Crosslinking Structures of Gelatin Hydrogels Crosslinked with Genipin or a Water-Soluble Carbodiimide

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ABSTRACT: It was suggested in our previous studies that carbodiimide- and genipin-crosslinked gelatin hydrogels could be used as bioadhesives to overcome the cytotoxicity problem associated with formaldehyde-crosslinked gelatin hydrogels. In this study, we investigated the crosslinking structures of carbodiimide- and genipin-crosslinked gelatin hydrogels. We found that crosslinking gelatin hydrogels with carbodiimide or genipin could produce distinct crosslinking structures because of the differences in their crosslinking types. Carbodiimide could form intramolecular crosslinks within a gelatin molecule or short-range intermolecular crosslinks between two adjacent gelatin molecules. On the basis of gel permeation chromatography, we found that the polymerization of genipin molecules could occur under the conditions used in crosslinking gelatin hydrogels via a possible aldol condensation. Therefore, besides intramolecular and short-range intermolecular crosslinks, additional long-range intermolecular crosslinks could be introduced into genipin-crosslinked gelatin hydrogels. Crosslinking a gelatin hydrogel with carbodiimide was more rapid than crosslinking with genipin. Therefore, the gelation time for the carbodiimide-crosslinked gelatin hydrogels was significantly shorter than that of the genipin-crosslinked gelatin hydrogels. However, the cohesive (interconnected) structure of the carbodiimide-crosslinked gelatin hydrogels was readily broken because, unlike the genipin-crosslinked gelatin hydrogels, there were simply intramolecular and short-range intermolecular crosslinks present in the carbodiimide-crosslinked hydrogel. In the cytotoxicity study, the carbodiimide-crosslinked gelatin hydrogels were dissolved into small fragments in the cultural medium within 10 min. In contrast, the genipin-crosslinked gelatin hydrogels remained intact in the medium throughout the entire course of the study. Again, this may be attributed to the differences in their crosslinking structures. The genipin-crosslinked gelatin hydrogels were less cytotoxic than the carbodiimide-crosslinked gelatin hydrogels. J Appl Polym Sci 91: 4017–4026, 2004

Key words: adhesives; biopolymers; crosslinking

INTRODUCTION

Bioadhesives are used for tissue adhesion and hemostasis in surgery.[1,2] A gelatin–resorcinol mixture crosslinked with formaldehyde (GRF glue) was used for this purpose.[3–11] Although the bonding strength of the GRF glue to tissue was satisfactory, concerns about the cytotoxicity of formaldehyde were reported in the literature.[6,9] It was suggested that the cytotoxicity problem of the GRF glue could be overcome by a change in its crosslinking method.[12] The feasibility of using a water-soluble carbodiimide or genipin (GP) to crosslink a gelatin hydrogel for new bioadhesives was previously evaluated *in vitro* and *in vivo* by our group.[13,14]

The results obtained in the *in vitro* study indicated that the cytotoxicity of the carbodiimide- and GP-crosslinked glues were significantly lower than that of the GRF glue.[13] The GRF glue had the shortest gelation time and the greatest bonding strength to tissue of all the test adhesives. However, the GRF glue was less flexible than the carbodiimide- and GP-crosslinked glues. In the *in vivo* study conducted in a rat model to close skin wound lesions, it was found that the wounds treated by the carbodiimide- and GP-crosslinked glues induced significantly less inflammatory responses and recovered sooner than those treated by the GRF glue.[14] It was concluded that the GRF glue may be used as a tissue adhesive when the ability to bind tissue rapidly and tightly is required; the carbodiimide- and GP-crosslinked glues are preferable when the adhesive action must be accompanied by minimal cytotoxicity and stiffness in clinical applications.[13,14]

Carbodiimide is a condensation agent used between carboxylic acid and amino groups to form amide-type bonds.[15] Crosslinking a gelatin hydrogel with carbodiimide involves the activation of its carboxylic acid groups of glutamic or aspartic acid residues to give *O*-acylisourea groups, which form crosslinks after a reaction with its free amino groups of lysine or hydroxyline residues.[15] It was reported that the addition

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of *N*-hydroxysuccinimide (NHS) to a carbodiimide-containing solution was very effective in increasing the number of crosslinks introduced.[16]

GP can be obtained from its parent compound, geniposide, which may be isolated from the fruits of *Gardenia jasminoides* Ellis. GP and its related iridoid glucosides have been widely used as antiphlogistics and cholagogues in herbal medicine.[17] Additionally, it has been reported that GP can spontaneously react with amino acids or proteins to form dark blue pigments.[18,19] These dark blue pigments have been used in the fabrication of food dyes. As a naturally occurring crosslinking agent, GP has been used by our group to fix biological tissues or amino-group-containing biomaterials for biomedical applications.[20–22]

The previous discussion suggests that the crosslinking of a gelatin hydrogel with carbodiimide or GP may produce distinct crosslinking structures because of differences in their crosslinking types. Therefore, in this study, we further investigated the crosslinking structures of carbodiimide- (in the absence or presence of NHS) and GP-crosslinked gelatin hydrogels.

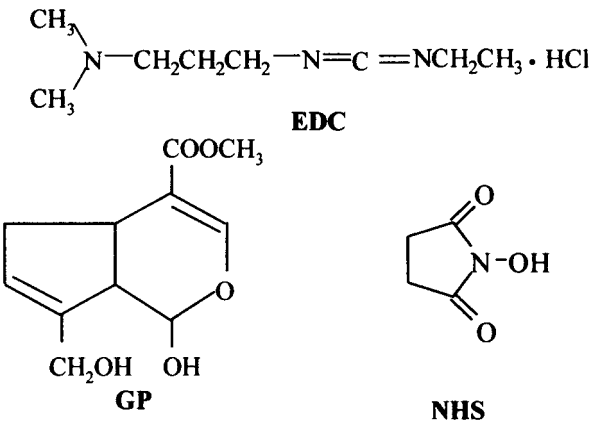


Figure 1 Chemical structures of EDC, GP, and NHS.

