, chemical stability, metabolic stability, and cell penetration, however, the peculiar peptide property instead of organic substance gives attraction in that it resembles the function of protein as small portion with selectivity and specificity and gives less toxicity resulted from degradation as amino acid and no accumulation in body. Thus peptide modifications to overcome the disadvantages have been studied with maximizing the profits[6-8].

Modification of peptide in many studies has been developed and utilized to enhance the activity by transforming the N- or C-terminal ends, introducing D-amino acids to peptide sequence, connecting side chains to each other in the peptide chain, forming disulfide bonds, inserting hydrazine moiety, cyclization through ligation methods, connecting cell penetrating peptide to biological active peptide, a method of introducing a peptide that does not exist in nature, glycosylation, or PEGylation[9-18]. PEGylation is not much used for a functional cosmetic ingredients but research related to pharmaceuticals has been progressed in drug development to improve the property of protein structure like erythropoietin conjugate, interferon-alpha conjugate, and epidermal growth factor conjugates[19-21].

Gallic acid (GA) is a kind of polyphenols known to main constituent of tannin which exists in a lot of medicinal herbs[22,23] such as Cornus, Schisandra chinensis, hawthorn, nut gall as well as grocery vegetables. Recently, GA was known that there is a free radical scavenging, anti-allergic, anti-inflammatory, anti-mutagenic, and anticancer activity, it has been developed as a lot of therapeutic agents against diseases using the same.

In this study, a transformation method of PEGylation at N-terminal to connect a complementary biomolecule to the collagen stimulator peptides that are being inten-

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sively studied as cosmetic raw materials is applied. Through this method, the PEG-peptide derivative gave rise to better properties of activity, stability, solubility, and absorption due to the PEG linker which is connected with the complementary biomolecule at the other side, the PEG linker was considered as a pivotal factor for a synergistic effect. Hence, the PEGylated peptide derivatives were synthesized in a high yield, and proposed in possibility and effectiveness as a future functional cosmetic material.

2. Experiments

2.1. Reagents and Instrument

All reagents used in this study were provided from GL Biochem of China and Sigma-Aldrich of USA, and TCI Tokyo, Kogyo. The reagents were used without a further purification. The synthesized substances were confirmed by ^{1}H-NMR and ^{13}C-NMR equipped in JEOL FT/NMR (500 Mz) Spectroscopy.

2.2.1. Synthesis of Protected Peptide Segment (GHK, 1a)

2-Chlorotritylchloride resin(34 mg, 50 μmol) was placed in a reaction vessel, and was shaking for 2-3 minutes after addition of 2 mL methylene chloride (MC). After removing the solvent, the protected 9-fluorenylmethoxycarbonyl(Fmoc) lysine (Fmoc-Lys(boc)-OH; K, 100 μmol) were dissolved in N,N'-dimethyl formamide (DMF) 1.5 mL and N,N'-diisopropylethylamine(DIPEA) 34 μL(200 μmol). The solution was added into the reaction vessel with a 2-chlorotrityl chloride resin, and then the mixture was shaking for 2 to 4 hours. After removing the solvent, the resin was washed by MC five times, and then the loading capacity was measured by taking a small amount of resin. To cap resin with unreacted moiety, MC/methanol/DIPEA(17/2/1) was added into the reaction vessel, and then repeated twice for 15 minutes. After capping resin with unreacted moiety, the reaction vessel was washed three times or more with MC. 20% Piperidine dissolved in DMF to cleave the Fmoc group was added into the reaction vessel twice for 10 minutes, and then the resin was sufficiently washed by MC to remove piperidine after draining the piperidine solution. The protected Fmoc (Fmoc-His-OH; H, 100 μmol), DIPEA 1-hydroxybenzotriazole(HOBt) 14 mg(100 μmol), and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl uroniumhexafluorophosphate(HBTU) 38 mg(100 μmol) dissolved in 1.5 mL DMF was added into the reaction vessel. The mixture was shaking for 2 to 4 hours, and then the solution was drained. After washing at least 3 times with MC, the histidine loading was confirmed by the Kaiser test. The Fmoc-protected glycine (Fmoc-Gly-OH: G, 100 μmol) was loaded as same as above procedure. The protected state of glycine-histidine –lysine(GHK) from the trityl resin was obtained as a yellow solid of 0.4 g by using 20% hexafluoroisopropanol(HFIP) in MC for 1 h.

