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Development of a photocurable gelatin-based gelation material for application to periodontal regeneration

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ABSTRACT

Novel photocurable materials, comprised mixed aqueous solutions of photoreactive gelatin – gelatin partially derivatized with eosin – and polyamine, which is a polymer of *N*,*N*-dimethylaminopropylacrylamide, were designed. The materials had appropriate viscosity for shape formation at 37 °C. When photoirradiated with a clinically used visible light source, the materials were almost completely converted to hydrogels to fix their shape in the desired form within several tenths of a second. Cells that were seeded on the eosin–gelatin-coated surface showed good proliferation. Negligible cytotoxicity of eosin–gelatin was confirmed when culturing was performed in the presence of a large amount of eosin–gelatin. The developed photocurable materials could be converted to a hydrogel with desired physical property change by using a convenient curing procedure of photoirradiation at any desired time and in any shape with minimal cytotoxicity, which was appropriate for the application in periodontal tissue regeneration.

1. Introduction

Recently, two major treatments for periodontal tissue regeneration that are based on different concepts have been performed widely and proved successful. Of these, the first is guided tissue regeneration (GTR) [1–3], in which a physical membrane barrier is placed to create space for periodontal tissue ingrowth; the other is Emdogain® gel treatment using enamel matrix derivatives (EMD) [4–6]. EMD promote the adherence of cementoblasts to the root surface [5]. Periodontal regeneration using Emdogain is evaluated as positive in almost all periodontal patients [6,7]. However, it is difficult for stability spaces for regeneration to be secured due to the behavior of GTR membrane as well as the investment even at treatment of the fluid Emdogain gel to obtain the desirable form. Both of these two regenerative methods are needed for formation of the desired acellular extrinsic fiber cementum instead of the cellular intrinsic fiber cementum, the so-called bone-like tissue. Therefore, ideally the fluidity of the gel should be controlled optimally according to the stage of the treatment.

Gelatin-based hydrogels are useful as biomedical materials [8]. Usually, hydrogelation of the gelatin is performed by a chemical crosslinking reaction using a crosslinking regent such as aldehydes, azide compounds, or carbodiimides. However, such gelation processes require careful washing for the removal of the crosslinking regents, which are potentially cytotoxic in nature due to the release of immobilized biologically active substances. Therefore, an alternative potent hydrogelation method—photo-induced crosslinking of gelatin was developed. In this method, gelatin is derivatized with photo- or radical-sensitive compounds. These photocrosslinkable gelatins have been investigated for use in a number of biomedical applications as a matrix for drug delivery [9,10], tissue adhesive glue, and wound dressing for surgery [11–13], a scaffold material in regeneration medicine, and a coating material for implantable medical devices [14–16]. These materials may be useful, particularly in dentistry and related fields, because of the popularity of treatments using visible light-curing units.

In this study, photoreactive gelatin (eosin–gelatin) was initially synthesized by the derivatization of eosin to gelatin for use as a core compound. Subsequently, the photogelation ability of photocurable materials comprised mixed aqueous solutions of eosin–gelatin and polyamine was evaluated. Finally, the preliminary physical and biological properties of the materials before and after photoirradiation were evaluated and the possibility of the application of the materials in periodontal regeneration is discussed.

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2. Materials and methods

Gelatin (obtained from swine skin, Mw: ca. 9.5×10^4, CP-925) was supplied by Jellice (Sendai, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC), eosin Y (sodium tetrabromofluorescein, $\lambda_{max} = 522$ nm, $\varepsilon = 9.9 \times 10^5$ dm^3 mol^{-1} cm^{-1} in water), 2,2′-azobis (isobutyronitrile) (AIBN), and *N*,*N*-dimethylformamide (DMF) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(*N*,*N*-Dimethylamino)propyl acrylamide (DMAPAAm) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and purified by distillation prior to use.

