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Preparation of Photocured Azidophenyl-Fish Gelatin and its Capturing of Human Epidermal Growth Factor on Titanium Plate

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ABSTRACT: Protein immobilization is important in various medical fields that involve the use of metal. Titanium has been often utilized in various medical fields based on its biocompatibility and noncytotoxicity. However, as an unmodified titanium surface is not suitable, dopamine must be coated on the titanium surface. Among gelatin sources, fish gelatin is more suitable in the human body and does not pose serious side effects compared to porcine gelatin. Therefore, a photoreactive azidophenyl group was introduced to fish gelatin to create azidophenyl-fish gelatin (azidophenyl-f.gel). The azidophenyl group was analyzed by ^{1}H-NMR, UV spectroscopy, and gel permeation chromatography. The curing ratio of azidophenyl-f.gel was measured using a micropatterning plate. Epidermal growth factor immobilization ability, cytotoxicity, and cell attachment tests were performed. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: gelatin; azidophenyl-fish gelatin; UV; EGF immobilization; dopamine; natural polymer

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INTRODUCTION

Gelatin, a biopolymer, is used in the food, cosmetic, pharmaceutical, and photographic industries.[1] Gelatin is also used in the medical field for drug delivery via microspheres and implant matrices. Further, gelatin has an advantage as a biomaterial for colloidal drug delivery.[2] The structure of gelatin provides protein binding sites for various selective functions.[3,4] Gelatin is prepared through hydrolysis of collagen. Collagen of animal origin has high antigenicity, whereas gelatin has a lower antigenicity than collagen.[5] Human and animal gelatins have disadvantages compared to that from fish. First, gelatin of human origin is expensive, while gelatin of animal origin can cause religious and social problems. Specifically, the use of gelatin from animal origin is prohibited in Islam, Judaism, and Hinduism.[6] However, it is generally acceptable to use gelatin of fish origin. Second, gelatin of animal origin can cause bovine spongiform encephalopathy (BSE; mad cow disease) and foot and mouth disease.[7,8] Moreover, as fish gelatin is often obtained from fish skin, which is a by-product of the fish processing industry, it is more environmentally friendly than animal gelatin.[9] Among fish gelatin, cold-water fish gelatin is especially useful because it has high solubility in cold water.[10]

Recently, protein immobilization on metal surfaces has received attention. In many applications, titanium is an available key material based on its biocompatibility, non-toxicity, and excellent corrosion resistance.[11,12] Therefore, titanium can be applied as a dental implant, stent, and hip-joint replacement device among its many applications.[13] However, an unmodified titanium surface is not suitable due to osseointegration.[14] And bioinerts like tissue engineering scaffold and implant need surface modification in order to promote biological response such as cell adhesion, cell proliferation, and antibacterial response.[12,15]

Azidophenyl-fish gelatin (azidophenyl-f.gel) was prepared using gelatin which is introduced azide compound. Azido group decomposes to nitrogen gas and nitrene when UV irradiates azide compounds. Nitrene is the most reactive group like carbene and so it involves various reactions. So nitrene of azidophenyl-f.gel can occur easily through cycloaddition reaction, C—H insertion, and N=N bond making reaction with dopamine. Therefore, dopamine coating titanium can be more effectively coated with azidophenyl-f.gel when it is irradiated by UV.

In this study, a titanium surface was coated with dopamine, which is a known neurotransmitter.[16] At pH 8.5, dopamine solution forms a polydopamine film on a titanium surface

through autopolymerization.[17] This method is effective for surface conjugation.

Protein immobilization on a metal surface is used in chemical modification and scaffold encapsulation methods.[18,19] However, these methods have a limitation in that proteins cannot be effectively immobilized, which increases the possibility of chemical damage. Therefore, an effective protein immobilization method must be developed. Azidophenyl-f.gel was changed to film form upon UV irradiation. Photoimmobilization is simple since a light source is used for crosslinking. Proteins can be more stably immobilized using this method. In this study, human epidermal growth factor (EGF) was effectively immobilized on a dopamine-coated titanium surface using azidophenyl-f.gel crosslinked by UV irradiation.