2.2.2. Synthesis of Protected Peptide Segment (KTTKS, 1b)

The protected peptide segment, KTTK, was synthesized as same as the 1a synthetic method. Fmoc protected serine (Fmoc-Ser(tBu)-OH: S, 100 μmol) as the first amino acid to the reaction was used, and then threonine (Fmoc-Thr (tBu) -OH: T, 100 μmol), threonine (Fmoc-Thr(tBu)-OH: T, 100 μmol), lysine (Fmoc-Lys (boc)-OH: K, 100 μmol) were added sequentially. The protected state of lysine-threonine-threonine-lysine-serine (KTTKS) from the trityl resin was obtained as a yellow solid of 0.4 g by using 20% HFIP in MC for 1 h.

2.3. Synthesis of 3,4,5-triacetoxy Benzoic Acid

Sulfuric acid(32.0 μL) as a catalyst was added into the reaction flask prepared with adding gallic acid (5.00 g, 0.100 mol) and acetic anhydride(17.0 mL, 176 mmol), and then the flask was stirred at room temperature for 20 min. The flask solution was changed to a yellow suspension. To remove excess the acetic anhydride 100 mL distilled water was added into the flask, and then a white solid was precipitated in the solution after stirring for 2-3 h. The white solid was filtered, and then washed with distilled water several times. The dried product, 3,4,5-trimethoxybenzoic acid, was obtained in vacuum desiccator (8.0 g, yield: 93.0%).

2.4. Synthesis and Purification of Partially Oxidized α-Hydroxy-ω-carboxyl Polyethylene Glycol (HO-PEG-COOH)

Polyethylene glycol(PEG) (25.0 g, 0.0125 mol, molecular weight 2,000) in 150 mL toluene was added into a dry three-necked flask equipped with thermometer and Dean stark, and then was stirred at 110°C to remove water. After it cooled to room temperature,

potassium *tert*- butoxide $(CH_3)_3COK$ 1.70 g (0.015 mol) was slowly added, and then stirred for a while. Ethyl bromo acetate 1.75 mL (0.015 mol) was added into the salt flask, and then the mixture was stirred for 12 h. The solution was filtered with a filter paper, and then the solution was concentrated under reduced pressure. Addition of 1 N NaOH 50.0 mL into the solution provided the hydrolyzed product after stirring at an ambient temperature for 40-60 min. The solution was adjusted to pH 3 by adding 6 N HCl solution, and then extracted by MC 500 mL. The MC solution was concentrated by evaporator. The concentrated filtrate was precipitated by addition of cold ether. It was obtained as a white powder mixture of poly ethylene glycol(OH-PEG-OH), α-hydroxy-ω-carboxyl poly ethylene glycol(OH-PEG- COOH), dicarboxylated poly ethylene glycol(HOOC-PEG-COOH). The partially oxidized HO-PEG-COOH was purified by Sephadex DEAE. The product identified by ^1H and ^{13}C NMR was 64% yield. ^1H NMR(DMSO-d_6) : δ 2.0 (1H, s). 3.54 (4H, m), 3.56 (2H, d) 3.70 (2H, m), 4.31(2H, s), 11.0 (1H, s); ^{13}C NMR(DMSO-d_6): δ 61.4(1C, s), 70.0(2C, s), 72.7(1C, s), 173.0(1C, s).

