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Enhancement of visible light-induced gelation of photocurable gelatin by addition of polymeric amine

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Abstract

We evaluated the effect of an electron donor on photogelation of photocurable gelatin, which is gelatin partially derivatized with eosin (eosin–gelatin). As an electron donor, ascorbic acid, 2-(*N*,*N*-dimethylamino)ethyl methacrylate (DMAEMA), and three kinds of radical polymerized amines such as poly(*N*,*N*-dimethylacrylamide-co-2-(*N*,*N*-dimethylamino)ethyl methacrylate) (poly(DMAAm-co-DMAEMA)), poly(*N*,*N*-dimethylacrylamide-co-3-(*N*,*N*-dimethylamino)propyl acrylamide) (poly(DMAAm-co-DMAPAAm)), and poly(3-(*N*,*N*-dimethylamino)propyl acrylamide) (polyDMAPAAm) were examined. Upon photo irradiation at the wavelength ranging from 400 to 520 nm with low illumination intensity (7.7×10^3 lx), no gel was obtained from 20 wt.% of a viscous aqueous solution of the eosin–gelatin even by adding with ascorbic acid. Whereas in the presence of monomeric amine (DMAEMA, 3.0 wt.%), gel formation occurred by radical recombination between eosin groups incorporated into the gelatin. When the polymeric amines were added to the eosin–gelatin solution, gelation was markedly enhanced due to cross-linking of gelatins through polymeric amines in addition to direct bonding between gelatins. An increase in amine unit content in the polymeric amines resulted in increased gel yield and reduced swelling degree of water. In the presence of polyDMAPAAm, almost all gelatins were converted relatively rigid hydrogel. Application for a topical hemostatic glue was preliminary performed in rat injured model. A rat liver injured in laparotomy was coated with the aqueous eosin–gelatin solution containing polyDMAPAAm. Upon irradiation, the solution was immediately converted to a swollen gel, which was tightly adhered to the liver tissue and concomitantly hemostasis was completed with little tissue damage.

Keywords: Gelatin; Eosin; Visible light; Electron donor; Hemostatic glue

1. Introduction

Gelatin-based hydrogels, which are water-swollen three-dimensional cross-linked polymer, have been investigated for a number of biomedical applications as a matrix for drug delivery [1–7], a tissue adhesive glue [8–11] and a wound dressing [12–14] for surgery, a scaffold material for regeneration medicine [15–18], and a coating material for implantable medical devices [19,20]. Usually, hydrogelation of gelatin was performed by chemical cross-linking reaction using a cross-linking reagent such as aldehydes (e.g. glutaraldehyde), azide compounds, or carbodiimides. However, the careful washing process is needed in such gelation to remove the cross-linking reagents that are potentially cytotoxicity in nature in which immobilized biological active substances may release.

On the other hand, as an alternative, potent hydrogelation method, photo-induced cross-linking system of gelatin was developed [10,11,21]. In the system, gelatin was derivertized with photo- or radical-sensitive compounds such as benzophenone, styrene, and xanthene dyes (e.g. fluorescein sodium salt, eosin Y, and rose bengal). However, still now, a

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photocross-linkable material, which can store too long time without denature and simultaneously be gelated with the visible light irradiation of low illumination intensity without thermal damage, is not developed.

In this study, as continuation of our study on the development of photochemical cross-linking system of gelatin, a novel higher effective visible light-induced hydrogelation system was designed by modification of the previously developed photogelation system using an eosin-derivatized gelatin, which has the highest gelation efficiency among the xanthene dyes-derivatized gelatins [11,21]. To modify, we focused attention on an electron donor for an additive. Amine compounds such as triethanolamine or 2-(*N*,*N*-dimethylamino)ethyl methacrylate (DMAEMA) has been widely utilized as an electron donor in the photoreaction of eosin [22,23] or camphorquinone (CQ) [24,25]. In the presence of amine, accumulated evidence suggests that α-amino radicals produce from such amines following electron transfer and deprotonation [25,26]. Therefore, it is highly expected that the gelation will be enhanced if amine compound serves both as an electron donor and a cross-linking agent [27]. In this study, several polymeric amines were prepared as an electron donor, and photogelation characteristics were examined from gel yield and water swelling property, and subsequently, their potential in vivo application for hemostasis in rat liver model was demonstrated.

