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Promotion of cell adhesion by low-molecular-weight hydrogel by Lys based amphiphile

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ABSTRACT

Hydrogels formed by low-molecular hydrogelators have been used as anti-microbial agents and cell-attachment materials. However the biomedical application of low-molecular gelators is slowly progressing compared to the hydrogels formed by polymer hydrogelator that is applied to biomedical application such as tissue engineering and biomedical regions. To obtain a simple molecular model for potent and prospective usage of low-molecular hydrogelators, we designed a Lys-based hydrogelator which was mimic to the poly cationic poly-L-lysine that promotes cells to attach to a plastic plate nonspecifically. The gel-coating led to cause 10-fold cell attachment compared to no-coating well. Also five-time cells were attached to the well compared to the poly-L-lysine coating. From the competitive assay, these hydrogels could interact with cells through electrostatic interaction between positive charge from $-NH_3^+$ in the hydrogelator and negative charge from substances on the cell surface such as glycosaminoglycans. This strong adhesive ability can be useful for the tissue engineering and molecular glue regions using low-molecular hydrogels in the future.

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1. Introduction

Many surfactants and amphiphiles can self-aggregate resulting micelle, lamellae structure followed by forming fiber in a solution. In some cases, this fiber structure leads to form complicated three-dimensional (3D) network so that solvent molecules are taken into the space of the 3D network to form a gel known as one of soft matters [1]. When the organic solvents are taken into the network, that is called as “organogel” and when the water solvents are taken into that, that is called as “hydrogel” [2–4]. Lots of reports about low-molecular gelators prepared by amphiphiles and surfactant-like structure have been reported both forming organogels and hydrogels. These low-molecular gelators are applied to oil absorbents, drug delivery, electronics, and so on [5–7]. Hydrogels formed by low-molecular hydrogelators are used as anti-microbial agents and cell-attachment materials [8–10]. However the biomedical application of low-molecular gelators are slowly progressing compared to the hydrogels formed by polymer hydrogels such as alginate, poly vinyl alcohol that is applied to biomedical application such as tissue engineering [11–14]. The application of low-molecular hydrogelator is limited to its toxicity against human cell, that is, biocompatibility and unstable state of the hydrogel comes from its supramolecular dynamics [10].

To obtain a simple molecular model for potent and prospective leverage of low-molecular hydrogelators to more biomedical regions, we designed a Lys-based hydrogelator which was mimic to the poly cationic poly-L-lysine that promotes cells to attach to a plastic plate nonspecifically through interaction with anionic substances such as glycosaminoglycan on the cell surface.

In this study, we synthesized Lys-based amphiphilic compound H-L-Lys(ε-NH_2)-$NHC_{18}H_{37}\cdot 2TFA$ (**1**) forming hydrogel which was viscous fluid to attach to the plastic 96-well plate easily. And we employed these characteristics to the adhesive matters for cell adhesion that means we tried to attach cells to the plastic plate using the hydrogel formed by compound **1**. From the results, the gel-coating led to cause 10-fold cell attachment compared to the control of no-coating well. These attachment cells were also examined by MTT assay that showed 10,000 to 20,000 attached cells were alive on the well. This hydrogel could attach more cells than other conventional coating materials. It was observed that about five-time cells were attached to the well compared to the poly-L-lysine coating. From the competitive assay, these hydrogels could interact with cells through electrostatic interaction between positive charge from $-NH_3^+$ in compound **1** and negative charged substances on the cell surface such as glycosaminoglycans. This strong adhesive ability is useful for the tissue engineering and various biomedical fields. Our research allows simple Lys-based amphiphile to have a strong ability to attach to cells and to the plastic plate, which might be a good model for biomedical region especially tissue engineering and molecular glue regions using low-molecular hydrogels in the future.