2.1. Preparation of eosin–gelatin

A typical procedure for the synthesis of eosin–gelatin is as follows. A PBS(−) solution (pH 7.4, 40 mL) of eosin Y (0.3 g) and a PBS(−) solution (200 mL) of WSC (0.4 g) were mixed and stirred at 0 °C for 2 h. A PBS(−) solution (100 mL) of gelatin (5 g) was added to the aqueous solution and stirred at room temperature for 24 h. Using a seamless cellulose tube (Dialysis Membrane, size 36, Viskase Co., Ill, USA), the reaction mixture was dialyzed under flowing water for 3 days and lyophilized using a freeze dryer (FRD-82M, Asahi Techno Glass Co., Chiba, Japan) to yield the eosin-derivatized gelatin. The number of eosin groups derivatized into a gelatin molecule (degree of derivatization: DD) was 2.8 per molecule, as determined by the UV–vis spectra (UV-1700, Shimadzu Co., Kyoto, Japan). Eosin–gelatins with an eosin content ranging from 0.4 to 14.2 were synthesized by changing the reaction ratio of eosin and gelatin.

2.2. Preparation of polyamine

Polyamine, which is poly(*N*,*N*-dimethylaminopropylacrylamide), was synthesized by a conventional radical polymerization technique. A glass tube containing a mixture of *N*,*N*-dimethylaminopropylacrylamide (2 g), AIBN (21 mg), and DMF (6 mL) was sealed under reduced pressure after three freeze-pump-thaw cycles. After shaking at 60 °C for 24 h, the precipitate that was obtained by the addition of ether (500 mL) was separated from the solution by filtration. Reprecipitation was carried out three times in an ethanol–diethyl ether system. After the last precipitate was dried under reduced pressure, polyamine was obtained (1.6 g, 80% yield). The number-average molecular weight (Mn) and polydispersity (Mw/Mn) determined by GPC analysis (HPLC-8020 calibrated with poly(ethylene glycol), column: TSKgel α-3000 and α-5000, Tosoh Co., Tokyo, Japan) were found to be ca. 23,000 and 2.2, respectively.

2.3. Photogelation of the eosin–gelatin solutions

Photogelation of the eosin–gelatin solutions (50 mg) containing eosin–gelatin (20 wt%, weight of the solid content: W_{solid}) and polyamine (5 wt%) was performed on a polystyrene petri dish by photoirradiation, using an 80-W halogen lamp (Tokuso Power Light, Tokuyama Co., Tokyo, Japan) for a predetermined time (40, 60, and 120 s). The gel obtained was immersed in distilled water (ca. 40 mL) at room temperature for 2 h and then weighed (W_{wet}) after the careful removal of excess water. The vacuum-dried gel was weighed (W_{dry}). The gel yield (%) was calculated using the following equation:

$$\text{Gel yield}\,(\%) = \frac{W_{dry}}{W_{solid}} \times 100.$$

The degree of swelling (DS) was calculated as follows:

$$\text{DS} = \frac{W_{wet} - W_{dry}}{W_{dry}}.$$

2.4. Viscosity of the eosin–gelatin solutions

The viscosity of the aqueous eosin–gelatin solutions (0.4 mL) was measured at 37 °C by using a rotating viscometer (TV-22, TOKIMEC, Tokyo, Japan). The concentration range of the eosin–gelatins was set from 5% to 25%. Emdogain gel (Seikagaku Co., Tokyo, Japan) was used as the control.

2.5. The mechanical strength of the photogelated eosin–gelatin

The compressive force of the photogelated eosin–gelatins was determined by irradiating the aqueous solutions of the eosin–gelatins (0.1 mL) for 40 s; this force was measured using an apparatus that was originally designed by Murayama and Omata (College of Engineering, Nihon University). The concentration range of the eosin–gelatins was set from 5% to 25%.

Since the data of the physical measurements were reproducible ($n = 5$, S.D. < 5%), only the average values have been described.

