In Situ Simultaneous Protein−Polysaccharide Bioconjugation and Hydrogelation Using Horseradish Peroxidase

Shinji Sakai,*,† Tomohiro Matsuyama,‡ Keisuke Hirose,‡ and Koei Kawakami‡

Division of Chemical Engineering, Department of Materials Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka, 560-8531, Japan, and Department of Chemical Engineering, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

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We propose the peroxidase-catalyzed simultaneous conjugation and hydrogelation of polysaccharide and protein derivatives, each possessing phenolic hydroxyl (Ph) moieties, as a novel route for obtaining protein−polysaccharide conjugate hydrogels. We used alginate, gelatin, and albumin derivatives bearing Ph moieties (Alg-Ph, Gela-Ph, and Alb-Ph) to demonstrate the feasibility. The gelation time of conjugate gels decreased with decreasing H_2O_2 concentration and with increasing horseradish peroxidase concentration. Gelation time was controllable from a few seconds to 6 min. The repulsion force detected at 40% compression of a conjugate gel obtained from a mixture of Alg-Ph and Gela-Ph at 1.0% (w/v), respectively, was more than 2.8 times larger than that detected for gels produced from 3.0% (w/v) Gela-Ph or 2.0% (w/v) Alg-Ph alone. Cell adhesiveness of gels was tunable by changing the type of protein derivative. A gel from Gela-Ph and Alg-Ph showed higher cell adhesiveness than Alg-Ph gel, but a gel produced from Alb-Ph and Alg-Ph showed a lower cell adhesiveness than Alg-Ph gel. The conjugate gel was degradable by degrading alginate molecules using the nonproteolytic enzyme alginate lyase. The tunable gelation, mechanical properties, and cell adhesiveness of polysaccharide−protein conjugate hydrogels obtained through peroxidase-catalyzed gelation indicates great potential for a wide range of applications, such as scaffolds for tissue engineering and carriers for drug delivery system.

Introduction

Hydrogels are attractive materials for a wide range of biopharmaceutical and biomedical applications.[1,2] A variety of approaches for obtaining hydrogels have been reported for these applications,[3–5] with the notable recent emergence of enzyme-mediated gelation.[6–11] An attractive aspect of enzymatic gelation is of the mild reaction conditions suitable for biomacromolecules and living cells. Recently, the horseradish peroxidase (HRP)-catalyzed gelation system has been studied as a novel and effective route for obtaining hydrogels. HRP is the enzyme that catalyzes the oxidation of donors using H_2O_2, resulting in polyphenols linked at the aromatic ring by C−C and C−O coupling of phenolic hydroxyl (Ph) moieties. The versatility of the HRP-catalyzed gelation system has been shown by the gelation of polysaccharide derivatives bearing Ph moieties such as hyaluronic acid,[8,9] sodium alginate,[12] dextran,[11] chitosan,[10] and sodium carboxymethylcellulose.[13,14] More recently, we revealed that protein hydrogels can be prepared using the same methodology by introducing Ph moieties in gelatin.[15] On the basis of these previous reports, our main question is whether we can obtain protein−polysaccharide conjugate hydrogels via the HRP-catalyzed cross-linking reaction in a solution containing both protein and polysaccharide derivatives bearing Ph moieties. No report has yet shown preparation of protein-polysaccharide conjugates using HRP (Scheme 1) despite the huge potential of the resultant conjugated protein−polysaccharide materials.

Hydrogels derived from proteins and polysaccharides are similar to the extracellular matrices of tissues composed of various amino acids and sugar-based macromolecules. The development of hydrogels composed of protein−polysaccharide conjugates is challenging. A variety of studies has been reported for developing synthetic protein−polysaccharide conjugates. It has been well established that the functional properties of proteins, such as emulsifying properties[16] and thermal stability,[17] can be further improved by covalent bonding with polysaccharides. In addition, protein−polysaccharide conjugate vaccines are widely recognized as more effective than polysaccharide vaccines for bacterial infection diseases.[18] In tissue engineering applications, the conjugation of proteins and polysaccharides is effective for controlling degradation behavior, mechanical properties, and cell adhesion of scaffolds.[19,20] In this article, we report simultaneous protein−polysaccharide bioconjugation and hydrogelation through HRP-catalyzed cross-linking reactions. We studied the gelation and mechanical properties of resultant gels using sodium alginate and gelatin derivatives containing Ph moieties (Alg-Ph and Gela-Ph). In addition, we studied the effect of bioconjugation of Alg-Ph and protein derivatives on the behavior of cells attached onto the resultant gels using Gela-Ph and an albumin derivative with Ph moieties (Alb-Ph). It is known that the surfaces covered with gelatin have a high cell adhesiveness.[4] In contrast, covering surfaces with albumin suppresses cellular adhesion because the albumin layer inhibits adsorption of proteins, including those that support cellular adhesion.[21] It is well-recognized that controlling the cellular response to implanted materials is an important issue in tissue regeneration and integration.[22] As one potential application of the protein−polysaccharide hydrogels, we harvested a cell sheet from a confluent monolayer formed on a Gela-Ph + Alg-Ph hydrogel by degrading alginate molecules within the conjugate system using alginate lyase.