2.5. Synthesis of PEGylated 3,4,5-Triacetoxybenzoic Acid (2)

3,4,5-Trimethoxybenzoic acid(0.863 g, 3.00 mmol) and methylene chloride (MC) 5.00 mL in a dried 50 mL round bottom flask was stirred at room temperature, and quickly followed by addition of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) 0.575 g (3.00 mmol) and 4-dimethylaminopyridine (DMAP) 0.010 g (0.080 mmol) under nitrogen. The α-hydroxy-ω-carboxyl poly ethylene glycol (HO-PEG-COOH) 5.00 g (2.50 mmol) dissolved in methylene chloride 5 mL was added slowly into the mixture solution, and then stirred for 24 h. The excess of 3,4,5-triacetoxybenzoic acid from the product was removed on a separatory funnel containing 20.0 mL of distilled water. A white solid material, 3,4,5-triacetoxybenzoic PEGylated acid [GA(OAc)-PEG] was obtained in 75.0% yield (5.23 g). The product was identified by ^1H and ^{13}C NMR. ^1H NMR(DMSO-d_6) : δ 2.08 (9H, t), 3.54 (4H, s), 3.83 (2H, m), 4.31 (2H, s), 4.42 (2H, s), 7.56 (2H, s); ^{13}C NMR(DMSO-d_6): δ 20.3 (3C, s), 64.3 (1C, s), 70.0 (4C, t), 118.6 (2C, s), 127.8 (1C, s), 144.8 (1C, s), 146.4 (2C, s), 166.0 (1C, s), 169.0 (3C, s), 173.0 (1C, s)

2.6.1. Synthesis of PEGylated GHK Derivative (3a)

PEGylated 3,4,5-triacetoxybenzoic acid [GA(OAc)-PEG-COOH] with a mixture solvent of MC and DMF in a reaction flask was stirring, and followed by *N,N'*-diisopropylcarbodiimide (DIC) and HOBt. The mixture solution was added into a reaction vessel containing GHK-resin. The molar ratio of reactants was used as protected GHK-resin:GA(OAc)-PEG-COOH:DIC:HOBT (1:4:4:4). After passing 12 h reaction period, the reaction was confirmed by the Kaiser test until there is no color change. The product, GA(OAc)-PEG-GHK, was released from the dried resin by a cleavage solution made by trifluoroacetic acid (TFA) / 1,2-ethanedithiol (EDT) / triisopropylsilane (TIS) / H_2O (9.5 / 0.2 / 0.1 / 0.2). The product was obtained as 45.0% yield by a routine peptide cleavage procedure. The product was identified by ^1H and ^{13}C NMR. ^1H NMR(DMSO-d_6): δ 1.29 (2H, s), 1.55 (2H, s), 1.78(2H, s), 2.65 (2H, s), 2.92 (1H, s), 3.54 (4H, s), 3.83 (2H ,s), 4.09 (2H, s), 4.26 (2H ,m), 4.42 (2H, m), 4.46 (1H, m), 4.92 (2H, m), 5.0 (3H, m), 6.83 (2H, s), 6.87 (1H, m), 6.97 (1H, m); ^{13}C NMR(DMSO-d_6): δ 21.1(3C,s), 30.7 (1C, s), 31.8 (1C, t), 42.1 (2C, d), 53.0 (1C, s), 55.1 (1C, s), 127.5 (2C, s), 148.3 (1C, s), 170.8 (1C, s), 171.8 (1C, s), 174.9 (1C, s), 64.3 (1C, s), 69.4 (4C, d), 109.7 (2C, s), 125.5 (1C, s), 140.3 (1C, s), 148.6 (2C, s), 166.0 (1C, s), 169.0(1C, s),173.0 (1C, s).