2. Materials and methods

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC), gelatin (from bovine bone, M_w: ca. 9.5×10^4), eosin Y (sodium tetrabromofluorescein, λ_{max} = 522 nm, $\varepsilon = 9.9 \times 10^5$ dm^3 mol^{-1} cm^{-1} in water), *N*,*N*-dimethylacrylamide (DMAAm), 2-(*N*,*N*-dimethylamino)ethyl methacrylate (DMAEMA), 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). DMAAm, DMAEMA, and DMAPAAm were purified by distillation before use.

2.2. Synthesis of eosin-derivatized gelatin (eosin–gelatin)

Eosin-derivatized gelatin (eosin–gelatin) was synthesized according to the procedure reported previously [11,21]. Briefly, an aqueous solution (40 mL) of eosin Y (1.2 g, 1.7×10^{-3} mol) and an aqueous solution (200 mL) of WSC (1.6 g, 8.7×10^{-3} mol) was mixed and stirred at 0 °C for 30 min and at room temperature for 1 h. To the mixture an aqueous solution (60 mL) of gelatin (5.0 g, 5.3×10^{-5} mol) was added. After stirring at room temperature for 1 day, the reaction mixture was dialyzed using a seamless cellulose tube (dialysis membrane, size 36, molecular weight cut off: 12,000) under flowing water for 3 days and lyophilized with a freeze dryer (FRD-82 M, Asahi Techno Glass Co., Chiba, Japan) to give the eosin-derivatized gelatin (4.4 g, 88% yield) as a light red powder. The number of eosin groups derivatized into a gelatin molecule, determined by UV–vis spectra, was 6.4 per molecule.

2.3. Synthesis of polymeric amines

Two kinds of copolymers (poly(DMAAm-co-DMAEMA); 3 and poly(DMAAm-co-DMAPAAm); 4) and one homopolymer (polyDMAPAAm; 5) were synthesized by a conventional radical polymerization. A typical procedure of poly(DMAAm-co-DMAPAAm) (4a) is as follows. Into a two-necked reaction vessel were added DMAAm (0.79 g, 8.0×10^{-3} mol), DMAPAAm (0.31 g, 2.0×10^{-3} mol), AIBN (16 mg, 1.0×10^{-4} mol), and DMF (10 mL). After stirring at 70 °C for 2 h under N_2 atmosphere, the solvent was removed in vacuo to give a crude product. Reprecipitation was carried out three times in an ethanol–diethyl ether system. After the last precipitate was dried under reduced pressure, 4a was obtained (1.1 g, 97% yield). The number-average molecular weight (M_n) and polydispersity (M_w/M_n) determined by GPC analysis were ca. 8.3×10^3 and 2.6, respectively. The content of DMAPAAm unit in the copolymer, determined by ^{1}H NMR spectra was 22 mol%. ^{1}H NMR (D_2O): δ 1.22–1.78 (br, 3.91 H), 2.09 (s, 2.74 H, $-CH_2-N(CH_3)_2$), 2.25 (m, 1.37 H), 2.78-3.10 (m, 4.17 H). Copolymer (4b) with 46 mol% of DMAPAAm unit content was synthesized by changing the monomer ratio ([DMAAm]:[DMAPAAm] = 1:1). DMAPAAm unit content, M_n, and M_w/M_n were 46, 7.6×10^3, and 1.8.

Poly(DMAAm-co-DMAEMA) (3) were synthesized from DMAAm and DMAEMA. DMAEMA unit content, M_n, and M_w/M_n were 28, 1.1×10^4, and 2.5 for 3a, and 41, 1.6×10^4, and 1.9 for 3b, respectively. 3a; ^{1}H NMR (D_2O): δ 0.80–2.15 (m, 2.84 H), 2.21 (s, 1.68 H, $-CH_2-N(CH_3)_2$), 2.62 (br, 0.56 H, $-CH_2-N(CH_3)_2$), 2.80–3.10 (m, 5.04 H), 4.08 (br, 0.56 H, $-O-CH_2-CH_2-$). PolyDMAPAAm (5) was synthesized from DMAPAAm. M_n was 8.5×10^3, and M_w/M_n was 2.6. ^{1}H NMR (D_2O): δ 1.22–1.64 (br, 5 H), 1.91 (br, 1 H, $-NH-CH_2-CH_2-$), 2.07 (s, 6 H, $-CH_2-N(CH_3)_2$), 2.22 (t, 2 H), 3.03 (br, 2 H, $-NH-CH_2-CH_2-$).