EXPERIMENTAL

Preparation of the test groups

The gelatin hydrogels (80 mg/mL; from porcine skin, 300 Bloom, Sigma Chemical Co., St. Louis, MO) were divided into five groups for crosslinking at 37°C: (1) without any crosslinking (fresh), (2) with crosslinking in GP (0.004*M*; Challenge Bioproducts, Taicung, Taiwan) buffered with phosphate-buffered saline (PBS; pH 7.4), (3) with crosslinking in a water-soluble carbodiimide [1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC); 0.004*M*] buffered with 2-(*N*-morpholino)-ethanesulfonic acid (MES; pH 5.5), (4) with crosslinking in EDC with NHS (EDC/NHS; 0.004*M* EDC and 0.0016*M* NHS; buffered with MES), and (5) with crosslinking in EDC/NHS for 3 h and then in GP until the hydrogel becomes rubberlike (EDC/NHS–GP). Samples of each test group were taken out at distinct elapsed crosslinking times. The chemical structures of the crosslinking agents used in this study are shown in Figure 1.

Extent of crosslinking

The extent of crosslinking for each test group was determined by the monitoring of changes in the free-amino-group content in the test gelatin hydrogel with the ninhydrin assay. The test sample first was lyophilized for 24 h and then was weighed (~3 mg). Subsequently, the lyophilized sample was heated with a ninhydrin solution (2 wt %) at 100°C for 20 min. After the heating with ninhydrin, the optical absorbance of the solution was recorded with a spectrophotometer at a wavelength of 570 nm (UV-150-02, Shimadzu Corp., Kyoto, Japan) with glycine at various known concentrations as a standard. The number of free amino groups in the test sample, after heating with ninhydrin, was proportional to the optical absorbance of the solution.[23]

Gel permeation chromatography (GPC)

The results reported by Touyama and coworkers[24,25] suggested that GP molecules could be polymerized before crosslinking with amino groups. Whether GP molecules could be polymerized before crosslinking was tested in this study with GPC. The molecular weights of polymerized GP molecules were determined by GPC with a Waters 600 system high-performance liquid chromatograph (Milford, MA) equipped with a GPC column (15062, Jordi, Bellingham, MA), and a refractive index detector (RI2000, Schamback, Bad Honnef, Germany). A solvent composed of *N*,*N*-dimethylformamide and distilled and deionized water in a volumetric ratio of 4:1 was delivered by a high-performance-liquid-chromatograph pump (P-100, TSP, Riviera Beach, FL). The solvent flow rate was 0.8 mL/min at the ambient temperature. Poly(ethylene glycol) (molecular weight = 600–20,000) standards were used to establish a molecular weight calibration curve.

The gelation time of each test group was investigated and compared. Additionally, the cytotoxicity of each test group was evaluated *in vitro* with 3T3 fibroblasts.

Gelation time measurements

After the addition of the crosslinking agent into each test group, the polymerization of the gelatin hydrogel took place. The polymerization of a gelatin hydrogel is distinguished by the occurrence of gelation at some point in crosslinking.[26] At that point, the adhesive

loses fluidity, or its viscosity increases abruptly. After gelation, the adhesive becomes rubberlike and is no longer workable.[1,2] Consequently, bioadhesives must be applied to tissues and used before gelation. The viscosity of each test group was measured with a viscometer (M/95-320, Brookfield Engineering Laboratories, Inc., Stoughton, MA) at a constant rotation speed of 50 rpm in a 37°C water bath. The duration required for the viscosity to increase abruptly was defined as the gelation time for the test adhesive.

Cytotoxicity study

A recognized disadvantage of a chemically modified bioadhesive is its potential toxic effects; a recipient may be exposed to the adhesive itself or its residues, such as the remaining crosslinking agent and particles degraded from the adhesive. The cytotoxicity of each test group was evaluated *in vitro* with an established mouse-derived cell line of 3T3 fibroblasts (Balb/3T3 C1A31-1-1) in the log phase of growth. Each test sample (0.1 mL) was placed in the centers of wells in a 24-well plate. Subsequently, 3T3 fibroblasts (5×10^4 cells) were seeded evenly in the wells with test adhesives in 1 mL of Dulbecco's modified eagle's medium (430-2800EG, Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT). The cell culture was maintained in a humidified incubator at 37°C with 10% CO_2 in air. After 24 h of culturing, the medium was removed, and the cells cultured in the vicinity of each test adhesive were photographed with an inverted light microscope.