2.6.2. Synthesis of PEGylated KTTKS Derivative (3b)

It was prepared as same as the 3a procedure. The difference was to use KTTKS-resin instead of GHK-resin, and the structure of obtained product was confirmed by ^1H and ^{13}C NMR.(yield 42.0%) ^1H NMR(DMSO-d_6): δ 1.21 (6H, m), 1.29 (4H, m), 1.55 (4H, m), 1.79 (4H, m), 2.0 (7H, s), 2.56 (4H, m), 3.54 (4H, s), 3.83 (2H, s), 4.24 (2H, m), 4.42 (2H, m), 4.53 (2H, m), 4.61 (2H, m), 5.0 (3H, s), 6.83 (2H, s), 8.0 (5H, s); ^{13}C NMR(DMSO-d_6): δ 18.9 (2C, s), 31.6 (2C, d), 42.1 (2C, s), 53.8 (1C, s), 57.0 (1C, s), 58.7 (2C, s), 60.9 (1C, s), 67.6 (2C, s), 171.1 (4C, s), 174.9 (1C, s), 64.3 (1C, s), 69.4 (4C, d), 109.7 (2C, s), 125.5 (1C, s), 140.3 (1C, s), 148.6 (2C, s), 166.0 (1C, s), 169.0 (1C, s).

2.7. Preparation of Nano-structure

GA-PEG-GHK and GA-PEG-KTTKS was dissolved

as a 0.5% concentration of distilled water respectively, and then calcium chloride was added slowly as half equivalent of the PEGylated peptides. A nano-structure was prepared by stirring with 150 rpm for 10 min. The nano-particle was obtained after centrifugation and dryness.

2.8. Measurement of Biological Activity

2.8.1. Cell Viability

Cell viability test of the synthesized peptide PEGylated derivatives was performed by MTT assay using (2-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) chemical into human keratinocyte cell, HaCaT cell. The cytotoxic degree was expressed as a percentage relative to the absorption intensity of control group using pure water.

2.8.2. Effect of NO Synthesis

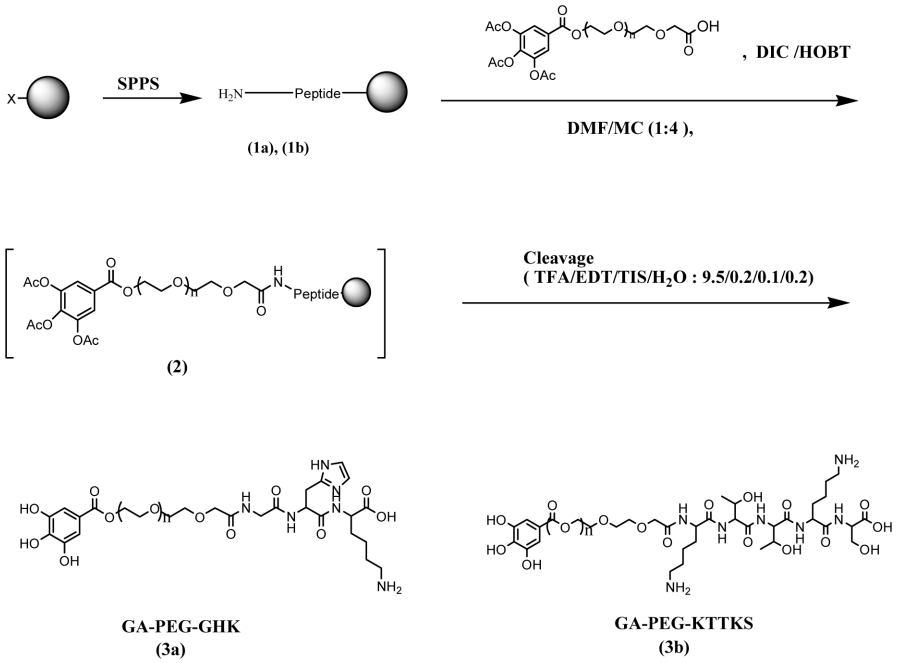
The 1×10^5 cells of RAW264.7 suspended in DMEM medium was inoculated into 24 well plate. Briefly, The peptide derivatives according to concentration was treated after the cells were cultured until cells reached 80% confluence. In order to induce iNOS, LPS (1 μg/mL) and various concentrations of the peptide derivatives were added after 1 h. Nitrite accumulation in the medium was measured at 24 h incubation after the application of LPS. Nitrite was measured in the optical density at 570 nm after adding 100 μL of Griess reagent to 100 μL samples of medium, and shaking the mixture for 10 min.