2.4. General methods

^{1}H NMR spectra in D_2O were recorded with a 300 MHz NMR spectrometer (GEMINI 300, Varian, Palo Alto, CA) using tetramethylsilane (0 ppm) as an internal standard. Gel permeation chromatographic (GPC) analysis in DMF was carried out with a HPLC-8020 instrument (Tosoh Co., Tokyo, Japan) (column: Tosoh TSKgel α-3000 and -5000). The columns were calibrated with narrow molecular weight distribution poly(ethylene glycol) (PEG) standards. UV–vis

spectra in H_2O were recorded with a UV–vis spectrophotometer (UV-1700, Shimadzu Co., Kyoto, Japan). Visible light irradiation ranging from 400 to 520 nm was carried out using an 80 W halogen lamp (Tokuso power lite, Tokuyama Co., Tokyo, Japan). Illuminance of visible light as measured with an illuminometer (T-1H, Konica Minolta Holdings Inc., Tokyo, Japan) was 7.7×10^3 lx.

2.5. Gel yield and degree of swelling

Onto a polystyrene petri dish, 100 mg of an aqueous solution of eosin–gelatin (20 wt.%, weight of the solid content: W_{solid}) mixed with an electron donor (1–5, 0.3 or 3.0 wt.%) was placed, and irradiated up to 10 min. The gel obtained was immersed into distilled water (ca. 40 mL) at 37 °C for 24 h, and then weighed (W_{wet}) after removal of excess water carefully. 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:

$$DS = \frac{(W_{wet} - W_{dry})}{W_{dry}}$$

As the data of the gel yield and degree of swelling were reproducible ($n = 5$, standard deviation (S.D.) < 5%), only the average values are described.

2.6. Hemostasis of liver tissue

Hemostasis studies were performed using Wistar rats (average weight, 250 g). A liver mechanically injured with a trephine (diameter; 2 mm) in laparotomy was applied with ca. 20 μL of an aqueous solution of eosin–gelatin (20 wt.%) mixed with 5 (3.0 wt.%). The coated surface was photo-irradiated for 1 min and then washed with a saline solution. After the predetermined periods, the liver with surrounding tissue was harvested, fixed with 10% formalin neutral buffer solution (pH 7.4), dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at a thickness of 5 μm.

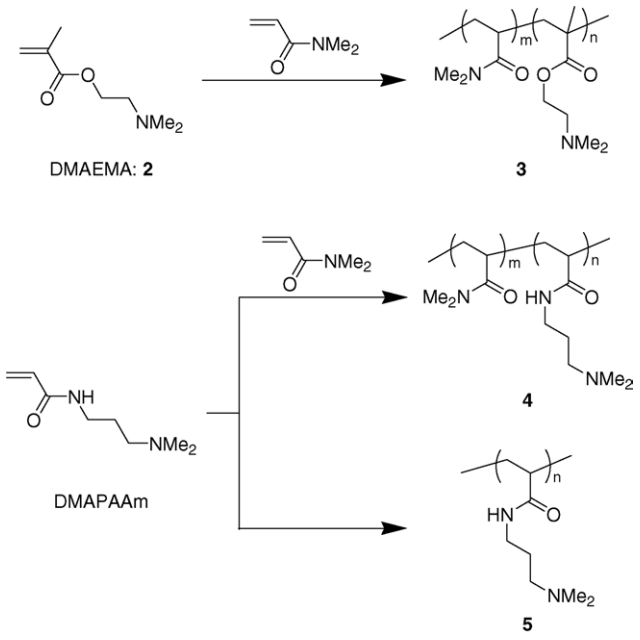

Fig. 1. Preparation schemes of polymeric amines.

After staining with hematoxylin–eosin, observation was performed under a light microscope (Eclipse E1000M, Nikon, Tokyo, Japan).