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2. Experimental

2.1. Materials

2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium (HBTU) was purchased from Merck & Co., Inc. (NJ, USA). *N*-hydroxybenzotriazole (HOBt), *tert*-butoxycarbonyl (Boc) protected amino acid, 2,2,2-trifluoroacetic acid (TFA), poly-L-lysine hydrochloride, and sodium poly-L-glutamate were available from Peptide Institute (Osaka, Japan). *N*,*N*-diisopropylethylamine (DIEA) and type I collagen from salmon skin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

2.2. Synthesis of compound

The hydrogelator **1** was synthesized from H-L-Lys(ε-NH_2)-OH·HCl as a starting material. Firstly, Boc-L-Lys(ε-Boc)-OH was synthesized by adding Boc_2O (4.8 g, 22 mmol) to the stirring solution of H-L-Lys (ε-NH_2)-OH·HCl (1.8 g, 10 mmol) in 30 mL of dioxane-water (2:1, v/v) and 30 mL 1 M NaOH. After being stirred at room temperature for 2 h, the solvents were concentrated. The resulting oil was washed with ethyl acetate and dried over anhydrous Na_2SO_4. The transparent oil of Boc-L-Lys(ε-Boc)-OH was obtained after concentrating the ethyl acetate phase (3.4 g, quantitative yield). Boc-L-Lys(ε-Boc)-$NHC_{18}H_{37}$ was synthesized by adding HBTU (3.1 g, 8.4 mmol) and HOBt (1.1 g, 8.4 mmol) to the stirring solution of Boc-L-Lys(ε-Boc)-OH (2.4 g, 7 mmol) and $C_{18}H_{37}NH_2$ (1.8 g, 7 mmol) in 100 mL of *N*, *N*-dimethylformamide (DMF) and 2.4 mL DIEA. After being stirred at 0 °C for 17 h, the solvents were concentrated. The resulting precipitate was washed with water and petroleum ether, and recrystalized from ethanol/water to give Boc-L-Lys(ε-Boc)-$NHC_{18}H_{37}$ as a white solid (2.8 g, 68% yield). H-L-Lys(ε-NH_2)-$NHC_{18}H_{37}$·2TFA (**1**) was obtained by dissolving Boc-L-Lys(ε-Boc)-$NHC_{18}H_{37}$ (600 mg, 1 mmol) in TFA. After being stirred at room temperature for 30 min, the TFA was removed in a stream of nitrogen. The resulting white precipitate was washed with hexane to give the hydrogelator **1** (620 mg, quantitative yield). The compounds were judged to be of >95% purity based on TLC homogeneity and ^{1}H NMR analyses. ^{1}H NMR (300 MHz, $(D_3C)_2S{=}O$) δ 0.85 (t, J = 6.6 Hz, 3H), 1.17–1.32 (m, 32H), 1.42–1.53 (m, 4H), 1.63–1.71 (m, 2H), 2.72 (t, J = 9.0 Hz, 2H), 3.11 (q, J = 6.0 Hz, 2H), 3.66 (d, J = 6.0 Hz, 1H), 7.70 (br s, 2H), 8.08 (d, J = 3.0 Hz, 2H), 8.40 (t, J = 6.0 Hz, 1H); MS (MALDI) m/z calcd for $C_{24}H_{51}N_3O$ (M + H) 398.41, found 398.40.

2.3. Gelation test and microscopic observation

A procedure for gelation testing was as follows: 40 mg H-L-Lys (ε-NH_2)-$NHC_{18}H_{37}$·2TFA was mixed with 1 mL PBS (40 mg/mL) in a vial with a screw cap [3]. The mixture was heated at 60 °C until the solid was dissolved (pH adjusted to 7–8 with 25% NaOH solution). The resulting solution was cooled at 25 °C for 30 min. The gelation was checked visually by observing no running down fluid when inverting the vial. The optical analysis of the hydrogel was performed by using a polarized optical microscope (Leica DMI3000B, Leica Microsystems, Wetzlar, Germany) [15,16]. The hydrogel on a slide glass was squeezed with a cover glass. The hydrogel sample was kept at room temperature for 30 min before observation. The Scanning Electron Microscopy (SEM) measurement was performed on a field emission microscope (JSM6510, JEOL, Tokyo, Japan) with the accelerating voltage of 20 kV. The gel sample was freeze-dried and the fractured specimen was coated with Au [15].