2.8.3. Effect of Anti-wrinkle

The anti-wrinkle effect was performed on the synthesized PEGylated peptide derivative. Human skin dermal fibroblasts (HDF) were washed twice with PBS, and then treated with various concentration of the PEGylated peptide derivative to assay the effect. The HDF cells was irradiated for 40 min by UVA lamp with energy density of 6.30 J/cm. After irradiating for 72 h, the protein level of MMP-1 in the culture medium was analyzed by ELISA(enzyme-linked immunosorbent assay) method. Test analysis was performed according to the manual of manufacturer.

3. Results and Discussion

In this study, a possibility of new functional cosmetic materials was shown by biological studies, structural analysis, and synthetic approach of oligopeptide derivative with a PEG linker. The oligopeptide derivative is



Scheme 1. Solide phase peptide synthesis of PEGylated peptide derivatives.

composed as two parts, oligopeptide with collagen stimulation activity and GA with anti-oxidative, anti-allergic, anti-cancer, and anti-inflammatory activities.

In the synthetic approach for multifunctional peptide derivative the partial insertion of carboxylic group into PEG was accomplished by using potassium *tert*-butoxide and ethylbromoacetate, and followed by hydrolysis of NaOH. However, the optimal condition was examined to improve the yield of carboxylic group insertion at one end of PEG because temperature control and water removal greatly affected the yield of the product. It was important to increase the yield that Dean Stark apparatus was required to remove a small amount of water, and the reaction temperature has to maintain to below 30.0°C while adding ethylbromoacetate dropwise. Otherwise, in above 30.0°C, all oxidized HOOC-PEG-COOH at both ends was generated more. The product was obtained as a white powder in a yield 64%.

In order to join the GA that represents a biological effect on PEG the 3,4,5-triacetoxybenzoic acid was prepared from gallic acid by using acetic anhydride. The [GA(OAc)-PEG] was synthesized in 75.0% yield from the prepared 3,4,5-triacetoxybenzoic acid by using a coupling reagent, EDC and DMAP.

Like above, the prepared [GA(OAc)-PEG] was coupled at 30°C with GHK or KTTKS peptide sequences attached on resin with anti-wrinkle activity by using a coupling reagent, DIC and HOBt. During the coupling

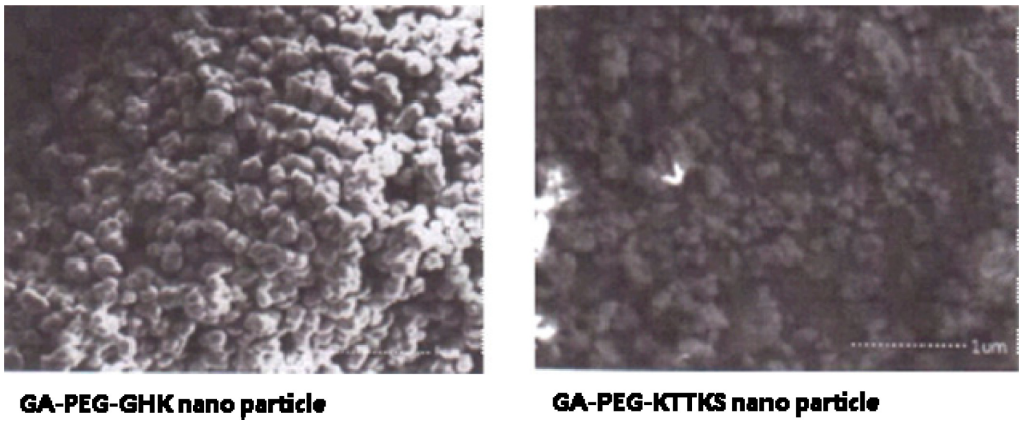


Fig. 1. SEM images of PEGylated peptide nano particles.