3. Results

3.1. Photogelation and swelling properties

As an electron donor, two kinds of monomeric compounds such as ascorbic acid (1) and DMAEMA (2), and three kinds of polymeric amines such as poly(DMAAm-co-DMAEMA) (3), poly(DMAAm-co-DMAPAAm) (4), and polyDMAPAAm (5) were used. The polymeric amines were prepared by a conventional radical polymerization using AIBN as an initiator (Fig. 1). Table 1 lists the preparation conditions and characteristics of the obtained copolymers (3 and 4). All polymers had almost same molecular weight (M_n: ca. 1×10^4) and were well water-soluble. ^{1}H NMR spectral

Table 1
Syntheses (total monomer concentration = 100 g/L in DMF, [monomer]/[initiator] = 100, 70°, C2 h) and characteristics of 3 and 4

Feed mole ratio	Poly(DMAAm-co-DMAEMA)				
DMAAm:DMAEMA	Conversion(%)	$M_n(\times 10^4)$[a]	M_w/M_n[a]	Contents of DMAEMA unit (mol%)[b]	Code
80:20	71	1.1	2.5	28	3a
67:33	44	1.6	1.9	41	3b
Feed mole ratio	Poly(DMAAm-co-DMAPAAm)				
DMAAm:DMAPAAm	Conversion(%)	$M_n(\times 10^3)$[a]	M_w/M_n[a]	Contents of DMAPAAm unit (mol%)[b]	Code
80:20	97	8.3	2.6	22	4a
50:50	77	7.6	1.8	46	4b

[a] Determined by GPC measurements (calibrated with poly(ethylene glycol); eluent, DMF).
[b] Determined by ^{1}H NMR analyses.

Table 2
Gelation characteristics of eosin–gelatin

run	electron donor	concentration(wt.%)	light source[a]	irradiation time(min)	gel yield(%)[b]	degree of swelling[b]
1[c]	1	0.3	A	1	62	14
2	1	0.3	B	10	0	–
3	1	3.0	B	10	0	–
4	2	3.0	B	10	11	72
5	3a	3.0	B	10	20	21
6	3b	3.0	B	10	28	16
7	4a	3.0	B	10	17	5.7
8	4b	3.0	B	10	24	2.4
9	5	3.0	B	1	68	6.4
10	5	3.0	B	5	90	6.1

Concentration in water was 20 wt.%.
[a] A: 150 W xenon lamp (illuminance: 99,000 lx); B: 80 W halogen lamp (illuminance: 7700 lx).
[b] Determined by gravimetrically; S.D. < 5% ($n = 5$).
[c] Previous work (Ref. [11]).

analyses showed that in each kind of copolymers content of amine unit was adjusted from 20 to 50 mol%.

In our previous study, when extremely small amount (0.3 wt.%) of ascorbic acid (1) was added to an aqueous solution (20 wt.%) of eosin–gelatin, which was prepared by partial derivatization of eosin to lysine residues of gelatin (degree of derivatization: 6.4 per molecule), ca. 60% of gel was formed even without degassing after 1 min of irradiation with high power light intensity using a 150 W xenon lamp (illuminance: 9.9×10^4 lx) (run 1 in Table 2). However, upon irradiation with reduced light intensity (illuminance: 7.7×10^3 lx) of an 80 W halogen lamp used clinically in dentistry, no gel was obtained even after longer irradiation time (up to 10 min) and using a large amounts of 1 (3.0 wt.%) (runs 2 and 3).

On the other hand, in the presence of monomeric amine (2, 3.0 wt.%) as an electron donor, gel formation was confirmed in ca. 10% yield after 10 min of irradiation with the halogen lamp (run 4). When the polymeric amines (3.0 wt.%), which are multiply aminated water-soluble polymers, were mixed with the gelatin solution, gelation was further enhanced. The increasing gel yield and reducing degree of swelling occurred with an increase in the amine unit content in both polymeric amines (3 and 4) copolymerized with DMAAm, which is water-soluble monomer and does not function as an electron donor (runs 5–8). There was little change in gel yield with the difference of type of amine but degree of swelling was markedly decreased in DMAPAAm than DMAEMA. In homopolymer of DMAPAAm (5) higher yield of gel with relatively low degree of swelling was obtained within a very short period of irradiation time (1 min, run 9). After 5 min of irradiation, almost all gelatins were converted to hydrogel (run 10).