2.6. Cell culture

Mouse vascular endothelial cells (ECs, F2 line) were used as model cells; these were allowed to grow in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen Co., Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), a mixture of penicillin (200 units/mL), and streptomycin (200 mg/mL). The ambient was water-saturated 5% CO_2/95% air, and the temperature was maintained at 37 °C. The culture medium was changed twice on a weekly basis.

2.7. Cell proliferation on the eosin–gelatin coated surfaces

ECs were seeded at a density of 1×10^5 cells/well up to 96 h after the bottom of each well of a 24-multi-well tissue culture plate was coated with the eosin–gelatins (1 wt% aqueous solution). Samples ($n = 3$) were observed by phase-contrast microscopy, and the number of adherent ECs/unit area was counted.

2.8. Cytotoxicity of the eosin–gelatin

ECs were precultured for 72 h in 24-well tissue culture microplates (IWAKI, Chiba, Japan) at an initial density of 1×10^6 cells/well. To each well, 20 wt% of the eosin–gelatin medium (0.2 mL) was added. After 30 min of incubation under standard cell culture conditions at 37 °C in water-saturated 5% CO_2/95% air, the medium that was supplemented with the eosin–gelatin was replaced with PBS(−). Cytotoxicity was evaluated based on the cellular shape of the selected samples ($n = 2$), as examined by phase-contrast microscopy (Nikon, Diaphoto, Tokyo, Japan).

3. Results

Eosin–gelatin was synthesized by derivatization of eosin, which is a visible light reactive xanthene dye, to amino groups of lysine residues in gelatin using a condensation agent (Fig. 1A). By changing the ratio of eosin and gelatin, a range of the eosin content from 0.4 to 14.2 groups per gelatin molecule was obtained. Since the maximum number of amino groups that are available for derivatization was approximately 40 per molecule, the percentage of