EXPERIMENTAL

Materials

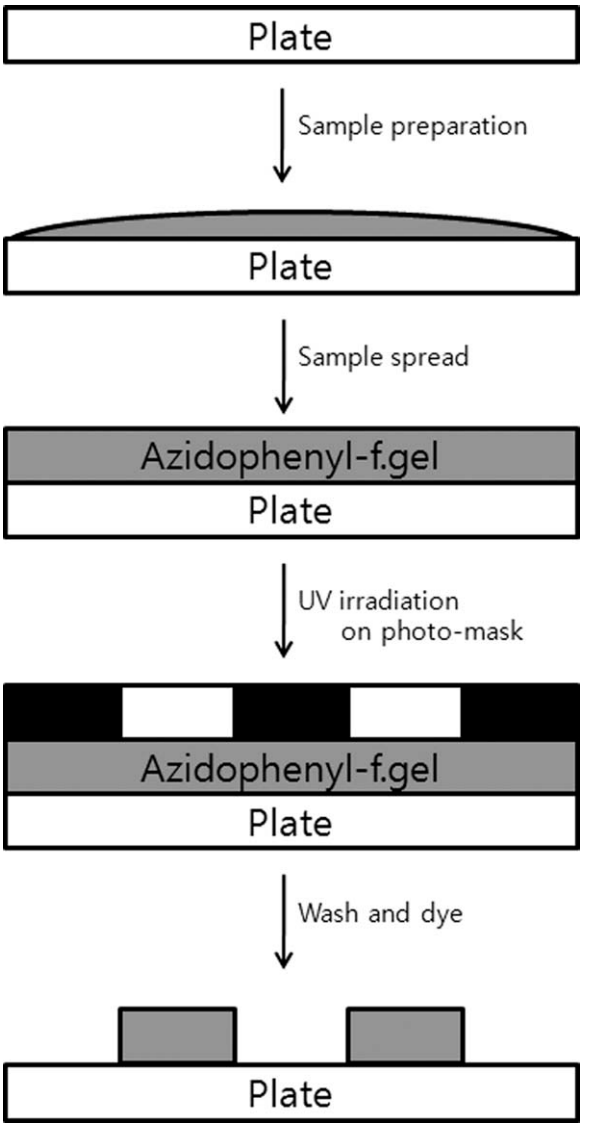
Fish gelatin powder (gelatin from cold water fish skin) was purchased from Sigma-Aldrich (Flanders, NJ). Sodium hydroxide, hydrochloric acid, acetone, and ether were purchased from Duksan Pure Chemical (Seoul, South Korea). *N*-Hydroxysuccinimide, *N, N*-dicyclohexylcarbodiimide, and hexane were purchased from Wako Pure Chem. Ind. (Tokyo, Japan). 1,4-Dioxane was purchased from Samchun Pure Chemical Co. (Seoul, Korea), and 4-azidobenzoic acid was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA), and coomassie protein assay reagent was purchased from Perbio Science (Cheshire, UK). Dopamine hydrochloride (3,4-dihydroxylphenethylamine hydrochloride) was obtained from Wako Pure Chem. Ind., and recombinant human EGF and monoclonal anti-human EGF antibodies were purchased from R & D Systems (Minneapolis, MN). Secondary fluorescein isothiocynate (FITC)-conjugated antibody was obtained from Cappel Research Reagent (Costa Mesa, CA). Dulbecco's Modified Eagle's Medium (DMEM), EDTA-trypsin, penicillin-streptomycin, and phosphate buffered saline (PBS) were purchased from Wako Pure Chem. Ind. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Moregate Inc. (Australia & New Zealand). A cell counting kit was obtained from Wako Pure Chem. Ind., and polymethacryl-PEG was purchased from Aldrich (New Jersey, USA). The UV irradiation machine used was a UV Spot Light Source L5662 from Hamamatsu (Japan, Shizuoka).

Preparation of N-4-(Azido)-Benzoyloxysuccinimide

N-Hydroxysuccinimide (2.1 g) was dissolved in 1,4-dioxane (21 mL), and 4-azidobenzoic acid (3 g) was added to the solution at room temperature. *N, N*-dicyclohexylcarbodiimide (DCC, 3.8 g) dissolved in 1,4-dioxane (15 mL) was added dropwise at 4°C, followed by stirring for 24 h at room temperature. After filtration, the solution was evaporated and dissolved in 1,4-dioxane (50 mL) and ether (50 mL). The brown precipitate, *N*-4-(azido)-benzoyloxysuccinimide (ABS), formed at 4°C after 24 h. The precipitate was washed using ether and then vacuum dried.