* To whom correspondence should be addressed. Tel: +81-6-6850-6252. Fax: +81-6-6850-6254. E-mail: sakai@cheng.es.osaka-u.ac.jp.
† Osaka University.
‡ Kyushu University.

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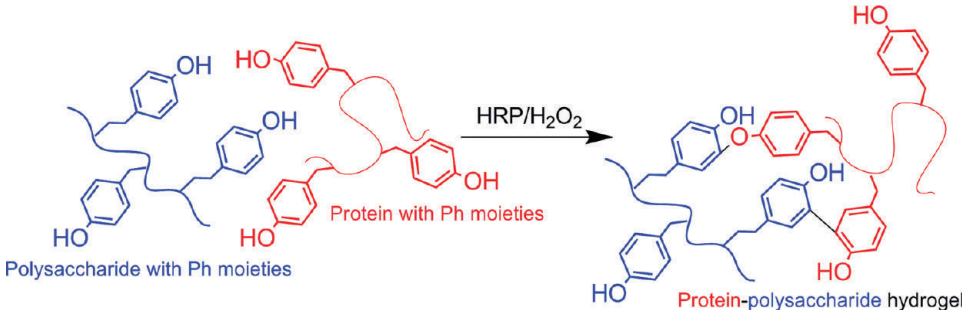
Scheme 1. Schematic of Simultaneous Protein−Polysaccharide Conjugation and Hydrogelation by the HRP-Catalyzed Oxidation Reaction



Materials and Methods

Materials. I-1G sodium alginate with a high content of guluronic acid and molecular weight of 70 00 was purchased from Kimica (Tokyo, Japan). Gelatin (type A from porcine skin, 300 Bloom), tyramine hydrochloride, and alginate lyase from *Flavobacterium* sp. were obtained from Sigma Chemicals (St. Louis, MO). Bovine albumin, HRP (260 units/mg), and *N*-hydroxysulfosuccinimide (NHS) were obtained from Wako Chemicals (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from the Peptide Institute (Osaka, Japan). Aqueous H_2O_2 solution (30% (w/w)) was obtained from Kanto Chemicals (Tokyo, Japan). Mouse fibroblast L929 (RCB1451), provided by the Riken Cell Bank (Tsukuba, Japan), was used to evaluate cellular adhesion and proliferation on gels. The cells were grown in minimum essential medium (MEM, Wako Chem., Osaka, Japan) supplemented with 5.0% (v/v) fetal bovine serum, 1×10^5 units/l penicillin, and 100 mg/L streptomycin. The cells were cultured under a humidified atmosphere at 37 °C with 5% CO_2.

Synthesis of Alg-Ph, Gela-Ph, and Alb-Ph. Alg-Ph, Gela-Ph, and Alb-Ph were synthesized by combining sodium alginate, gelatin, or bovine albumin with tyramine hydrochloride via the carbodiimide-mediated condensation of the carboxyl moieties of the molecules and amino moieties of tyramine, as previously reported, using EDC and NHS.[12,15] Ph moieties incorporated into 1 mg of sodium alginate, gelatin, and bovine albumin were measured using an ultraviolet−visible spectrometer (UV-2500, Shimadzu, Kyoto, Japan) at an absorbance of 275 nm, giving (1.5, 1.4, and 1.4) $\times 10^{-7}$ Ph moieties/mg derivative, respectively.