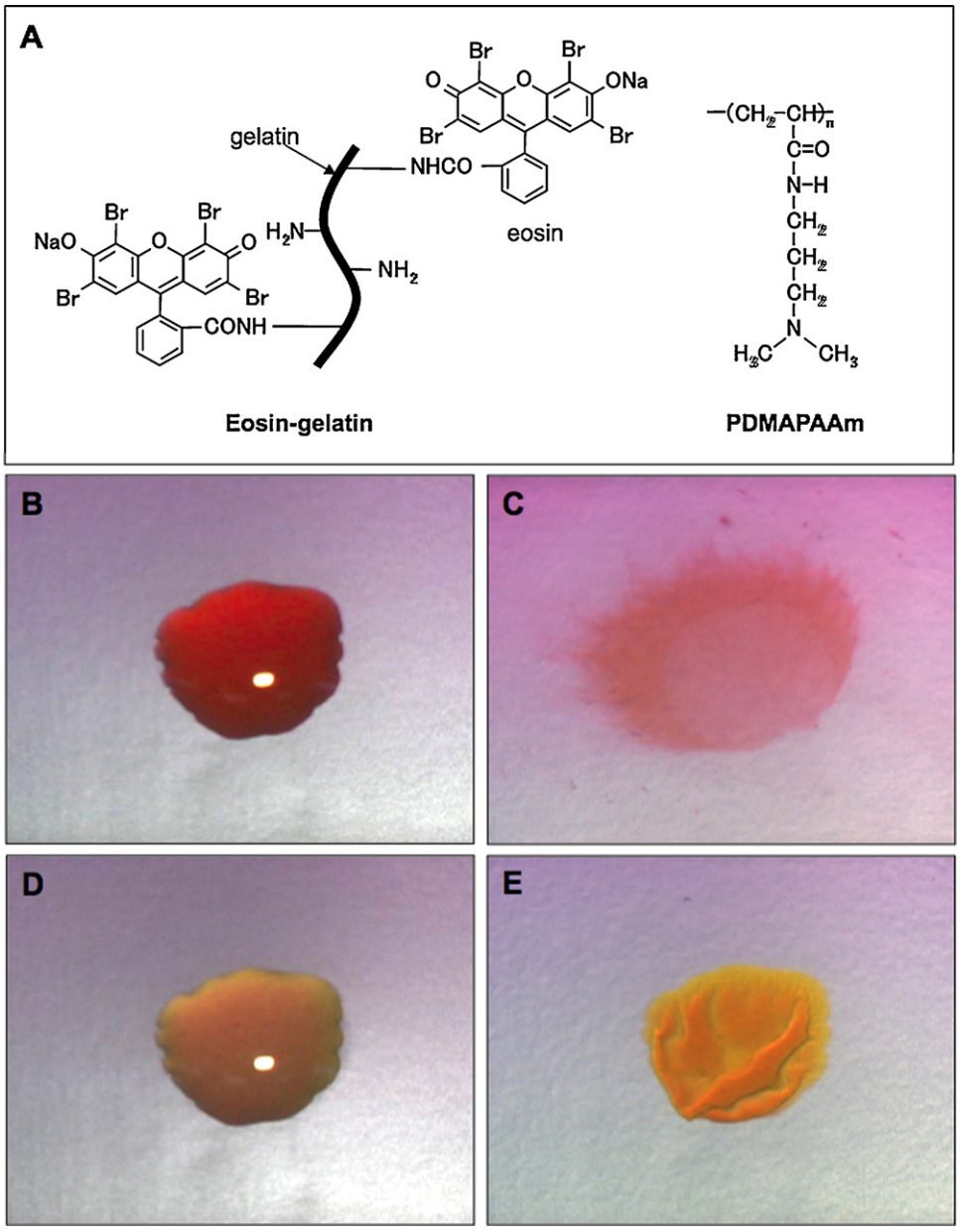

Fig. 1. (A) The chemical structures of eosin–gelatin and polyamine (PDMAPAAm) as a proton donor used for enhancing the photoradical-producing reaction of eosin. Eosin–gelatin was synthesized by derivatization of eosin to amino groups of lysine residues in gelatin. Photos of the photocurable materials, including eosin–gelatins (20 wt%) and PDMAPAAm (5 wt%) before (B) and after (D) 40 s-visible light irradiation. Upon immersion into water the solution was swelled to produce hydrogel by irradiation (E), whereas almost all solution without irradiation was washed out (C).

derivatization ranged from approximately 1% to 35%. On the other hand, polyamine (PDMAPAAm) was synthesized by conventional radical polymerization of *N*,*N*-dimethylaminopropylacrylamide (Fig. 1A). The photocurable materials were prepared by dissolving the eosin–gelatin (5–25%) into distilled water with PDMAPAAm (5%).

The viscosity of the photocurable materials increased with the concentration of the eosin–gelatin (Table 1). At the concentration above 20%, appropriate viscosity for forming the desired shape was obtained under 37 °C.

3.1. Photogelation

Visible light irradiation of the photocurable materials (Fig. 1B) produced hydrogels (Fig. 1D and E) even at 40 s with the color change from red to yellow, indicating the disappearance of eosin. However, no gel was obtained from the aqueous solutions of eosin–gelatins without polyamine or non-derivatized gelatin with polyamine even after irradiation (Fig. 1C). The solutions were immediately dissolved in water at room temperature.

Fig. 2A and B shows the yield of gels produced by a 40-s irradiation to the photocurable materials and the degree of swelling against the eosin content. A high gel yield over approximately 80% was obtained under the eosin content of approximately 3 (Fig. 2A). On the other hand, above the eosin content of approximately 4, the gel yield was markedly reduced to approximately 20%. Content-dependency was also observed in the degree of swelling (Fig. 2B). That is, a relatively high degree of swelling was observed at a low eosin content, under approximately 3, and