RESULTS AND DISCUSSION

Through previous long-term pharmaceutical and medical applications, it is known that gelatin is biodegradable, biocompatible, nontoxic, and noncarcinogenic.[27,28] It has been suggested that gelatin, denatured triple-helical collagenous molecules, can be used for tissue adhesion and hemostasis in surgery.[1,2] Nevertheless, in order for its bonding strength to be increased and its degradation rate to be controlled, a gelatin hydrogel has to be chemically modified by a crosslinking agent before it can be used as a bioadhesive.[1,2]

Crosslinking mechanism

The mechanism of crosslinking with carbodiimide involves the formation of an *O*-acylisourea derivative between carbodiimide and the carboxylic acid groups of glutamic and aspartic acid residues in gelatin molecules, followed by a nucleophilic substitution with an amino group, most often ϵ-NH_2 of a lysine residue.[29] No spacer is introduced for this amide-bond-type crosslinking.[30] However, Nakajima and Ikada[31] reported that amide formation from carboxyl and amino groups in aqueous media in the presence of carbodiimide required the formation of carboxylic anhydride as an intermediate. Hence, carbodiimide may couple carboxylic acid and amino groups that are located within 1.0 nm of one another.[32] Therefore, carbodiimide may form intramolecular crosslinks within a gelatin molecule or short-range intermolecular crosslinks between two adjacent gelatin molecules as long as the crosslinked carboxylic acid and amino groups are less than 1.0 nm apart. However, it has been reported that the highly reactive *O*-acylisourea group may be rearranged into a stable *N*-acylurea group, a side branch attached to the modified peptide.[16] In the presence of NHS, the *O*-acylisourea group is converted into the NHS-activated carboxylic acid group, which is less susceptible to hydrolysis than the *O*-acylisourea group. Therefore, the addition of NHS to the carbodiimide-containing solution is effective in increasing the number of crosslinks introduced into collagen-based materials.[16]

The reaction mechanism of amino-group-containing compounds with GP has been discussed in the literature.[24,25] It has been proposed that a GP-amino-group monomer is formed though a nucleophilic attack by amino-group-containing compounds such as gelatin on the third carbon of GP. This is followed by the opening of the GP ring, and an aldehyde group is formed. Subsequently, the resulting aldehyde group is attacked by the attached secondary amino group. Dimerization occurs during the second stage, perhaps by a radical reaction. Accordingly, GP may bridge peptide chains that have a distance of up to 1.6–2.5 nm[33] by introducing a dimeric GP spacer with a cyclic structure.[34] Therefore, GP may form intramolecular crosslinks within a gelatin molecule and short-range intermolecular crosslinks between two adjacent gelatin molecules.

Touyama and coworkers[24,25] studied the structures of the intermediates leading to a blue pigment produced from GP and methylamine, the simplest primary amine. The blue pigment that formed was found to be a mixture of polymers on the basis of its chromatographic behavior and unanalyzable ^{13}C-NMR spectrum. The polymers were reported to have an average molecular weight of 8970 ± 600 on the basis of osmotic pressure measurements. On the basis their observations, it can be speculated that GP molecules may be polymerized before crosslinking with amino-group-containing compounds.

Figure 2 shows GPC data for a GP solution buffered in PBS used in crosslinking gelatin hydrogels at various elapsed times after the solution was prepared. The GPC data indicated that the polymerization of GP monomers (molecular weight = 226) occurred in PBS. The polymers were found to have molecular weights of 3900–7900 and to consist, on average, of 30–40

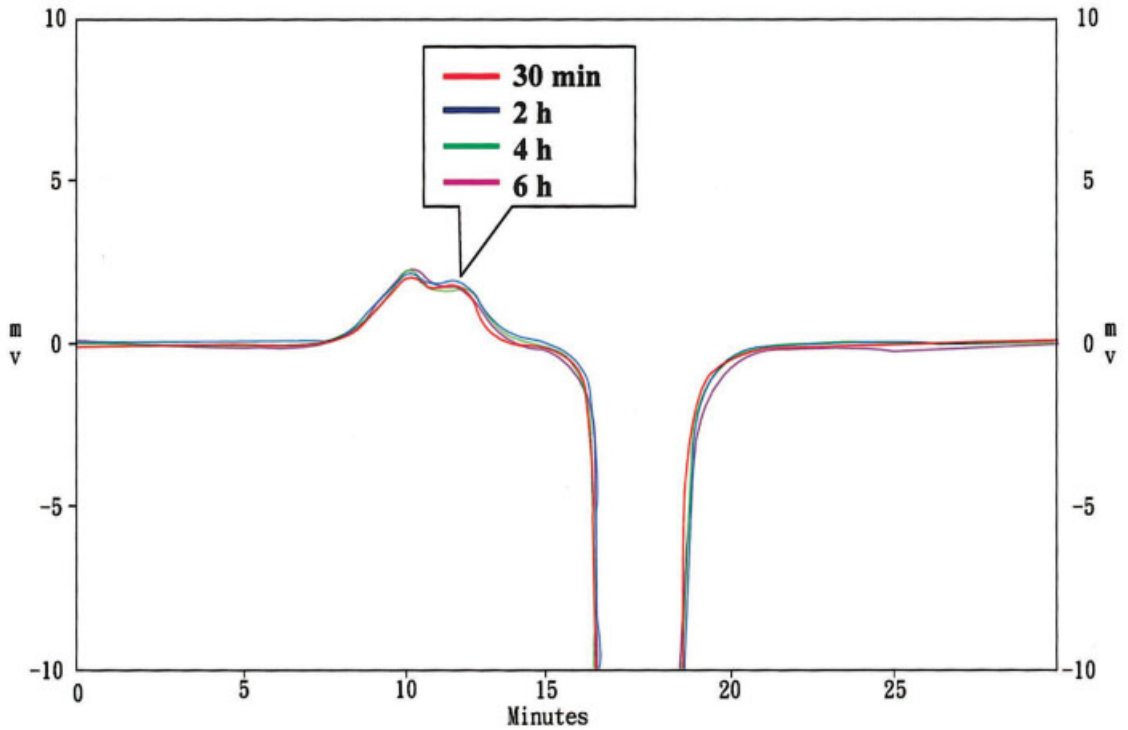


Figure 2 GPC chromatograms of a GP solution buffered in PBS and used in crosslinking gelatin hydrogels for distinct times after preparation.

monomer units. Additionally, it was noted that the polymerization of GP monomers in PBS could take place in a short time (within 30 min) because the nucleophile OH^- in PBS could attack GP molecules and open the GP ring to form an aldehyde group, as described earlier. Subsequently, the resulting aldehyde groups could be polymerized via an aldol condensation. This implied that GP molecules could be polymerized before crosslinking amino-group-containing compounds through the introduction of an oligomeric GP spacer (long-range intermolecular crosslinks).