3.2. Photoreactivity of electron donors with eosin

Absorption spectral changes of an aqueous eosin–gelatin solution (2.0×10^{-2} wt.%) by photo-irradiation with halogen lamp were observed (Fig. 2). In the absence of an electron donor, a gradual reduction of the maximal absorption wavelength at 522 nm derived from eosin was observed time-dependently. On the other hand, upon addition of small amount of ascorbic acid (3.0×10^{-3} wt.%) to the eosin–gelatin solution, higher rate of the absorption decreasing, indicating destruction of conjugation structure of eosin, was observed but about 60% of eosin was remained even after 5 min of irradiation. In the presence of polymeric amine 5 (3.0×10^{-3} wt.%), the absorption derived from eosin was mostly disappeared after irradiation for 5 min.

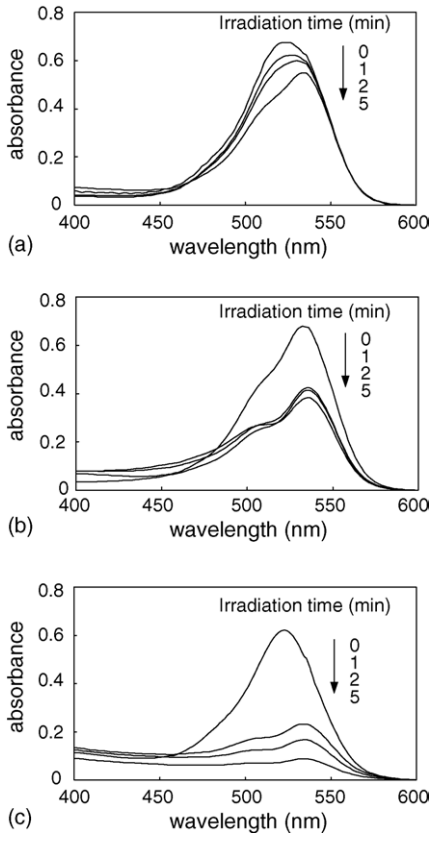

Fig. 2. Absorption spectral changes of an aqueous eosin–gelatin solution (2.0×10^{-2} wt.%) induced by irradiation with halogen lamp (a), with 1 (3.0×10^{-3} wt.%) (b), or 5 (3.0×10^{-3} wt.%) (c).