Synthesis of Azidophenyl-Fish Gelatin

Fish gelatin powder (2 g) dissolved in milli-Q water was added to 1.5*N* NaOH solution at pH 11 and room temperature. After 4 h, an ABS solution was prepared with ABS (1.7 g) dissolved in 1,4-dioxane (15 mL). The ABS solution was then added to the fish gelatin solution, which was kept at room temperature for 6 h and 60°C for 48 h. The azido-f.gel solution was adjusted to pH 7 using 1*N* HCl solution and dialyzed (cut-off; 1000 Da, Spectrum Laboratories, Rancho Dominguez, California) for 30 h in milli-Q water, which was changed every 3 h. The solvent of the azidophenyl-f.gel solution was evaporated, and the powder was washed four times using acetone and twice using ether. Azidophenyl-f.gel powder was dried in a vacuum drying oven.



Scheme 1. Illustration of fabricating azidophenyl-f.gel micropattern. 5% of azidophenyl-f.gel solution was cast (20 μL) on the plate. And UV light irradiated for 60 s. To remove uncross-linked azidophenyl-f.gel, the plate was washed by milli-Q water for 10 min.

Dopamine Coating on Titanium

A glass plate coated with titanium (Ulvac Inc. pressure, 2×10^{-5} Pa) was washed using hexane, 6*N* HCl solution, and milli-Q water three times for 10 min each in a sonicator. Dopamine was melted in 10 m*M* Tris-buffer solution (2 mg/mL) and adjusted to pH 8.5 using 0.5*N* NaOH solution. A glass plate

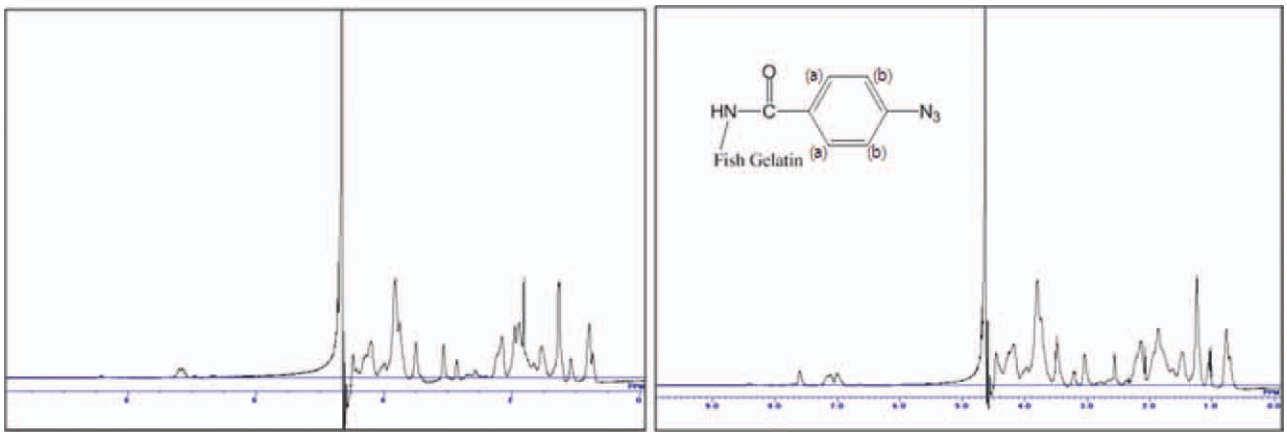


Figure 1. Fish gelatin and azidophenyl-f.gel were analyzed by ^{1}H-NMR spectroscopy. The azidophenyl group of azidophenyl-f.gel demonstrated two specific peaks at 6–8 ppm. These peaks prove that the azidophenyl group was successfully introduced to fish gelatin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

coated with titanium was shaken in dopamine solution for 24 h, followed by washing three times using milli-Q water (10 min) and drying.

Structural Analysis Using ^{1}H-NMR Spectroscopy

Fish gelatin and azidophenyl-f.gel (15 mg) were dissolved in D_2O (600 μL). Each solution was analyzed by ^{1}H-NMR spectroscopy (JEOL 400, 400 MHz, Japan Magnetic Technology, Japan).