Crosslinking structure

Figure 3(a–e) illustrates the presumed crosslinking structure of each test group based on the aforementioned discussion. For the gelatin hydrogel without crosslinking (fresh), only physical entanglements exist among gelatin molecules in the hydrogel [Fig. 3(a)]. After crosslinking, besides physical entanglements, GP may chemically react with amino groups within a gelatin molecule (intramolecular crosslinks) or between two adjacent gelatin molecules [short-range intermolecular crosslinks; Fig. 3(b)]. In addition, long-range intermolecular crosslinks may be introduced between gelatin molecules via the polymerization of GP molecules before crosslinking [oligomeric crosslink; Fig. 3(b)]. However, intramolecular and short-range intermolecular crosslinks with some pendant side branches (*N*-acylurea groups) may be found in the EDC-crosslinked gelatin hydrogel [Fig. 3(c)]. However, there are only intramolecular and short-range intermolecular crosslinks in the EDC/NHS-crosslinked gelatin hydrogel because the use of NHS can prevent the *N*-acyl shift of the *O*-acylisourea group [Fig. 3(d)]. The addition of GP to the EDC/NHS-crosslinked hydrogel may further establish long-range intermolecular crosslinks in the EDC/NHS–GP-crosslinked gelatin hydrogel [Fig. 3(e)].

Extent of crosslinking

The reductions in the free-amino-group content at distinct elapsed crosslinking times for each test group were determined by the ninhydrin assay and were used as a measure of the extent of crosslinking.[32,35] After the crosslinking, it was noted that the reductions in the free-amino-group content for all test groups were significant in comparison with a fresh group (Fig. 4). This indicated that both GP and carbodiimide were effective crosslinking agents.

The free-amino-group contents of the EDC- and EDC/NHS-crosslinked gelatin hydrogels declined significantly within 30 min of crosslinking and stayed at their minimal values subsequently. This indicated that the crosslinking of a gelatin hydrogel with carbodiimide in the absence or presence of NHS was essentially