Gelation Time. Gelation times of solutions were determined in calcium-free Krebs Ringer Hepes buffer solutions (CF-KRH, pH 7.4) containing Gela-Ph and/or Alg-Ph. The solutions were poured in the wells of a 48-well plate at 500 μL/well. Subsequently, 50 μL of HRP solution was poured in each well and stirred at 80 rpm using magnetic stirrer bars (10 mm length, 4 mm diameter). Finally, 50 μL of H_2O_2 solution was poured in each well as stirring continued. The formation of the gel state was signaled when the magnetic stirring was hindered and the surface of the solution swelled. The concentrations of Gela-Ph and Alg-Ph in the mixture solutions were 1.0% (w/v). Concentrations of each derivative in single-component solutions were 1.0 (w/v) or 2.0% (w/v). The concentrations of H_2O_2 and HRP were varied to determine the effects of these parameters on gelation time.

Mechanical Properties. The CF-KRH solution (pH 7.0) containing either or both of Gela-Ph and Alg-Ph (dissolved) was mixed with 1/11 vol of concentrated solutions of HRP and H_2O_2. The concentrations of Gela-Ph and Alg-Ph in the mixture solutions were 1.0% (w/v). The concentrations of each derivative in single-component solutions were 3.0 (w/v) and 2.0% (w/v), respectively. Concentrations of HRP and H_2O_2 in the final mixture solution were 1 unit/mL and 15 mM, respectively. Immediately after mixing, the solutions were poured in molds (15 mm width × 15 mm length × 5 mm depth) at 1.0 mL/well. After 12 h of standing at room temperature, gels were collected from the molds. Compression−repulsion force profiles for the specimens were measured using a Table-Top material tester (EZ-Test-500N, Shimadzu, Kyoto, Japan) at a compression rate of 5 mm/min.

Cell Adhesion and Proliferation on Gel Sheets. The CF-KRH solution (pH 7.0) with Alg-Ph and/or one of protein derivatives, Gela-Ph and Alb-Ph (dissolved), was mixed with 1/11 vol of concentrated solutions of HRP and H_2O_2 in a six-well cell culture dish. The volume of the mixture in each well was 1 mL. Immediately after mixing, the mixture solutions were spread to cover the bottoms of the wells completely before gelation. After 4 h of standing at 37 °C, CF-KRH was poured in each well at 5 mL/well. After additional standing for 4 h, the CF-KRH in each well was removed, and the wells were subsequently rinsed four times with cell culture medium to remove residual H_2O_2 and HRP. L929 cells suspended in MEM containing FBS were then seeded in each well at 5.0×10^5 or 5.0×10^4 cells/well to study adhesion of cells at 4 h after seeding and their growth profiles for 168 h, respectively. After an appropriate incubation period, the cells were recovered by treatment with trypsin to determine the number of cells adhering to the gels.

Alg-Ph Degradation. L929 cells were allowed to grow confluently on Gela-Ph + Alg-Ph gels in six-well cell culture dishes. To six wells containing medium, a medium containing concentrated alginate lyase was added to obtain a final concentration of 0.2 mg/mL. Changes in morphology after the addition of alginate lyase were observed using an optical microscope. After detaching cell sheets, they were gently aspirated using a glass pipet and transferred to a fresh cell culture dish to evaluate their reattachment onto other surfaces. Gela-Ph gel was used as a control.

Results and Discussion

Gelation Time. One feature of HRP-catalyzed gelation of solutions of polymers bearing Ph moieties is that the solutions are gellable within dozens of seconds, even within seconds, by controlling reaction conditions such as concentration of HRP and H_2O_2.[9,12,14] This behavior has been shown only for solutions containing a single component bearing Ph moieties.[9,12,14] In this study, first, we investigated whether we could control gelation time of a mixture of Alg-Ph and Gela-Ph. Figure 1 shows the dependence of gelation time on (a) H_2O_2 and (b) HRP concentrations for solutions containing Alg-Ph and Gela-Ph at 1.0% (w/v), respectively. Under the conditions of this study, gelation times of the mixture solutions were changed from seconds to ~6 min. The trends for the time required for gelation under different HRP and H_2O_2 concentrations were the same as those of systems containing a single component bearing Ph moieties.[10,12,14,15] Gelation time decreased with decreasing H_2O_2 concentration (Figure 1a) and increasing HRP concentration (Figure 1b). The longer gelation times resulting from higher concentrations of H_2O_2 can be explained by the deactivation of HRP by H_2O_2.[23,24] Comparing the times necessary for gelation between the solutions containing either or both of Gela-Ph and Alg-Ph at 1.0% (w/v), respectively, the time detected for the mixture solution (78 ± 6 s) was intermediate between those detected for 1.0% (w/v) Alg-Ph (19 ± 3 s) and 1.0% (w/v) Gela-