UV Spectrum

Azidophenyl-f.gel and fish gelatin were separately diluted in milli-Q water to prepare 0.07% azidophenyl-f.gel and 0.07% fish gelatin solutions. The UV absorbance of each solution was measured between 200 and 400 nm.

Gel Permeation Chromatography

Azidophenyl-f.gel was proven to contain an azidophenyl group based on the fact that the fish gelatin and azidophenyl-f.gel had different molecular weights. Samples were diluted in milli-Q water to sample concentrations of 1 mg/mL for fish gelatin and 3 mg/mL for azidophenyl-f.gel. The solutions were filtered using a millex-syringe-driven filter unit (PIFE, LCR, Millipore Corporation, MA). The measurements were performed using gel permeation chromatography (GPC DP – 8020; TOSHO, Japan). A TSK-GEL α series column model No. H0042 (Tosoh, Tokyo, Japan) was used at a 0.6 mLmin^{-1} velocity.

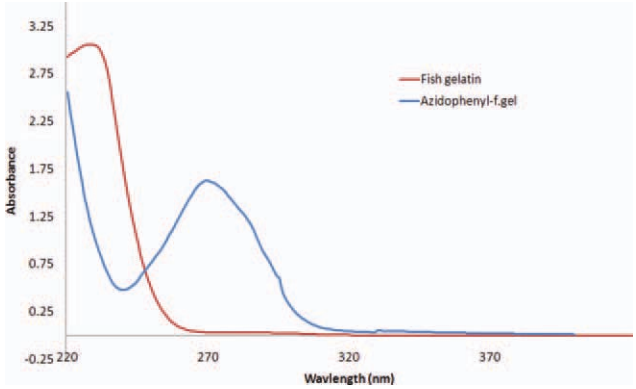


Figure 2. UV absorbance values of fish gelatin and azidophenyl-f.gel were measured at various UV wavelengths of 200–400 nm. The benzene group of azidophenyl-f.gel showed a specific absorbance at 270 nm, whereas fish gelatin did not show a specific azidophenyl group peak. This proves that azidophenyl-f.gel had an azidophenyl group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Curing Test Using UV Irradiation

Empty Thermanox plastic coverslips (NUNC, Rochester, NY) were weighed, and azido-f.gel solution of one of various concentrations (3%, 5%, and 7%) was spread onto each Thermanox plastic coverslip. UV light from a UV lamp (model L5662 UV spot light source, Hamamatsu Photonics, Hamamatsu, Japan) at one of several exposure times (0.5, 1, 1.5, and 2 min) was applied to the coverslips, which were then covered with azidophenyl-f.gel solution and weighed. Thermanox plastic coverslips coated with cured azido-f.gel were washed with milli-Q water for 10 min and then dried at room temperature and weighed.

$$\text{Degree of curing}(\%) = (\text{washed weight(g)}/\text{Initial weight(g)}) \times 100$$

$$\text{Initial weight} = (\text{weight of thermanox plastic coverslips coated with cured azidophenyl-f.gel solution}) - (\text{weight of empty thermanox plastic coverslips})$$

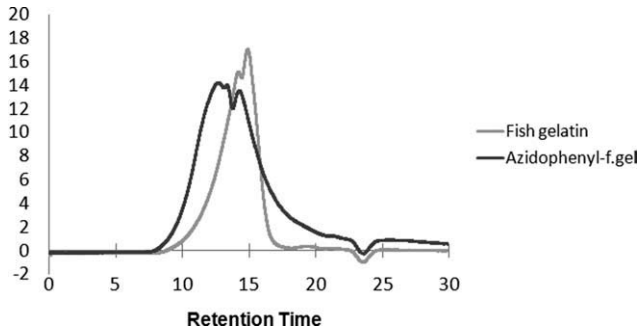


Figure 3. Gel permeation chromatography (GPC) of fish gelatin and azidophenyl-f.gel. Retention time of azidophenyl-f.gel (3 mg/mL) was 12.63, while that of fish gelatin was 14.89. This proves that azidophenyl-f.gel was synthesized successfully from fish gelatin.