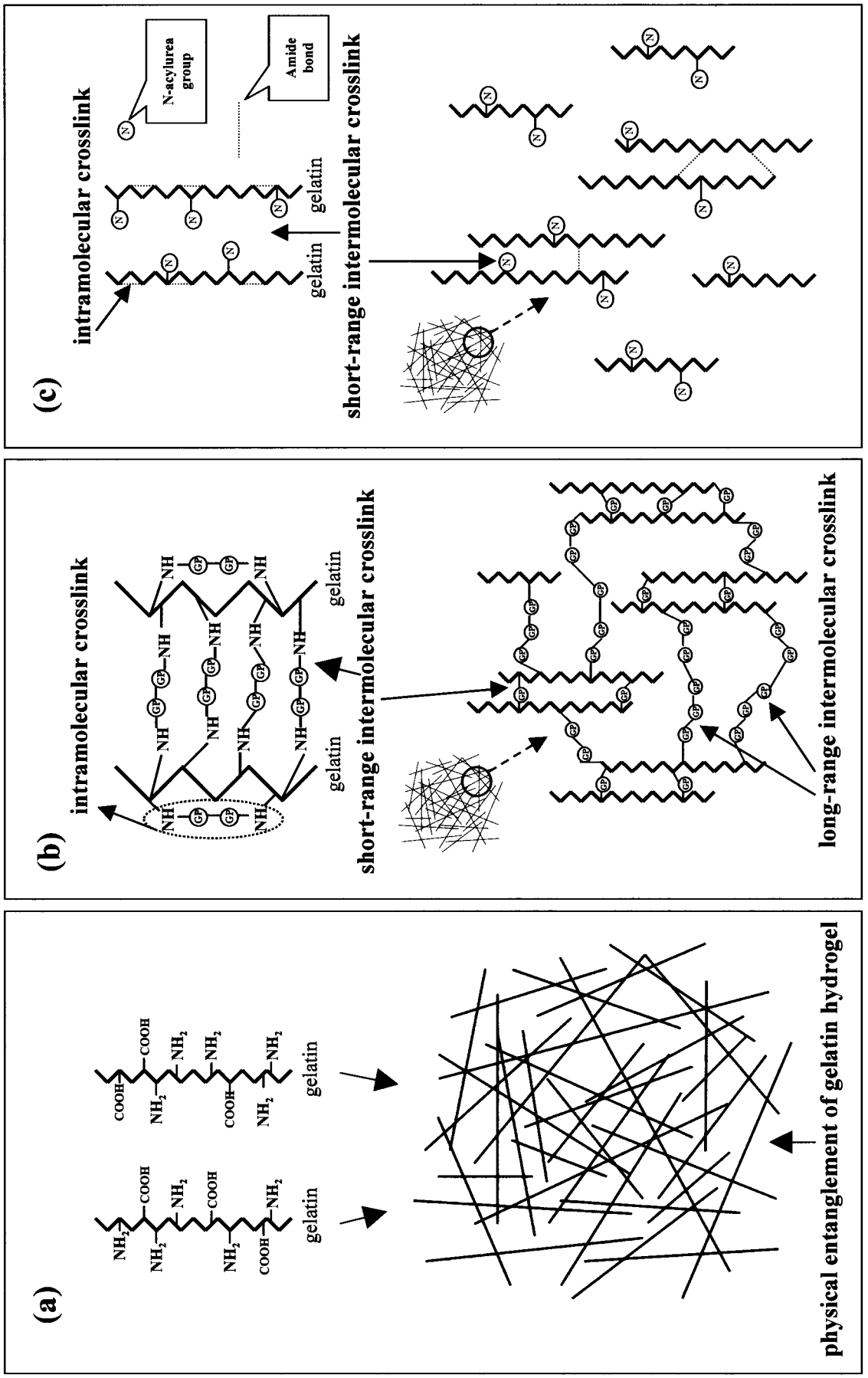
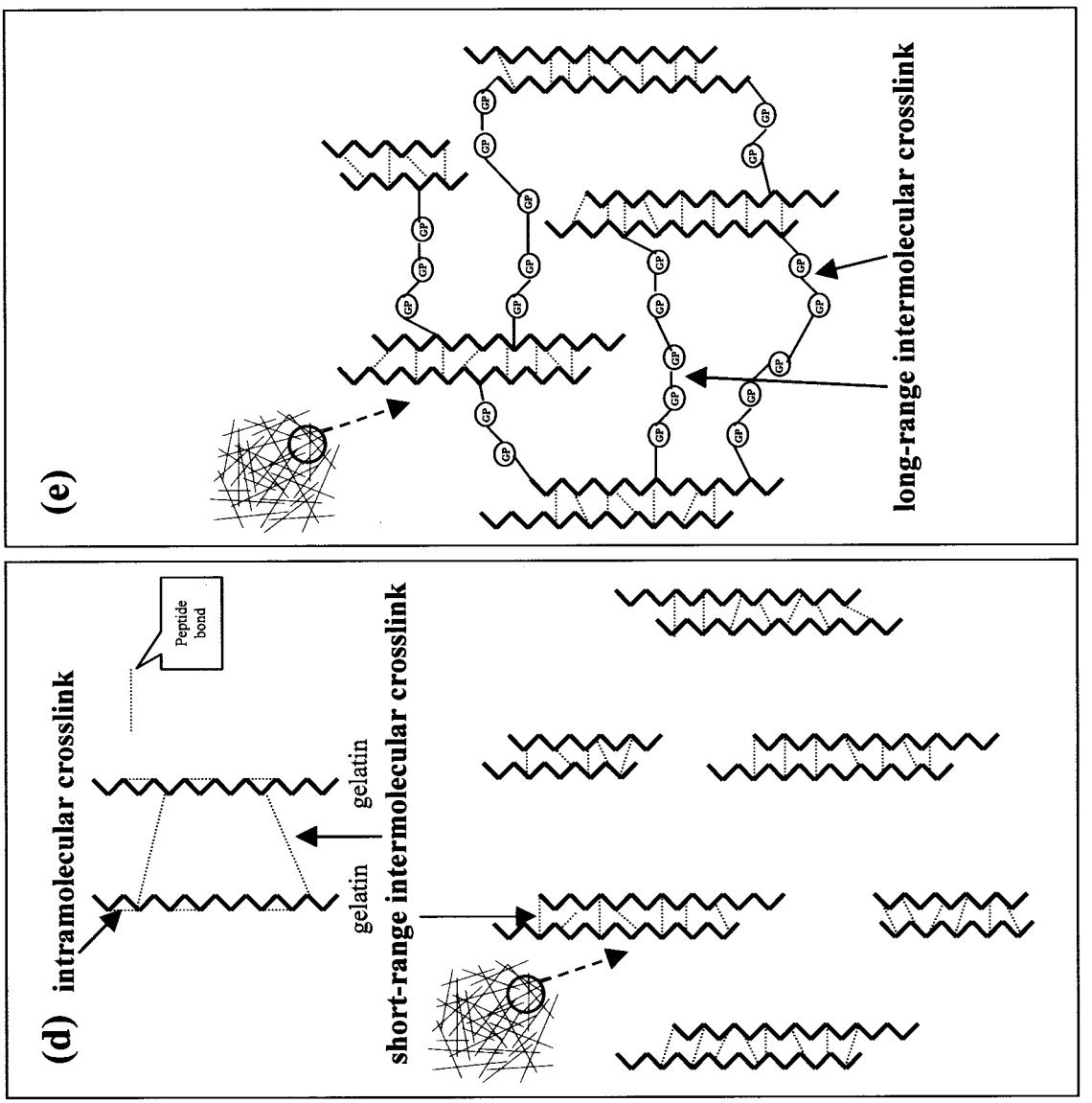


Figure 3 Schematic illustrations of the crosslinking structures presumed for (a) a fresh gelatin hydrogel (a gelatin hydrogel without crosslinking), (b) a GP-crosslinked gelatin hydrogel, (c) an EDC-crosslinked gelatin hydrogel, (d) an EDC/NHS-crosslinked gelatin hydrogel, and (e) an EDC/NHS–GP-crosslinked gelatin hydrogel.



completed within 30 min after the initiation of crosslinking. It has been reported that carbodiimide crosslinking is a rapid reaction.[16,30] In contrast, the reaction of GP with the free amino groups in a gelatin hydrogel was comparatively slow. However, the reduction in the free-amino-group content for the GP-crosslinked gelatin hydrogel was significant as the duration of crosslinking increased. After 3 h of crosslinking, the free-amino-group content of the GP-crosslinked gelatin hydrogel was significantly lower than that of the EDC- and EDC/NHS-crosslinked gelatin hydrogels.