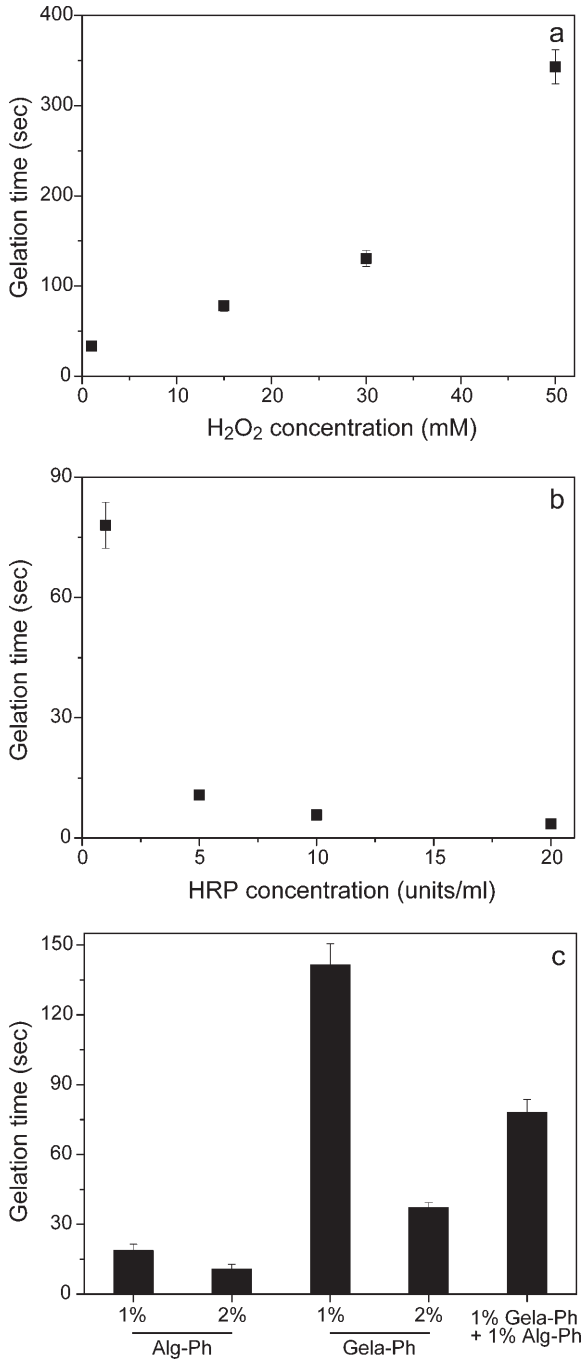


Figure 1. Dependence of gelation time for the mixture of Gela-Ph 1.0% (w/v) + Alg-Ph 1.0% (w/v) on (a) H_2O_2 concentration and (b) HRP concentration. Concentrations of HRP and H_2O_2 were fixed at (a) 1 unit/mL and (b) 15 mM, respectively. (c) Gelation time of a solution containing single or double components at 1.0 (w/v) or 2.0% (w/v) under 1 unit/mL HRP and 15 mM H_2O_2. Values represent the mean ($n = 4$) and standard deviation.

Ph (142 ± 9 s) (Figure 1c). Furthermore, the time necessary for gelation was more than twice that for 2.0% (w/v) single-component solutions despite the same content of polymers with a similar content of Ph moieties. These findings indicate that interactions between protein and polysaccharide, for example, electrostatic interactions, affect the gelation of mixture solutions.