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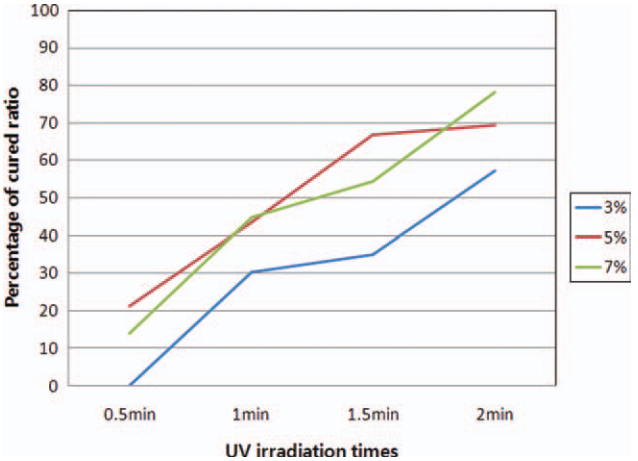

Figure 4. The curing ratio of azidophenyl-f.gel was affected by concentration and UV irradiation time. Azidophenyl-f.gel had high water solubility but changed to a thin film when it was UV irradiated. Its properties were affected by irradiation time and concentration. Various concentrations (3%, 5%, and 7%) and times (0.5, 1, 1.5, and 2 min) showed different percentages of cured f.gel, and a higher irradiation time and concentration yielded higher curing percentage. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Washed weight =(weight of thermanox plastic coverslips coated with cured azidophenyl-f.gel solution after washing) − (weight of empty thermanox plastic coverslips)

Micro-Patterning of Cured Azidophenyl-f.gel Solution

Azidophenyl-f.gel solution (5%) was spread onto Thermanox plastic coverslips. The coverslip was covered with a photomask (Toppan Printing, Tokyo, Japan), and irradiated with UV light for 1 min, followed by washing for 10 min using milli-Q water. Then, the remaining azidophenyl-f.gel patterning was dyed using Coomassie Blue solution and measured using a microscope (Olympus CKX 41, Olympus, Japan) and camera (CCD C5600, Olympus, Japan), as shown in Scheme 1.

EGF Immobilization Using Azidophenyl-f.gel on Dopamine-Coated Metal

Azidophenyl-f.gel (7%) was mixed with EGF (50 μg/mL) and patterning was performed using that sample. Titanium glass coated with dopamine and azidophenyl-f.gel was shaken in blocking solution (30 min) containing the primary antibody (24 h). The metal plate was washed three times using a 0.05% PBS/Tween-20 solution and then shaken with the secondary antibody (FITC) for 1 h. After washing, the plate was analyzed with a fluorescent microscope (Axio Vision, German) and camera (Cool SNAP HQ, Tokyo, Japan).

WST Assay Test

Azidophenyl-f.gel was dissolved in autoclaved pure milli-Q water (3%, 5%, and 7%), after which the solutions were filtered (PIFE, LCR, Millipore corporation, MA). MG-63 osteoblast cells were cultured in a 5% CO_2 incubator using DMEM media containing penicillin-streptomycin (1%) and FBS (5%). Cells were counted at a concentration of 4×10^4 cells/mL, seeded ($n = 5$) in a 96-well plate (DK-4000 Roskilde, Kamstrup Vej 90, Nunc A/S, Denmark), and grown in a 5% CO_2 incubator for 24 h. After cells were attached to the plate, the media was removed and new media was added (98 μL). One of the various concentrations of azidophenyl-f.gel solution (3%, 5%, and 7%) was added (2 μL) to each well of the 96-well plate. After the reactions proceeded for various times (12, 24, 36, and 48 h), media was removed from the 96-well plates, and 10% WST solution (50 μL) was added to each well. The plate was then incubated in a 5% CO_2 incubator for 1 h and analyzed using a microplate reader Model 680 (Bio-Rad, Richmond, CA) at 450 nm. In the formula, $OD_{450(sample)}$ indicates the absorbance of the azidophenyl-f.gel solution-treated plate, and $OD_{450(control)}$ indicates the absorbance of the nontreated plate.