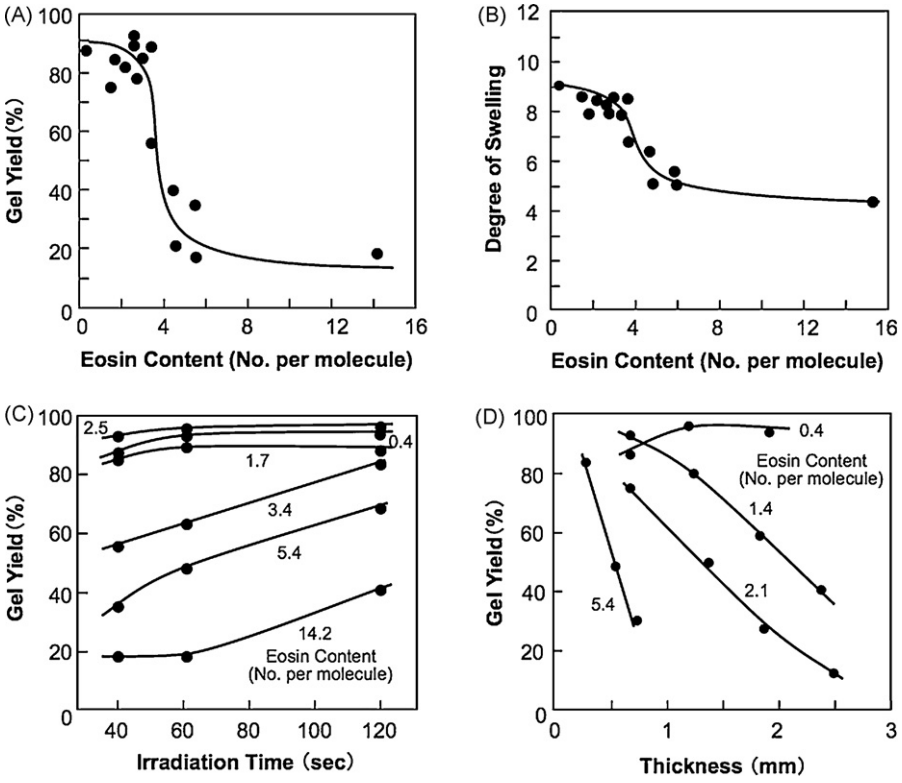

Fig. 2. The gelatin characteristics of the aqueous solutions of the eosin–gelatin. Relationships of the gel yield by 40 s irradiation (A) or the degree of swelling (B) with eosin content in the eosin–gelatin. A highly gel yield over approximately 80% was obtained under an eosin content of approximately 3. That is, a relatively high degree of swelling was observed at a low eosin content, under approximately 3, and at a high eosin content, the degree of swelling was relatively low. Relationships of the gel yield with irradiation time (C) or thickness of the eosin–gelatin solution (D). On prolonging the irradiation time, the gel yields increased irrespective of the eosin content. The thickness of the produced gels hardly depended on the eosin content. At the lowest eosin content of 0.4, the thickest gel was obtained following only 40 s of irradiation.

at a high eosin content, the degree of swelling was relatively low.

On prolonging the irradiation time, the gel yields increased irrespective of eosin content (Fig. 2C). Even at intermediate eosin content ranges from approximately 3 to 6, relatively high gel yields above 60% were obtained after 120 s of irradiation time. However, at the highest eosin content of 14.2, the gel yield continued to be low after a long irradiation time. The thickness of the produced gels also depended on eosin content (Fig. 2D). At the lowest eosin content of 0.4, the thickest gel was obtained; in this case, following only 40 s of irradiation, a 2-mm thick aqueous solution was completely converted to gel, whereas at a higher eosin content, gel yields were markedly decreased with an increase in the thickness of the aqueous solutions.

The compressive force of the hydrogels gradually increased with the gelatin concentration (Table 1). No detectable force was observed in all photocurable materials before irradiation and Emdogain gel. At an eosin–gelatin concentration of above 20%, the produced gels had adequate strength to retain their shape. The gel shape could be thus retained in the desired form.