Mechanical Properties. An attractive feature resulting from conjugation of proteins and polysaccharides is that the resultant gels show different mechanical properties from those observed in the gels obtained from either polysaccharide or protein alone.[19,20] Figure 2 shows the compression−repulsion force

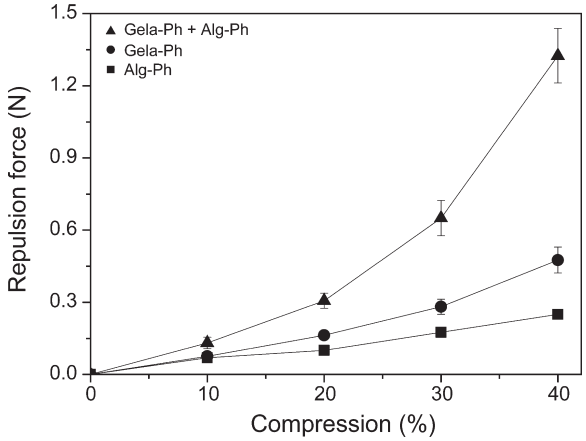


Figure 2. Compression−repulsion force profiles of gels prepared from 3.0% (w/v) Gela-Ph, 2.0% (w/v) Alg-Ph, and 1.0% (w/v) Gela-Ph + 1.0% (w/v) Alg-Ph solutions. Values represent the mean ($n = 4$) and standard deviation.

profiles, up to 40% compression, of the gels prepared from 3.0% (w/v) Gela-Ph solution, 2.0% (w/v) Alg-Ph solution, and a mixture solution containing Gela-Ph and Alg-Ph at 1.0% (w/v), respectively. The repulsion forces detected at each compression point were the highest for the gel prepared from the mixture solution. For example, the repulsion force detected at 40% compression for the gel obtained from the mixture of Gela-Ph and Alg-Ph (1.33 ± 0.11 N) was more than 2.8 times larger than those detected for the gels from Gela-Ph (0.48 ± 0.05 N) or Alg-Ph (0.25 N) alone. This increase in repulsion force detected for the gel prepared from the mixture solution was interpreted as a consequence of the interaction between alginate and gelatin derivatives. This result is in agreement with previous reports for a gel obtained from nonmodified gelatin and sodium-alginate.[25,26] The formation of intermolecular hydrogen bonds and ionic interactions between alginate and gelatin molecules after blending in an aqueous solution, resulting in the enhancement of mechanical strength, was reported for the resultant constructs, fibers,[26] and films.[25] From these results on mechanical properties, it can be concluded that interactions between polysaccharide and protein derivatives affect the mechanical properties of the resultant gels obtained through HRP-catalyzed cross-linking reaction and the time necessary for gelation.

Cell Adhesion and Proliferation on Gel Sheets. For evaluating successful conjugation and the possibility of controlling cellular response to protein−polysaccharide conjugate hydrogels, we prepared conjugate gels from a Gela-Ph + Alg-Ph mixture solution and an Alb-Ph + Alg-Ph mixture solution and seeded adherent L929 cells onto them. As previously reported,[15] the gel obtained from Gela-Ph alone showed very high cell adhesiveness: L929 cells adhered and spread (Figure 3c) and >98% of seeded cells adhered onto the gel at 4 h after seeding (Figure 4). In contrast, the gel obtained from Alb-Ph alone showed the lowest cell adhesiveness: L929 cells showed round shapes (Figure 3e) and only 13.8% of seeded cells adhered onto the gel at 4 h after seeding. Morphology of the cells on the Alg-Ph gel (Figure 3a) were almost the same as those on the Alb-Ph gel (Figure 3e), but the percentage of adhered cells on Alg-Ph gel was 36.9%. The higher cell adhesiveness of the Alg-Ph gel than the Alb-Ph gel can be recognized from the difference in morphologies at 72 h of cultivation (Figure 3b, f): the cells on the Alg-Ph gel were spread out, but those on Alb-Ph had a round shape. Alginate is known as an antiadhesive biopolymer[27] and has been used for suppressing cellular