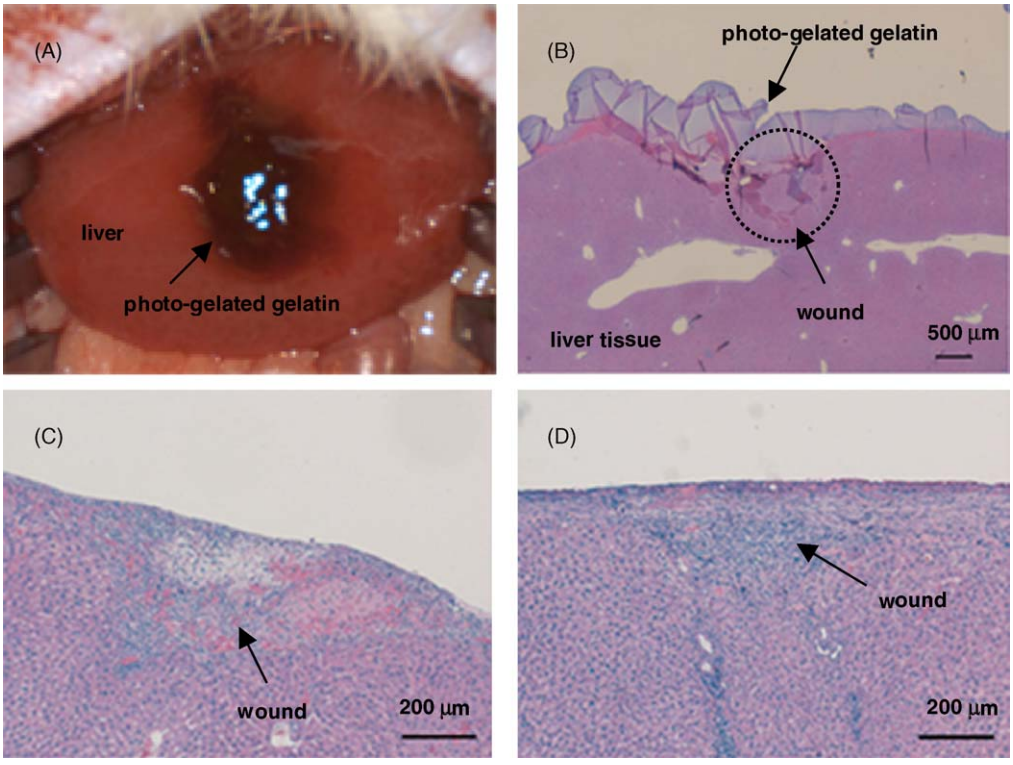

Fig. 3. In vivo application for hemostasis. Microscopic photo and histological microphotos of rat liver surface (hematoxylin–eosin stain); (A), (B) immediately after photogelation, (C) 3 days or (D) 7 days after surgery.

3.3. *In vivo application*

The preliminary in vivo application for hemostasis of liver was demonstrated using 20 wt.% aqueous solution of eosin–gelatin mixed with 3.0 wt.% of 5. Rat livers, mechanically injured in laparotomy, were coated with the viscous gelatin solution. After visible light irradiation to the coating part for 1 min, swollen gel was immediately produced on the bleeding spots. The gel layer tightly adhered on the liver surface even after washing with a saline solution, and little bleeding and no thermal damage were observed (Fig. 3A). Histological examination of the cross-sectional specimen stained with hematoxylin–eosin showed that the photocured gelatin film covered entirely the injured surface (Fig. 3B). Three days after surgery, inflammatory cells were observed at the local area of the injury, and not around the injury in liver tissue (Fig. 3C). At 1 week after surgery, fibroblast-like cells were covered the liver surface around the wound (Fig. 3D).

4. Discussion

In situ hydrogelation is expected as one of effective processes for medical applications such as wound healing material including a tissue adhesive glue and a tissue adhesion prevention material, and in situ three-dimensional tissue formation in regeneration medicine. For one of powerful tool in the hydrogelation, several photochemical cross-linking methods have been developed.

In our previous study [10,11,21], an aqueous solution of eosin–gelatin could convert to hydrogel in the presence of ascorbic acid, one of electron donors, by irradiation of visible light, which can penetrate thicker layer and is markedly harmless to cells and tissues in nature than ultraviolet light. The possible photogelation mechanism of eosin–gelatin in the presence of electron donor was shown in Fig. 4. From the reaction of ascorbic acid with eosin under photo-irradiation, radical form of eosin and ascorbyl-free radical (AFR) would be formed. The deformation of eosin induced by addition of ascorbic acid was confirmed by the absorption spectral change (Fig. 2). Pairs of AFR would disproportionate to form one molecule of dehydro-L-ascorbic acid (DHAA) and one ascorbic acid immediately, suggesting that ascorbic acid could not serve as cross-linking agent. In addition, it is reported that ascorbic acid has function as a strong radical scavenger [28,29]. Therefore, it is estimated that the gel produced by the recombination between radicals produced at the eosin groups in the side chains of gelatin only under irradiation with high power light source such as a 150 W xenon lamp (illuminance: 9.9×10^4 lx) (route I in Fig. 4).

In this study, to enhance gelation we developed a novel visible light-induced eosin–gelatin-based hydrogelation system, which was assisted by the addition of polymeric amines as another type of an electron donor. It is estimated that the amine compounds-associated hydrogelation was progressed