Table 1
Viscosity of the photocurable materials and compressive force of the hydrogels

Eosin content (no. per molecule)	Concentration of eosin–gelatin (wt%)	Viscosity (mPa s)	Compressive force (kPa)
0.4	5	–	2 ± 0
0.4	10	17 ± 9	24 ± 4
0.4	20	208 ± 39	39 ± 12
0.4	25	415 ± 86	58 ± 28
2.8	5	–	10 ± 3
2.8	10	32 ± 2	20 ± 6
2.8	20	503 ± 89	128 ± 120
2.8	25	2301 ± 408	149 ± 115

Concentration of PDMAPAAm in the photocurable materials was fixed at 5 wt%.
Viscosity was measured in 0.4 mL of the photocurable materials at 37 °C by using a rotating viscometer (TV-22, TOKIMEC).
The compressive force of the hydrogels obtained from 40 s irradiation to 0.1 mL of the photocurable materials was calculated based on the stress–strain relationships measured using an apparatus originally designed by Murayama and Omata.

3.2. Cellular responses to eosin–gelatin

Since the coated eosin–gelatin strongly adsorbed on the surface, proliferation of ECs on the coated surface was evaluated. After 3 h of incubation, the seeded ECs adhered to and elongated on the entire surface (Fig. 3A). This behavior was strikingly similar to that observed on the surface of culture dishes with or without swine skin gelatin coating, both of which were used as controls. On prolonged culture incubation, the cells on the eosin–gelatin-coated surface proliferated with a doubling time of approximately 24 h, similar to the control (Fig. 3B). After 96 h of incubation, the eosin–gelatin-coated surface was completely covered with a confluent monolayer of ECs.

On the other hand, even after addition of the medium containing eosin–gelatin on the confluent monolayer of ECs, negligible changes were noted in cell number and shape (Fig. 3C). However, with the addition of the eosin–gelatin having a high eosin content of 23, almost all the adhered cells were detached. Cellular toxicity was not recognized in the eosin–gelatins having low eosin content of 2.8.