In a study of crosslinking biological tissues with carbodiimide or GP conducted by our group,[36] it was found that the reduction in the free-amino-group content for the carbodiimide-crosslinked tissue was approximately 33%, whereas that for the GP-crosslinked tissue was approximately 90%. This was because carbodiimide could basically form intramolecular and short-range intermolecular crosslinks within collagen-based materials, and each crosslinking produced with carbodiimide simply consumed one free amino group. Therefore, the consumption of free amino groups in the carbodiimide-crosslinked tissue was limited. In contrast, besides intramolecular and short-range intermolecular crosslinks, GP may further establish long-range intermolecular crosslinks, and each crosslinking with GP may consume two free amino groups. Thus, the consumption of free amino groups in the GP-crosslinked tissue was significantly greater than in the carbodiimide-crosslinked tissue.

However, as indicated in Figure 4, the remaining amounts of free amino groups in the gelatin hydrogels were still abundant for all test groups. The reductions in the free-amino-group content for the EDC- and GP-crosslinked gelatin hydrogels were approximately 25 and 35%, respectively. For the EDC-crosslinked gelatin hydrogel, this was again because each crosslinking produced with carbodiimide simply consumed one free amino group. For the GP-crosslinked

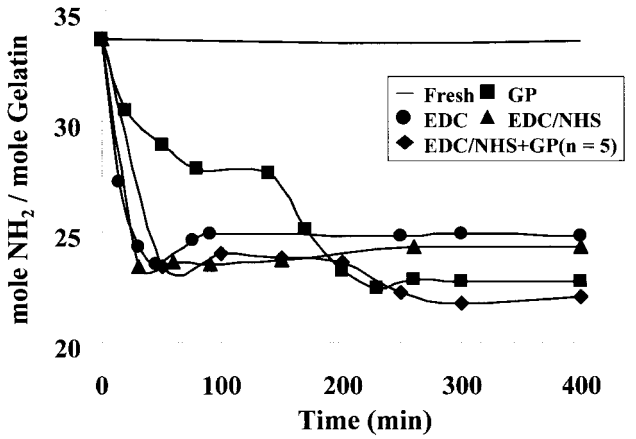


Figure 4 Free-amino-group content of each test group obtained at distinct elapsed crosslinking times ($n = 5$).

gelatin hydrogel, the distance between the adjacent gelatin hydrogels may have been too far apart to produce further crosslinking, even though the GP molecules could polymerize before crosslinking. Therefore, the consumption of free amino groups in the EDC- and GP-crosslinked gelatin hydrogels was limited.

After the addition of GP to the EDC/NHS-crosslinked hydrogel, the free-amino-group content of the EDC/NHS–GP-crosslinked gelatin hydrogel decreased further. This implied that GP could result in the formation of additional crosslinks (e.g., long-range intermolecular crosslinks) in the hydrogel after the completion of crosslinking with carbodiimide (the formation of intramolecular and short-range intermolecular crosslinks).

Gelation time

As illustrated in Figure 3(b–e), physical entanglements and chemical couplings may take place among gelatin molecules during crosslinking. In this fashion, a three-dimensional cohesive (interconnected) structure is created after gelation. The bonding strength of a test adhesive to tissue may be determined by the adhesive force between the adhesive and tissue or its cohesive force (intrinsic properties), whichever is smaller. As suggested in our previous study,[13] after the bonding strength measurements, the EDC- and GP-crosslinked gelatin adhesives were found to adhere firmly to the tissue surface and underwent cohesive failure during the bond breaking. This finding indicated that the adhesion forces of the EDC- and GP-crosslinked gelatin adhesives to tissue were greater than their cohesive forces. Therefore, the bonding strengths to tissue for the EDC- and GP-crosslinked gelatin adhesives were determined by their cohesive forces due to physical entanglement and chemical crosslinking.

The gelation time was defined as the duration required for the viscosity (or the cohesive force) of the test adhesive to increase abruptly. Figure 5(a–e) shows examples of the time course of the viscosity measurements for all test groups. The viscosity of the fresh group [with only physical entanglements among gelatin molecules; Fig. 3(a)] remained constant (5 cP) after a 24-h measurement with a viscometer [Fig. 5(a)]. Therefore, no gelation time could be determined for the fresh group. The viscosity of the GP-crosslinked gelatin hydrogel [with physical entanglements in addition to intramolecular crosslinks and short-range and long-range intermolecular crosslinks; Fig. 3(b)] increased abruptly in approximately 165 min [gelation time; Fig. 5(b)]. The increase in the viscosity was most remarkable just before gelation, and this indicated that the greatest bonding strength for the GP-crosslinked gelatin hydrogel occurred right at this moment. Subsequently, the hydrogel became rubberlike and was no longer workable. It was found in our previous study that the gelation time of the GP-crosslinked gelatin hydrogel could be shortened significantly to about 30 min if the concentration of GP was increased.[13]

In contrast, the viscosities of the EDC- and EDC/NHS-crosslinked gelatin hydrogels increased abruptly after the addition of the crosslinking agent [Fig. 5(c,d)]. This indicated that both the EDC- and EDC/NHS-crosslinked gelatin hydrogels were gelled in a short time because of their rapid reactions (Fig. 4). However, the peaks observed in the viscosity measurements for the EDC- and EDC/NHS-crosslinked gelatin hydrogels decreased shortly. As shown in Figure 3(c,d), unlike the GP-crosslinked gelatin hydrogel, there were simply intramolecular and short-range intermolecular crosslinks present in the EDC- and EDC/NHS-crosslinked hydrogels. Consequently, the cohesive structures of the EDC- and EDC/NHS-crosslinked gelatin hydrogels were readily broken by the shear force of the spindle in the viscometer during rotation. The increase in the viscosity for the EDC/NHS-crosslinked gelatin hydrogel was observed again after the addition of GP [EDC/NHS–GP-crosslinked hydrogel; Fig. 5(e)] because of the formation of long-range intermolecular crosslinks by polymerized GP molecules.