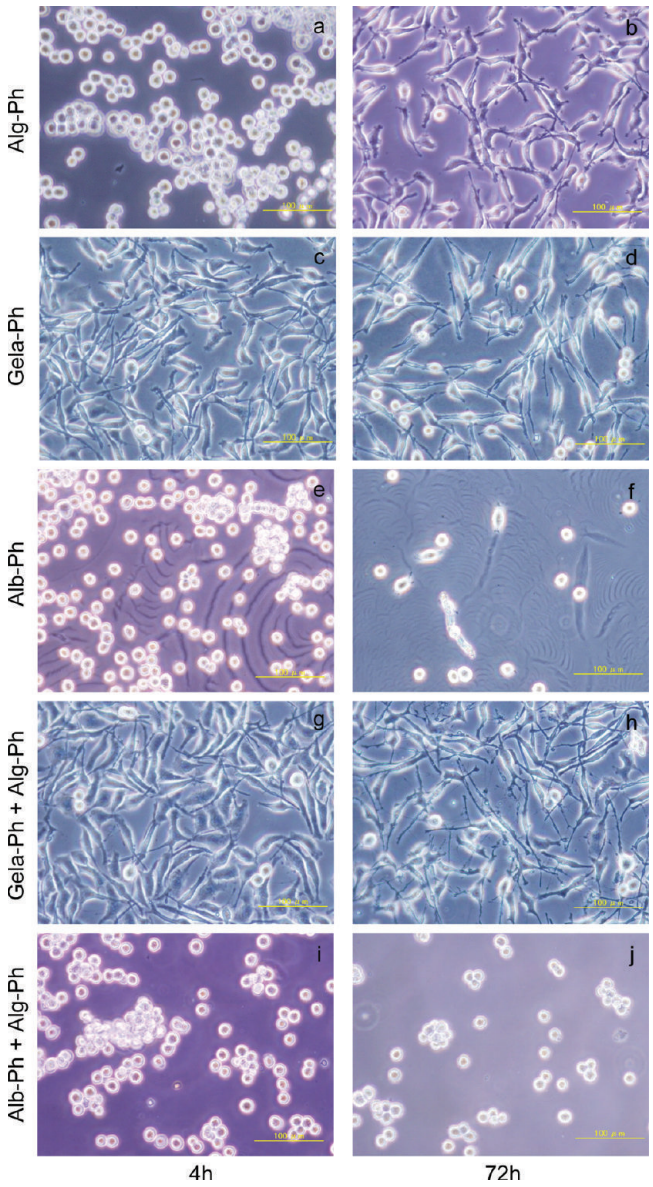


Figure 3. Photomicrographs of L929 cells on Gela-Ph, Alg-Ph, Alb-Ph, Gela-Ph + Alg-Ph, and Alb-Ph + Alg-Ph gels 4 h after seeding. Density of seeded cells: 5.0×10^5 (4 h) and 5.0×10^4 cells/well (72 h). Bars: 100 μm.

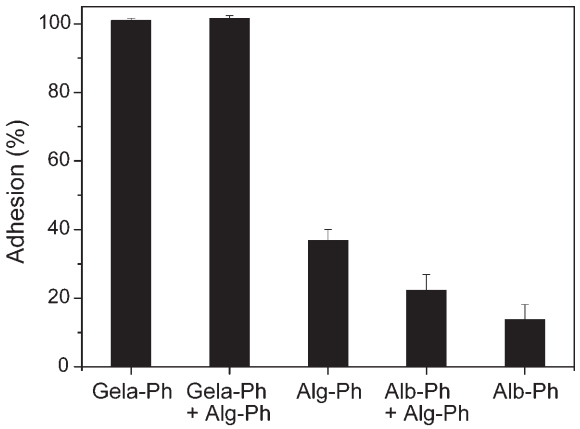


Figure 4. Percentages of adhering L929 cells on Gela-Ph, Alg-Ph, Alb-Ph, Gela-Ph + Alg-Ph, and Alb-Ph + Alg-Ph gels 4 h after seeding. Values represent the mean (n = 3 or 4) and standard deviation.

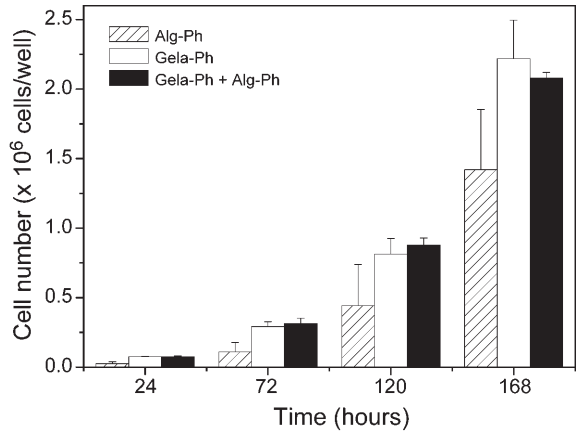


Figure 5. Growth profiles of L929 cells on Alg-Ph, Gela-Ph, and Gela-Ph + Alg-Ph gels. Values represent the mean (n = 3 or 4) and standard deviation.