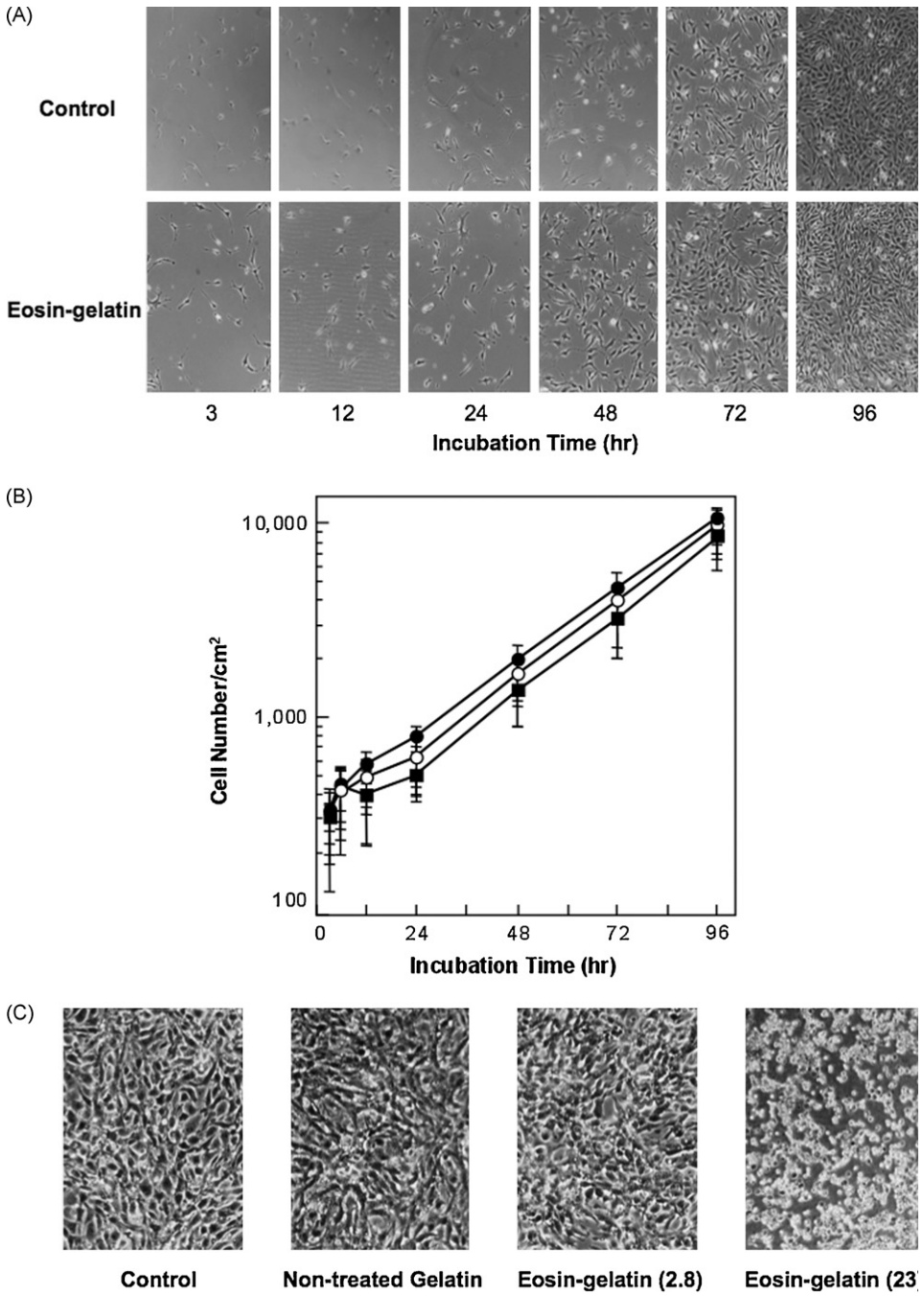

Fig. 3. (A) Phase-contrast micrographs of mouse vascular endothelial cells growth responses on tissue culture dishes with or without (control) coating of the eosin–gelatin (eosin content; 2.8). After 3 h of incubation, the seeded cells adhered and elongated on the both entire surfaces. The cells were firmly adhered and had proliferated on the eosin–gelatin-coated surface similar to the control. (B) Growth curves of mouse vascular endothelial cells adhered on tissue culture (■) dishes precoated with non-treated gelatin from swine skin (●) or the eosin–gelatin (eosin content; 2.8, ○). The cells on the eosin–gelatin-coated surface proliferated with a doubling time of approximately 24 h, similar to the control. (C) Phase-contrast micrographs of mouse vascular endothelial cells confluently adhered on tissue culture dishes after addition of aqueous solutions of the eosin–gelatins (eosin content; 2.8 or 23). Cytotoxicity was not recognized in the eosin–gelatin with low eosin content, whereas at high eosin-content almost all the adhered cells were detached.

4. Discussion

In this study, a novel photo-induced crosslinking system for periodontal regeneration by the application of photoreactive gelatins was attempted. This photocuring method has several advantages such as spatial and temporal control of the photocuring process, curing at room temperature, ease of fashioning, and minimal heat production. The most effective property of photocuring is *in situ* hydrogelation, by which three-dimensional tissues can be easily prepared in the desired shape. For the application of this technique in periodontal regeneration, a fast curing time is required even in thick samples, without any toxic effects exerted by either the materials or the curing process. Therefore, eosin – a xanthene dye which can produce radicals for crosslinking by visible light irradiation with low illumination intensity without any thermal damage – was selected as the photosensitive compound. The eosin was derivatized in the side chains of the gelatin by a water-soluble carbodiimide-induced condensation reaction

between the carboxyl groups in eosin and amino groups in gelatin. The eosin–gelatin obtained had eosin content ranging from 0.4 to 14.2. The eosin content was easily controlled by changing the reaction ratio between eosin and gelatin. In our previous study, when irradiated, an aqueous solution of eosin–gelatin with eosin content of 6.4 could produce hydrogel was reported. However, to achieve a hydrogelation of above 90% of the gel yield, at least 5 min of irradiation time was needed even in the presence of polyamine. Similar results were obtained in this study in the case of a high eosin content over approximately 4, when the gel yield was extremely low. On the other hand, at low eosin content under approximately 3, almost complete hydrogelation occurred.