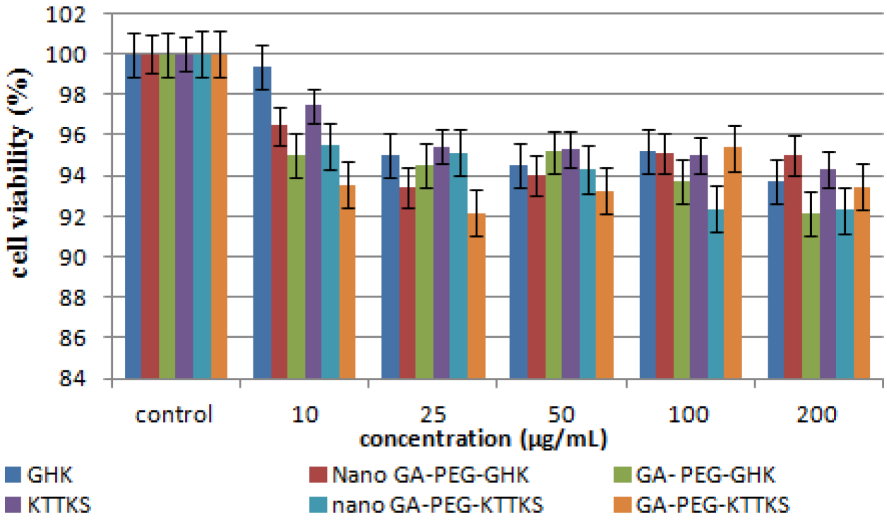


Fig. 2. Effects of peptide derivatives on the cell viability in HaCaT cell. All values are expressed as mean±S.D.

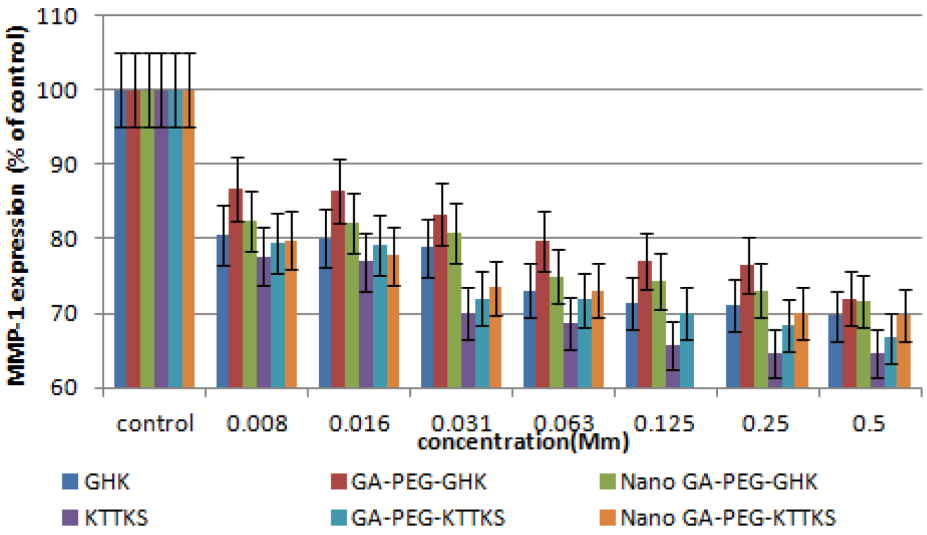


Fig. 3. Inhibitory effect on MMP-1 expression in human fibroblast cell by peptide derivatives. All values are expressed as mean±S.D.