adhesion on cell-enclosing microcapsules for cell therapy when they are implanted in vivo.[28,29] The moderate L929 cells adhesion can be explained by the increased hydrophobicity caused by the cross-linked Ph groups, resulting in a greater adsorption of cell adhesive proteins. We have showed for Alg-Ph and carboxymethyl cellulose bearing Ph moieties that cell adhesiveness of the gels obtained through the HRP-catalyzed cross-linking reaction between Ph moieties could be enhanced by increasing the content of cross-linked Ph moieties.[14,30] In this study, we prepared the Alg-Ph gel under the condition resulting in the moderate cell adhesiveness for evaluating the effect of conjugation with Gela-Ph and Alb-Ph. By conjugating Alg-Ph with Gela-Ph, the resultant gel showed a high cell adhesiveness, comparable to the Gela-Ph gel (Figure 4). Morphologies of the cells at 4 and 72 h of culture on the conjugate gel (Figure 3g,h) were also the same as those on the Gela-Ph gel (Figure 3c,d). Furthermore, the growth of cells on Gela-Ph + Alg-Ph gel was almost the same as that on the Gela-Ph gel (Figure 5). In contrast, by conjugating with Alb-Ph, the resultant gel showed lower adhesiveness (22.3%) than the Alg-Ph gel (36.9%) at 4 h after seeding. At 4 and 72 h of culture, the cells on the Alb-Ph + Alg-Ph mixture gel had a round shape (Figure 3i,j), the same with those observed on the Alb-Ph gel (Figure 3e,f). Considering the contrasting cell adhesiveness of the Gela-Ph and Alb-Ph gels, these results clearly demonstrate that HRP-catalyzed cross-linking of Ph moieties is an effective route for in situ simultaneous protein−polysaccharide conjugation and hydrogelation. In addition, it was also demonstrated that the cellular response to the polysaccharide−protein conjugate hydrogels is tunable by changing the type of protein used.

Alg-Ph Degradation. We have reported that a gel formed from Alg-Ph alone was degradable using alginate lyase without giving any obvious harmful effect to mammalian cells enclosed in and adhered onto the gels.[30−33] We examined the effect of degrading only Alg-Ph molecules in a conjugate system of Gela-Ph and Alg-Ph gel with a confluent monolayer of L929 cells (Figure 6a). By soaking in a medium containing alginate lyase at 0.2 mg/mL, complete detachment of the cell sheets was observed for the cells on Gela-Ph + Alg-Ph gel within several minutes (Figure 6b). When transferred to a fresh cell culture dish, the cell sheets, which could contact the bottom of the dish, resisted movement resulting from mild perturbation of the medium after ~30 min of transferring. At 1 h after transferring, obvious attachment and spreading of cells were observed (Figure 6c). Subsequently, the cells in the adhered cell sheet proliferated on the dish. In contrast, no detachment of the cell sheet was