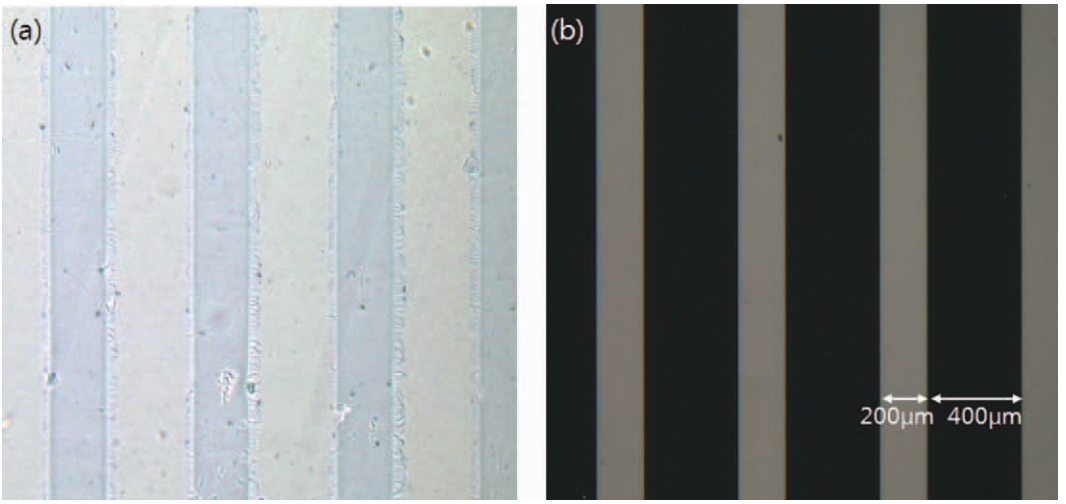

Figure 5. Micropatterning test of azidophenyl-f.gel was performed using a photomask. A micropattern plate had two different sections, white and dark (b). In the photomask, a white section allowed UV light to pass into the azidophenyl-f.gel sample, which was not possible in the dark sections. Only azidophenyl-f.gel that received UV (white section) was cured (a) and could be subsequently dyed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

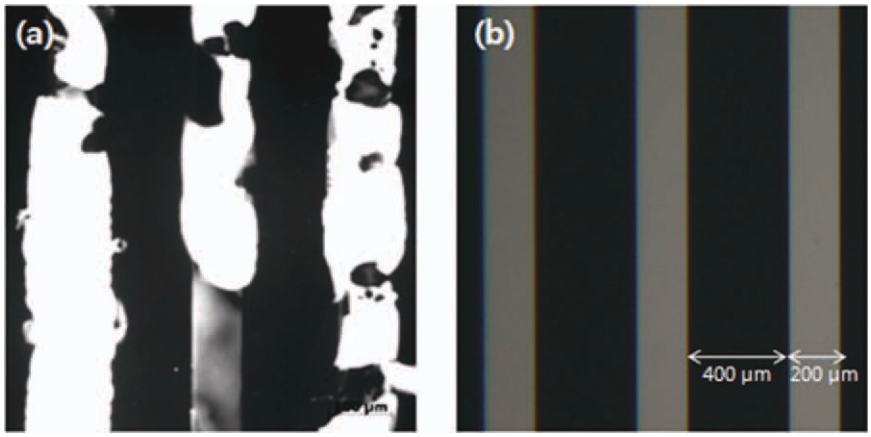

Figure 6. EGF capture test of azidophenyl-f.gel on metal coated with dopamine. White line showed micropatterned azidophenyl-f.gel on the dopamine-coated metal, which captured EGF (a). (b) A micropatterning plate.

$$\text{Cellviability}(\%) = \left(\text{OD}_{450(\text{sample})}/\text{OD}_{450(\text{control})}\right) \times 100$$

Cell Attachment Test

A glass plate coated with titanium was coated to use dopamine-like Dopamine coating on titanium section. Various concentrations of azidophenyl-f.gel (3%, 5%, and 7%) with EGF (50 μg/mL) or without EGF were spread onto the dopamine plate and UV was applied for 90 s, followed by washing for 10 min with milli-Q water. And each azidophenyl-f.gel coating plates were placed in a 12-well plate (DK-4000 Roskilde, Kamstrup Vej 90, Nunc A/S, Denmark). Mg-63 osteoblast cells cultured using DMEM media containing 5% FBS were added at a concentration of 4×10^4 cells/mL to 12-well plate. The plate was then incubated in a 5% CO_2 incubator for 24 h. After 24 h, each titan plate coated with azidophenyl-f.gel with EGF was subjected to microphotography (Olympus CKX 41, Olympus, Japan) using a camera (CCD C5600, Olympus, Japan).