The mechanism of hydrogelation was explained in our previous report [11,13–20]. In brief, upon irradiation, radicals were generated on both eosin and dimethylamine by proton transfer from amine to eosin. Recombination between the radicals produced covalent bonds. Therefore, gelatins were crosslinked by the covalent bonds between the eosin groups in the gelatin and dimethylamines in the polyamine, resulting in the formation of hydrogel. That is, eosin and amine groups could function as crosslinking points. It was considered that a greater number of crosslinking points resulted in higher gel yields with a lower degree of swelling. Therefore, prior to conducting this study, the possibility that the large number of eosin groups in gelatin and dimethylamines in polyamine could enhance hydrogel formation was considered. In another study, a high gel yield was obtained on increasing the number of dimethylamines in polyamine. On the other hand, increasing the number of eosin groups in gelatin resulted in a markedly reduced gel yield, as indicated in this study. Two reasons may account for this discrepancy. One of these is the absorption of light by eosin groups. Since eosin can produce a radical triggered only by light absorption, a large amount of eosin groups obstruct the transmittance of light energy. Thus, it was considered that photoreaction occurred only at the surface layer at a relatively high density due to the large number of eosin groups. Therefore, a relatively low degree of swelling was obtained even though the gel yield was extremely low. The other reason is the energy transfer between the eosin groups. When the eosin groups are located close to each other, the excitation energy of eosin can easily be transferred to the neighboring eosin in a ground state. The energy transfer inhibits the radical-formation reaction, resulting in a low hydrogelation yield. Therefore, to attain sufficient hydrogelation, eosin–gelatin having eosin content under approximately 3 is appropriate.

The composition of the photocurable material was fixed at 20 wt% of eosin–gelatin and 5 wt% of polyamine, based on the preliminary study findings. In this case, when the eosin–gelatin concentration was increased over 20 wt%, irrespective of eosin content, the viscosity of its aqueous solution increased such that it could not be handled at room temperature. On decreasing the polyamine concentration to less than 5 wt%, no photoreaction of eosin took place in the usual fashion. The obtained photocurable material had appropriate viscosity for shape formation even at room temperature.

The photocurable materials with a relatively high eosin content (approximately 6) have already seen biomedical applications in animal experiments. For example, when the liver of a rat that was injured in laparotomy was irradiated, coating with the photocurable material facilitated complete hemostasis with negligible tissue damage. In histological examinations, inflammatory cells were observed at the local site of the injury, indicating that negligible toxicity of the photocurable material was observed in the animal experiments. In this study, the photocurable materials were directly in contact with the cultured ECs, which were used as model cells in the evaluation of the cytotoxicity test by morphological observations. In the case of a low eosin content, minor shape changes were observed in the ECs, whereas a high eosin content resulted in the detachment of ECs. On the other hand, even on the addition of an aqueous eosin solution, minor changes in cell behavior were observed (data not shown). The toxicity was not a matter of concern in either gelatin or eosin. Therefore, the toxicity of eosin–gelatin might have resulted from the surface activity of eosin–gelatin, which was dependent on the balance of hydrophobic eosin groups and hydrophilic gelatin compounds.

We have developed a photocurable material with the potential for periodontal regeneration. The material before curing had an appropriate viscosity at 37 °C for handling and shape formation and showed negligible cytotoxicity; it could be converted to a hydrogel by using a convenient curing procedure of photoirradiation at any desired time and in any shape. Although this study was the first step toward *in vivo* experimentation, it is confident that the photocurable material has the great potential to be applied in periodontal defects.

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