2.4. Cell adhesion assay

Protocol for cell adhesion was designed based on a few of past reports [17–19]. A general procedure for cell adhesion assay was performed following three steps: coating, incubation, and quantitative analysis. Firstly, a procedure for coating was as follows: The viscous solution was prepared by heating the hydrogel containing the hydrogelator **1** (40 mg/mL) at 60 °C. The solutions of type I collagen (0.1 mg/mL), poly-L-lysine hydrochloride (poly(L-Lys)) (40 mg/mL), sodium poly-L-glutamate (poly(L-Glu)) (40 mg/mL), and sodium alginate (4 mg/mL) were prepared by diluting with PBS containing 1% DMSO. One-hundred microliter of each solution was added to 96-well plates and incubated for 1 h at 37 °C. The excessive hydrogel and the solutions were removed by washing with PBS three times, and the coating was completed. Secondly, a procedure for incubation was as follows: Jurkat T cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37 °C under humidified 5% CO_2. One hundred microliters of cell suspension in a culture medium was added to each well at a density of 1.0×10^6 cells/mL. After 5 h of incubation at 37 °C, non-adhered cells were removed by washing with PBS twice. Finally, a procedure for quantitative analysis was as follows: the adhered cells were stained with 0.5% crystal violet solution. After washing excess stain, 0.2% Triton-X solution was added to each well to elute the stain. The absorbance at 595 nm of the eluant was measured by the microplate reader (Bio-Rad model 680XR microplate reader, Bio-Rad Laboratories, Inc., CA, USA). Each assay was conducted in triplicate, and the means and standard derivations were calculated. The optical image of the adhered cells was taken by using an optical microscope (Nikon Eclipse TE2000-U, Nikon Instruments, NY, USA).

2.5. Cell viability assay

Cell viability was estimated by MTT assay. Instead of staining with crystal violet, 100 μl of culture medium and 20 μl of MTT solution dissolved in PBS (5 mg/mL) were added to the well. After incubation for 4 h at 37 °C, the supernatant solutions were removed carefully. To elute formazan, 200 μl of DMSO was added to the well. The absorbance at 595 nm of the eluant was measured by the microplate reader. Each assay was conducted in triplicate, and the means and standard derivations were calculated.

2.6. Competitive assay

A competitive assay was a similar cell adhesion assay in the presence of poly-L-lysine hydrochloride or sodium poly-L-glutamate solution as the inhibitors. After the 96-well plate was coated with the hydrogel, 50 μL of poly-L-lysine hydrochloride or sodium poly-L-glutamate solution (1 μg/mL, 100 μg/mL, and 10 mg/mL) was added to 96-well plates. One hundred microliters of cell suspension in a culture medium was added to each well at a density of 1.0×10^6 cells/mL. After 5 h of incubation at 37 °C, non-adhered cells were removed by washing with PBS twice. To prevent the cationic crystal violet from the stain of poly-L-glutamate attached to the hydrogel, 50 μL of poly-L-lysine hydrochloride solution (10 mg/mL) was added to the plates when incubated with the sodium poly-L-glutamate solution. After 10 min, the squelching mixture was removed. The adhered cells were measured by the crystal violet stain similarly. A percentage of cell adhesion was calculated as follows: [(OD_{595} in the presence of poly-L-lysine hydrochloride or sodium poly-L-glutamate solution / OD_{595} in the absence of poly-L-lysine hydrochloride or sodium poly-L-glutamate solution) × 100]. Each assay was conducted in triplicate, and the means and standard derivations were calculated.

3. Results and discussion

3.1. Hydrogelation ability

The chemical structure of synthesized compound **1** is shown in Fig. 1. To promote gelation, it is important to take a balance between crystallization and solubilization [4]. As a polar group for increasing

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