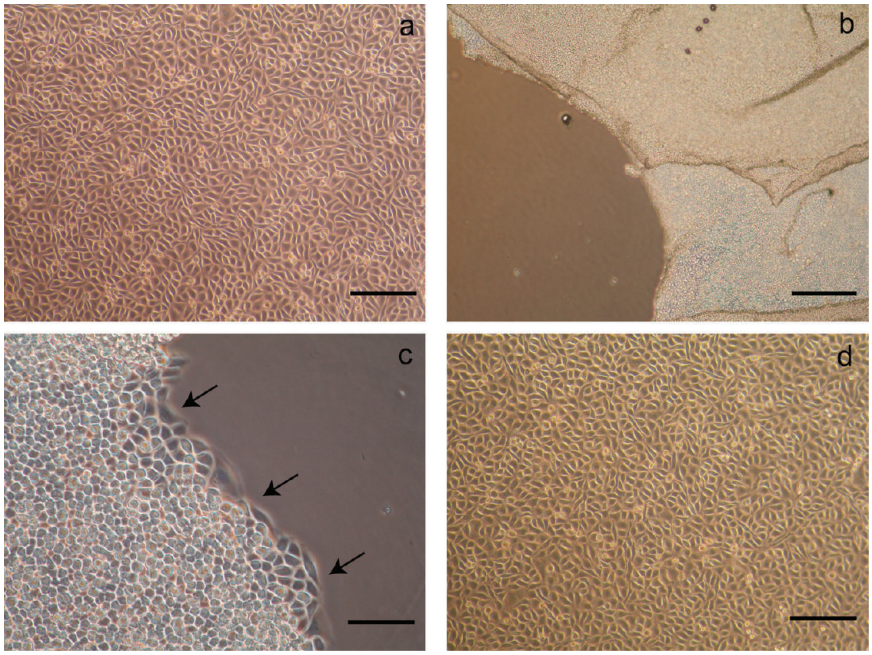


Figure 6. Photomicrographs of L929 cells cultured on (a−c) Gela-Ph + Alg-Ph gel and (d) Gela-Ph gel. (a) Confluent monolayer cells attached on Gela-Ph + Alg-Ph gel before soaking in medium containing alginate lyase. (b) Detached cell sheet after 5 min of soaking in the medium containing alginate lyase. (c) Adhered cell sheet on a fresh cell culture dish after 1 h of transferring. (d) Confluent monolayer cells nondetached from Gela-Ph gel after 1 h of soaking in the medium containing alginate lyase. Bars: (a,d) 100, (b) 250, and (c) 50 μm. The arrows in part c indicate cells adhered to the cell culture dish.

observed for the cells on the Gela-Ph gel, even after 1 h of exposure to the medium containing alginate lyase (Figure 6d). These results indicate that we could harvest cell sheet from the Gela-Ph + Alg-Ph conjugate gel using a harmless detachment process by degrading only the Alg-Ph molecules with alginate lyase but without using protease. This behavior would of course depend on the content of each component, but the result indicates that we can prepare a conjugate gel via the HRP-catalyzed cross-linking reaction that is degradable only by degrading the polysaccharide molecules. By utilizing the reported cytocompatible gelation condition[8,9,15] and a process exploiting in situ shape-conforming properties, we think a system using HRP-mediated conjugation accompanying gelation would be useful for fabricating cells sheets with complicated geometries by making impressions of the surfaces of organs to which the resultant cell sheets could be attached.

Successful synthesis of the derivatives of polysaccharides bearing Ph moieties was reported for hyaluronic acid,[8,9] dextran,[11] chitosan,[10] and sodium carboxymethylcellulose[13,14] as well as sodium alginate.[12] In addition, there is a wide variety of potential materials and polysaccharides subject to degrading enzymes and also many proteins, to which Ph moieties can be introduced. Furthermore, it is known that functions, for example, metabolic function and growth, of cells are affected by contacting proteins.[34,35] Considering this and the results of this study, we believe that simultaneous conjugation and hydrogelation of polysaccharides and protein derivatives bearing Ph moieties using HRP has huge potential for biomedical and biopharmaceutical applications.

Conclusions

We have developed protein−polysaccharide conjugate hydrogels from derivatives of alginate and proteins, gelatin and albumin, all possessing Ph moieties through HRP-catalyzed enzymatic reaction. The conjugate gels showed different characteristics in gelation time, mechanical properties, and cell adhesion compared with gels obtained from either alginate or protein derivatives alone. Even for a conjugation system using Gela-Ph and Alg-Ph, we could obtain a gel within dozens of seconds by controlling the concentrations of HRP and H_2O_2. Higher resistance to compression was observed when Gela-Ph was conjugated with Alg-Ph compared with gels formed from Alg-Ph or Gela-Ph alone. Cell adhesiveness of the Alg-Ph gel was increased by conjugating with Gela-Ph and decreased by conjugating with Alb-Ph. Exposure of a Gela-Ph + Alg-Ph conjugate gel with a confluent L929 cell layer to alginate lyase demonstrated that the conjugate gel was degradable by degrading alginate molecules. This degradation resulted in detachment of the cell sheet within several minutes of exposure. We believe that the possibility of controlling the gelation, mechanical properties, and cell adhesiveness of the polysaccharide−protein conjugated gels and the degradability by a polysaccharide degrading enzyme indicate that the HRP-catalyzed simultaneous conjugation and hydrogelation system would be useful for the applications such as scaffolds for tissue engineering and carriers for drug delivery system.

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