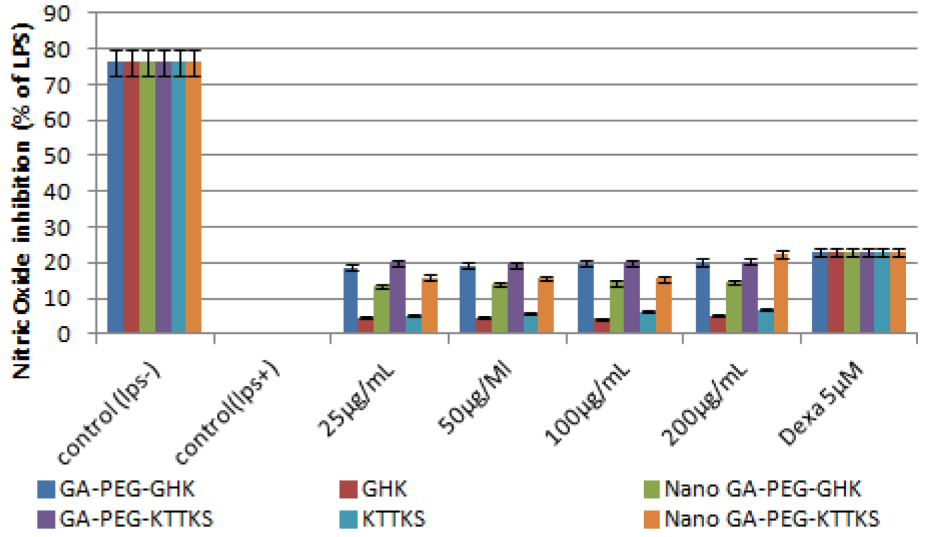


Fig. 4. Effects of peptide derivatives on NO production. All values are expressed as mean±S.D.

time the unreacted free amine moiety was confirmed as blue color by Kaiser test. When the PEGylated peptide derivatives were complete, treatment of the TFA cleavage solution to remove the peptide from the resin removed the side chain protection groups at the same time. The yields were 30-40% after purification.

The SEM pictures of the nano particles from the PEGylated peptide derivative showed like polydispersity like below pictures. The size of fine particles was 100 nm to 150 nm.

Treatment of 10-200 μg/mL into human keratinocyte HaCaT cells did not show a significant toxicity on comparison of the control group.

Generally, compounds presenting an MMP-1 (matrix metalloproteinase-1, collagenase) inhibitory activity are known to prevent skin aging like elasticity loss and skin dripping. In this experiment, the oligopeptides and PEGylated peptide derivatives were compared in MMP-

1 inhibitory activity. The PEGylated peptide derivatives showed the MMP-1 inhibitory activity in a concentration dependent manner, moreover, the activities were slightly less than those of oligopeptides. The result demonstrated that a modification attaching other bioactive molecule should may give a shielding effect to the original oligopeptide activity.

In order to observe the cause of NO reduction when oligopeptide, oligopeptide derivatives, or nano particles of oligopeptide derivative were treated because the phenomena might be occurred in the cell cytotoxicity, the cell viability through the MTT assay was compared in the peptide materials and LPS. It may be a evidence of no cell cytotoxicity that the peptide materials gave a similar cell viability(over 92%) in the range of 10-200 μg/mL concentration. Therefore, the reduced NO synthesis was thought to give rise to the anti-inflammatory effect of peptide materials.

As shown in Fig. 4, dexamethasone as a positive control showed 22.7% inhibition rate, and the nano particles were 16.53-22.33% as similar to the dexamethasone activity. In contrast GHK and KTTKS oligopeptides without the derivative moiety were 5.1%, 6,76%,respectively. The anti-inflammatory effect of PEGylated peptide derivatives seems to be increased due to introduction of Gallic acid. Hence, it is expected that a structural design analogs like PEGylated peptide derivatives giving synergical effect each other can be used as a good cosmetic material without skin problem.

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