The phenomenon of the reduction of the viscosity peak was more prominent for the EDC-crosslinked gelatin hydrogel than for the EDC/NHS-crosslinked gelatin hydrogel. Additionally, the viscosity of the EDC/NHS-crosslinked gelatin hydrogel (340 cP) was greater than that of the EDC-crosslinked gelatin hydrogel (70 cP) thereafter [Fig. 5(c,d)]. As shown in Figure 3(c), besides physical entanglements and intramolecular and short-range intermolecular crosslinks, there were some pendant side branches (the *N*-acylurea groups) attached to the modified gelatin molecules in the EDC-crosslinked hydrogel. In

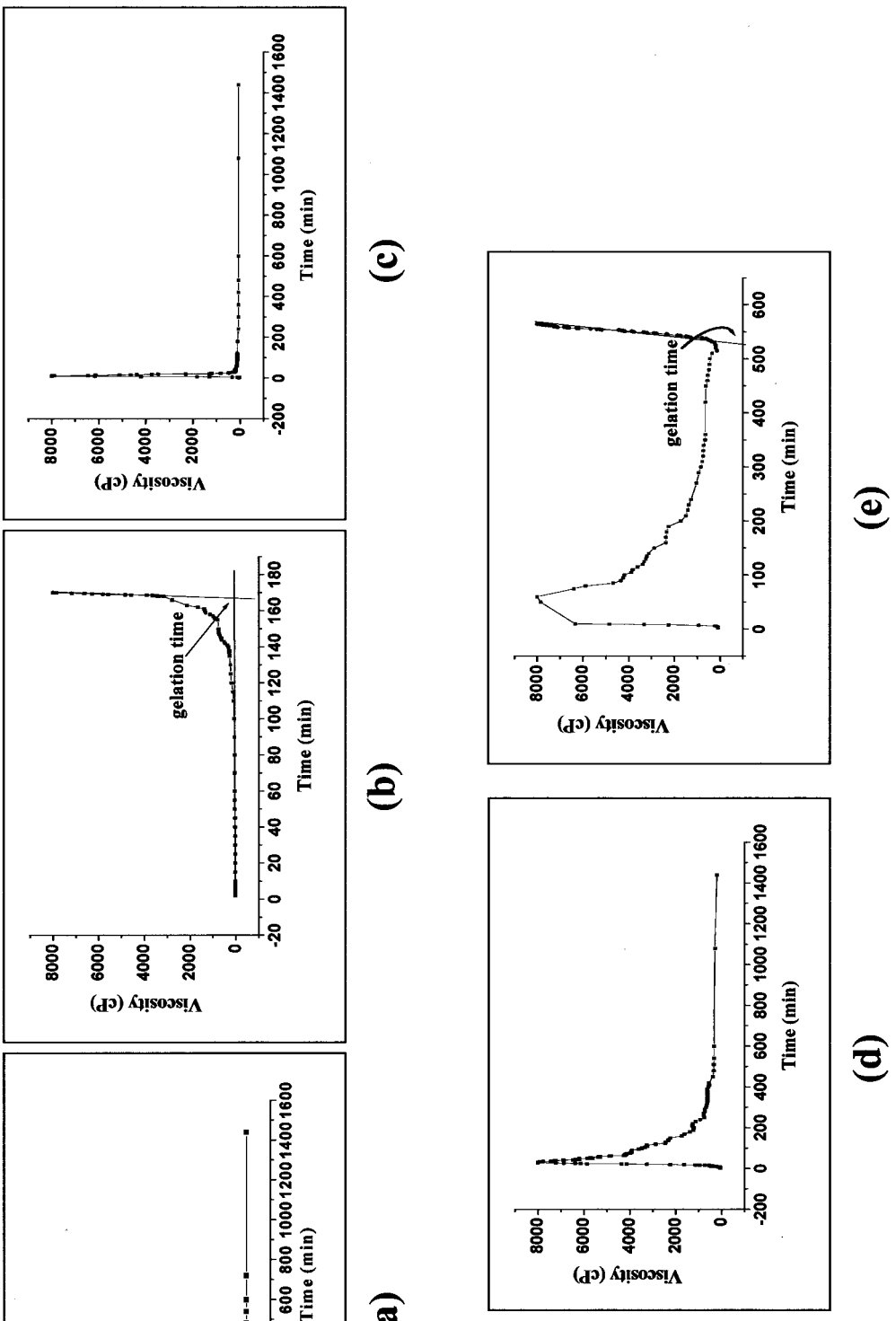


Figure 5 Examples of the time course in the measurement of the viscosity for (a) a fresh gelatin hydrogel, (b) a GP-crosslinked gelatin hydrogel, (c) an EDC-crosslinked gelatin hydrogel, (d) an EDC/NHS-crosslinked gelatin hydrogel, and (e) an EDC/NHS–GP-crosslinked gelatin hydrogel. The gelation time was defined as the duration required for the viscosity of the test adhesive to increase abruptly.