RESULTS AND DISCUSSION

Introduction of an Azidophenyl Group to Fish Gelatin

Azidophenyl-f.gel was synthesized using fish gelatin and so showed similar peaks to fish gelatin in ^{1}H-NMR (Figure 1). The azidophenyl group was substituted for a proton of the fish gelatin NH_2 group. The ^{1}H-NMR spectrum of azidophenyl-f.gel showed a benzene ring of the azidophenyl peaks at 6–8 ppm, whereas other peaks were similar to those in fish gelatin.

Fish gelatin contains diverse amino acids such as phenylalanine and glycine, but only the azidophenyl group has a specific absorbance at a UV wavelength of 270 nm. Therefore, fish gelatin and azidophenyl-f.gel were measured at various UV wavelengths. As shown in Figure 2, fish gelatin did not show any peak at a given wavelength, but azidophenyl-f.gel showed a peak representing an azidophenyl group at 270 nm.

The analysis of the azidophenyl-f.gel showed that an azidophenyl group was introduced to fish gelatin and not simply mixed into the solution. This observation was difficult to prove using only ^{1}H-NMR and UV spectrum, but the GPC result describes molecular weight via sample retention time. Fish gelatin and azidophenyl-f.gel had different molecular sizes, which allowed them to be separated using GPC. Fish gelatin containing an azidophenyl group had a higher molecular size than that of fish gelatin. In Figure 3, the peak of azidophenyl-f.gel (12.63 min) was measured at a shorter retention time than fish gelatin (14.89 min).

Through ^{1}H-NMR and GPC, it was shown that azidophenyl-f.gel was synthesized using fish gelatin.

Curing and Patterning Test of Azidophenyl-f.gel

At various concentrations (3%, 5%, and 7%) and times (0.5 min, 1 min, 1.5 min, and 2 min), azidophenyl-f.gel was tested with regard to curing degree. Azidophenyl-f.gel was affected by UV irradiation time and sample concentration (Figure 4). Azidophenyl-f.gel solution was well-cured at longer irradiation times and higher concentrations. Figure 4 shows that 7% azidophenyl-f.gel solution (2 min) was close to 80% cured, which constituted good conditions to change the azidophenyl-f.gel solution to a film.

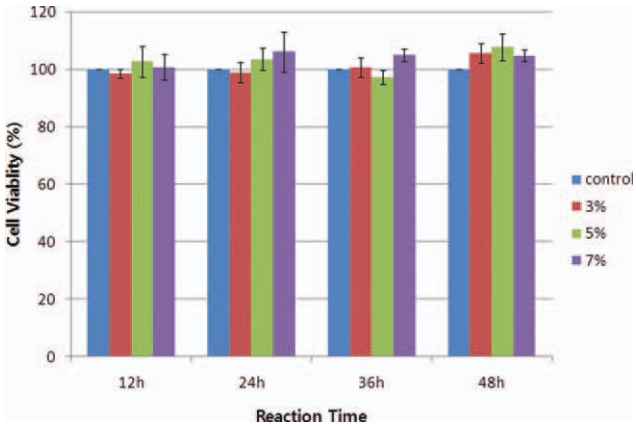

Figure 7. Cytotoxicity was evaluated using the WST test and various concentrations of azidophenyl-f.gel solution (3%, 5%, and 7%). Over 48 h, MG-63 osteoblast cells were cultured in a 5% CO_2 incubator and treated with azidophenyl-f.gel solution. After the specified reaction time, cell viability was measured using the WST test method. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