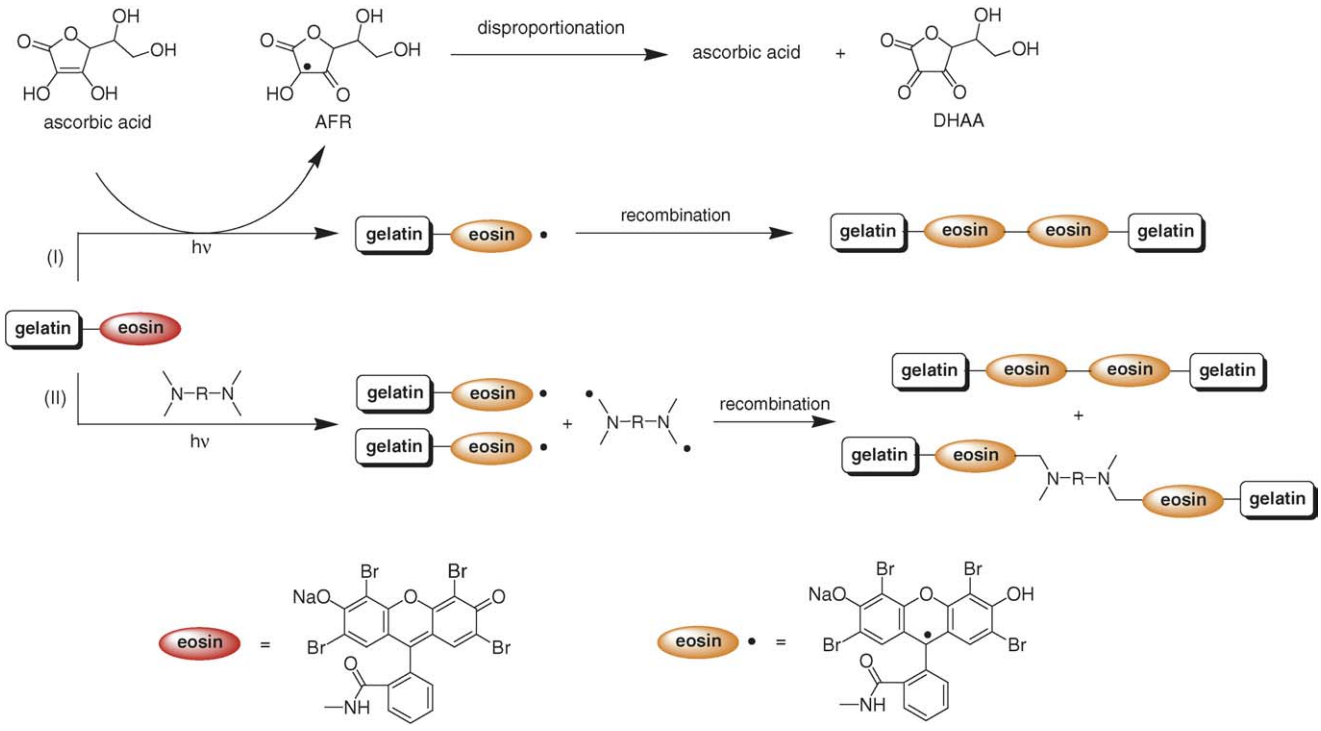

Fig. 4. A possible gelation mechanism of eosin–gelatin in the presence of ascorbic acid (route I) or amine (route II).

according to the route II in Fig. 4. That is, radicals both on eosin and amine compounds can be generated by irradiation [25,26]. Gelation was confirmed upon adding of monomeric amine even under irradiation with halogen lamp, suggesting that eosin radical would not be quenched with amine compound (run 4 in Table 2). Therefore, it was considered that a larger amount of eosin radicals were produced in the presence of amine, which induced the formation of hydrogel even upon irradiation of low intensity light. If two and more functioned amine compounds are used, those will be available as a cross-linking agent between gelatin molecules [27]. Indeed, when polymeric amines were used as an additive, gelation was more enhanced than monomeric amine (runs 4–6). In addition, an increasing gel yield was observed with an increase in the amine unit in polymeric amines (runs 5–6 and 7–9).

Hydrogelation was completed even by irradiation of visible light with low illumination intensity upon using homopolymer of DMAPAAm (5) as an electron donor (run 10 in Table 2). On the other hand, it was expected that if homopolymer of DMAEMA was used as an electron donor high gel yield was obtained similar in the homopolymer (5). However, the polyDMAEMA is thermally sensitive water-soluble polymer and has a lower critical solution temperature (LCST) of almost 40 °C. Therefore, it could not mix with gelatin since heating process over 60 °C is needed to obtain homogeneous solution.

In the preliminary animal studies on a rat liver, the aqueous eosin–gelatin solution containing polyDMAPAAm was immediately converted to a swollen gel, which was tightly adhered to the liver tissue and concomitantly hemostasis was completed with little tissue damage. The works to confirm the type of cells participating in tissue repair, the biodegradation rate of the gel, and the cytotoxicity of the gel degradation products were now in progress.

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