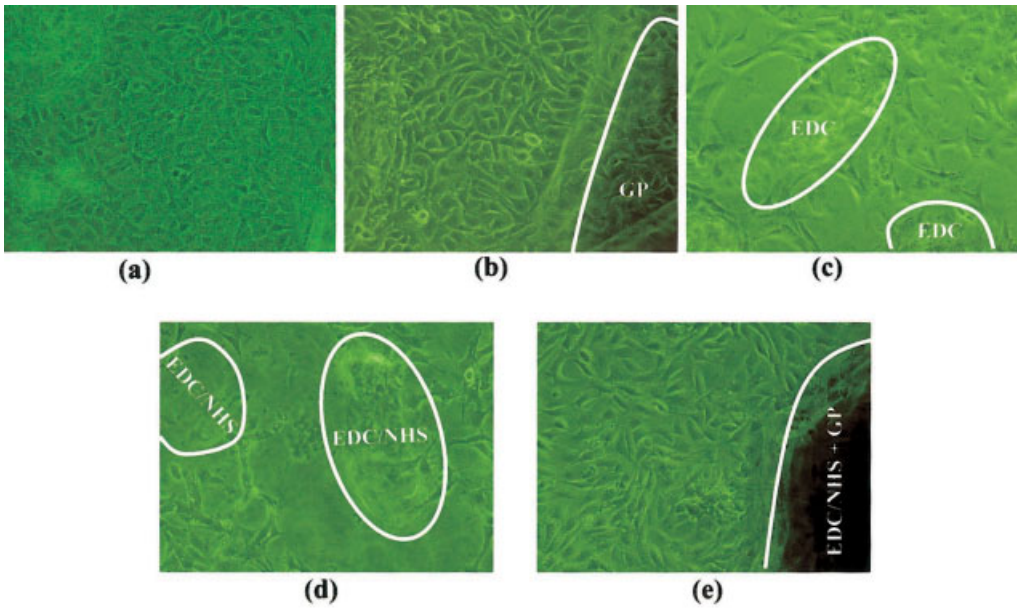


Figure 6 Photomicrographs (original magnification = 200×) of 3T3 fibroblasts cultured in the vicinity of (a) a fresh gelatin hydrogel, (b) a GP-crosslinked gelatin hydrogel, (c) an EDC-crosslinked gelatin hydrogel, (d) an EDC/NHS-crosslinked gelatin hydrogel, and (e) an EDC/NHS–GP-crosslinked gelatin hydrogel.

contrast, the addition of NHS to the carbodiimide-containing solution was effective in increasing the number of crosslinks introduced into the gelatin molecules [Fig. 3(d)]. Thus, the viscosity of the carbodiimide-crosslinked hydrogel in the presence of NHS (EDC/NHS) was significantly greater than that in the absence of NHS (EDC).

Cytotoxicity

The potential cytotoxic sources of a chemically crosslinked adhesive may be from residues, such as the remaining crosslinking agent and particles degraded from the adhesive. Cytotoxicity testing of chemical substances can be accomplished by either *in vivo* or *in vitro* experiments. Nishi et al.[37] reported that the results obtained in their *in vitro* measurements correlated well with those acquired in *in vivo* experiments in the cytotoxicity testing of chemical substances.

Figure 6(a–e) presents examples of photomicrographs of 3T3 fibroblasts cultured in the vicinity of each test group for 24 h. The fresh group (the gelatin hydrogel without crosslinking) was dissolved completely right after being placed in the culture medium [Fig. 6(a)], whereas the EDC- and EDC/NHS-crosslinked gelatin hydrogels were dissolved into small fragments within 10 min [Fig. 6(c,d)]. In contrast, the GP- and EDC/NHS–GP-crosslinked gelatin hydrogels remained intact in the medium throughout the entire course of the study [Fig. 6(b,e)]. Again, this was because there were only intramolecular and short-range intermolecular crosslinks in the EDC- and EDC/NHS-crosslinked gelatin hydrogels, whereas there were additional long-range intermolecular crosslinks within the GP-crosslinked gelatin hydrogels [Fig. 3(a–e)].

The cells cultured in the medium containing the fresh sample and in the vicinity of the GP-crosslinked gelatin hydrogel were confluent [Fig. 6(a,b)]. This indicated that the residues released form the GP-crosslinked gelatin hydrogel had no toxic effects on the seeded cells. The cytotoxicity of GP was previously studied by our group *in vitro* with 3T3 fibroblasts.[38] Glutaraldehyde was used as a control. The results obtained in the methyl thiazole tetrazolium (MTT) assay and the colony-forming assay suggested that the cytotoxicity of GP was significantly lower than that of glutaraldehyde. In contrast, the cells cultured in the vicinity of the EDC-, EDC/NHS-, and EDC/NHS–GP-crosslinked gelatin hydrogels were not as confluent [Fig. 6(c–e)]. In the polymerization of the EDC- and EDC/NHS-crosslinked gelatin hydrogels, most of the carbodiimide used was converted into urea derivatives, which have been reported to be relatively nontoxic.[2]

CONCLUSIONS

Carbodiimide (in the absence or presence of NHS) may form intramolecular crosslinks within a gelatin molecule or short-range intermolecular crosslinks between two adjacent gelatin molecules. Besides intramolecular and short-range intermolecular

crosslinks, additional long-range intermolecular crosslinks may be introduced into a GP-crosslinked gelatin hydrogel via the polymerization of GP molecules before crosslinking. A GP-crosslinked gelatin hydrogel is less cytotoxic than a carbodiimide-crosslinked gelatin hydrogel.

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