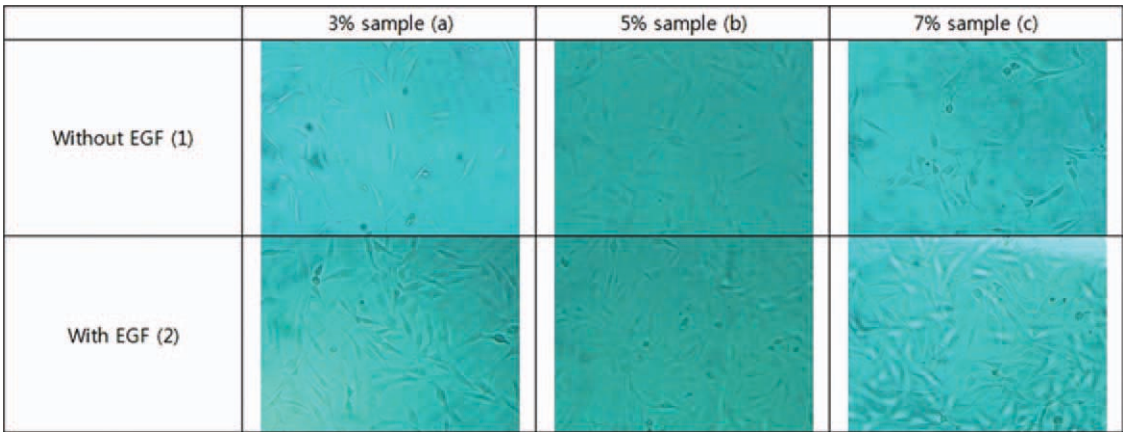

Figure 8. MG-63 osteoblast cell was attached on different concentrations of azidophenyl-f.gel. Azidophenyl-f.gel surface immobilized EGF was increased cell attachment then nonimmobilized EGF surface. And then, as the azidophenyl-f.gel concentration increased, the number of attached cells also increased. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A micropatterning test demonstrated that azidophenyl-f.gel solutions were cured only upon UV irradiation. The micropatterning plate had different sections of dark and white colors [Figure 5(b)]. A dark section blocked UV irradiation from the azidophenyl-f.gel solution, whereas the white section could not protect the sample from UV light. As shown in Figure 5(a), cured azidophenyl-f.gel was present only in the white sections, whereas the areas covered by the dark sections were cleaned by washing. Azidophenyl-f.gel film was dyed using the protein dye, Coomassie Blue. Only the areas within the white sections of the micropatterning plate, which were irradiated by UV and cured, were visualized.

EGF-Capturing Capacity

When osteoblast cells demonstrated growth, EGF was present, and azidophenyl-f.gel was cured by UV irradiation. Thus, azidophenyl-f.gel, which was mixed with EGF, was cured on the titan plates coated with dopamine, and its EGF-capturing capacity was measured using an antibody. The EGF-specific antibody only bound EGF, which was captured by the cured azidophenyl-f.gel. If cured azidophenyl-f.gel did not possess an EGF-capturing capacity, all the EGF would have been washed out. However, EGF was captured by cured azidophenyl-f.gel (Figure 6) and showed the same pattern as that with a photomask. Therefore, cured azidophenyl-f.gel on titan metal-coated dopamine has an EGF-capturing capacity.

Cytotoxicity Test of Azidophenyl-f.gel

For medical field applications, azidophenyl-f.gel possesses noncytotoxic properties. To verify this property, MG-63 osteoblast cells were cultured, and a cytotoxicity test was performed. Azidophenyl-f.gel at concentrations of 3%, 5%, and 7% was utilized with MG-63 osteoblast cells. Cell visibility remained relatively constant among the various concentrations of azidophenyl-f.gel-treated and nontreated cells (Figure 7). Thus, azidophenyl-f.gel solutions did not affect cell viability.

Cell Attachment Test

MG-63 cells should attach on the azidophenyl-f.gel immobilized surface in order to apply stent and implant coating material. So MG-63 cells were seeded on the azidophenyl-f.gel coated surface and observed by microscope. As a result, the more concentrations of azidophenyl-f.gel, the more cell attachment occurs (Figure 8). So Mg-63 cells can attach not only the azidophenyl-f.gel, but also photo immobilized with EGF surface.

CONCLUSIONS

Azidophenyl-f.gel was synthesized using fish gelatin and was shown to have an EGF immobilization capacity on the titan surface coated with dopamine. Further, azidophenyl-f.gel showed noncytotoxicity to MG-63 osteoblast cells. Therefore, the protein immobilization characteristic of azidophenyl-f.gel can be applied in the medical field.

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