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Control of cellular adhesiveness in an alginate-based hydrogel by varying peroxidase and H_2O_2 concentrations during gelation

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ABSTRACT

An aqueous solution of alginate possessing phenolic hydroxyl (Alg-Ph) groups is gellable via a horseradish peroxidase (HRP)-catalyzed oxidative crosslinking reaction between Ph groups, consuming H_2O_2 as an electron acceptor. This study evaluates the effect of H_2O_2 and HRP concentrations on cellular adhesiveness and proliferation on the resultant enzymatically crosslinked Alg-Ph gels. After 4 h of seeding, 81.1% of L929 fibroblast cells adhere to an Alg-Ph hydrogel prepared with 1 U ml^{-1} HRP and 1 mM H_2O_2. Increasing the concentration of H_2O_2 to 15 mM decreases the percentage of adhering cells to 28.4%. The cellular adhesion at this H_2O_2 concentration is increased to 82.6% by increasing the HRP concentration to 10 U ml^{-1}. The cells adhering to the Alg-Ph hydrogels with higher cellular adhesiveness establish a confluent monolayer during 168 h of culture. A cell sheet can then be harvested within 5 min of immersion in a medium containing alginate lyase at 1.0 mg ml^{-1}. The harvested cell sheet re-adhere, and the cells contained in the sheet proliferate after being transferred to another cell culture dish.

1. Introduction

Enzymatic crosslinking has attracted attention as a novel route for fabricating hydrogels for biomedical applications, such as drug delivery or tissue engineering, because the mild reaction conditions involved are compatible with biomacromolecules and mammalian cells [1–4]. Horseradish peroxidase (HRP) and transglutaminase are two enzymes that have been used for enzymatic hydrogel preparation, and the former is used in the present paper. HRP is an oxidoreductase which catalyzes the oxidation of a variety of substances, using H_2O_2 as an electron acceptor [5]. In HRP-catalyzed gelation systems, the enzyme catalyzes the oxidation of phenolic hydroxyl (Ph) groups, resulting in polyphenols linked at the aromatic ring by C–C and C–O coupling between the Ph groups (Fig. 1). In pioneering work using this system for biomedical applications, Kurisawa et al. [2] subcutaneously injected a solution of a hyaluronic acid derivative possessing Ph groups into mice, along with peroxidase and H_2O_2, to demonstrate feasibility. The effectiveness of incorporating Ph groups into polymers for HRP-catalyzed gelation has been shown for other biocompatible materials, e.g., dextran [6], alginate [7], carboxymethylcellulose (CMC) [8], chitosan [9] and gelatin [3].

The mechanical properties of gels synthesized by HRP catalysis systems are affected by the Ph group content and the concentration of HRP, H_2O_2 and the polymers bearing the Ph groups [7,10]. In addition, it was reported that controlling the content of Ph groups in CMC was effective in controlling cellular adhesion and proliferation on the resultant gel [11]. Notably, a hydrogel obtained from CMC, which is known to be an anti-adhesive biopolymer [12,13], showed cellular adhesiveness after gelation via crosslinking of Ph groups [11,14]. The acquisition of cellular adhesiveness was explained by the enhancement of hydrophobicity caused by the crosslinking of Ph groups, resulting in greater adsorption of cell-adhesive protein [11].

The aim of this study was to determine whether cellular adhesiveness could be generated in alginate, also known as anti-adhesive biopolymer, and to explore the effects of HRP and H_2O_2 concentration during peroxidase-catalyzed gelation on the cellular adhesiveness of the resultant gels. Alginates are an interesting family of polymers which have been studied for a wide variety of biomedical applications [15] owing to their high biocompatibility and low toxicity and the relative ease with which they can gelate with divalent cations under very mild conditions compatible with biomacromolecules and living cells [16]. It was demonstrated that alginate incorporating Ph groups (Alg-Ph) at <10% of the total uronic acid units in the alginate molecule was gellable either by the conventional route, using divalent cations, or by the HRP-catalyzed reaction alone, as well as through both reactions [7]. One limitation of using native alginate gels for culturing mammalian cells is that lower cell-adhesiveness results, which is important because adhesion of cells is a strict requirement for their survival. Rowley et al. [17] covalently incorporated RGD-containing cell adhesion ligands into alginate molecules and showed promotion of cellular adhesion

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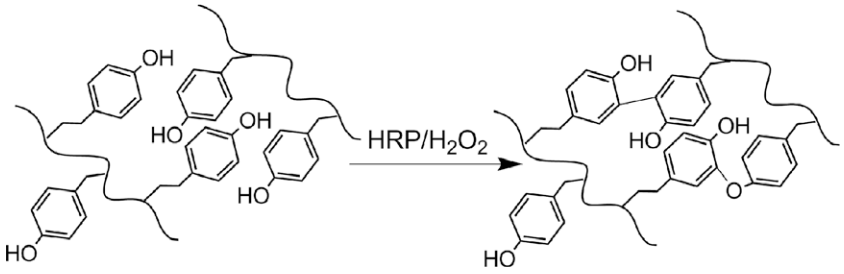

Fig. 1. Formation of hydrogels composed of polymer molecules possessing Ph groups by HRP-catalyzed oxidation reaction.

and proliferation. Another limitation of conventional, ionically gelled, native alginate gels is that they possess limited mechanical stability under physiological conditions [16]. Alginate gels possessing crosslinks formed by the HRP-catalyzed reaction showed higher stability than those that were crosslinked by divalent ions alone [7]. This study determined the effect of HRP and H_2O_2 concentrations during HRP-catalyzed reaction on the adhesion and proliferation of adherent cells. It also evaluated their effects on the mechanical properties of the resultant gels. In addition, the potential application of enzymatically crosslinked alginate gels as a substrate for cell sheet technology is described.

2. Materials and methods

2.1. Materials

Sodium alginate with a high guluronic acid content and a molecular weight of 70,000 (Kimica I-1G) was purchased from Kimica (Tokyo, Japan). Tyramine hydrochloride and 30% (w/w) H_2O_2 aqueous solution were obtained from Sigma (MO, USA) and Kanto Chemicals (Tokyo, Japan), respectively. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Peptide Institute (Osaka, Japan). HRP (170 U mg^{-1}) and hydroxysulfosuccinimide (NHS) were obtained from Wako Chemicals (Osaka, Japan). L929 fibroblasts (RCB1451), provided by the Riken Cell Bank (Tsukuba, Japan), were used to evaluate cellular adhesion and proliferation. They were grown in minimum essential medium (MEM; Wako Chemicals, Osaka, Japan) supplemented with 5% (v/v) fetal bovine serum, 1×10^5 U L^{-1} penicillin and 100 mg L^{-1} streptomycin. The cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2.

2.2. Synthesis of Alg-Ph

Synthesis of Alg-Ph containing 4.4 Ph groups per 100 U of uronic acid in the alginate molecules was based on a previously reported method [7]. Briefly, sodium alginate was dissolved in 50 mM morpholinoethanesulfonic acid buffer (pH 6.0) at 1.0% (w/v). Tyramine hydrochloride was added to the solution at a weight ratio of sodium alginate to tyramine hydrochloride of 1:0.703. To this solution, NHS and EDC were added at a weight ratio of 1:0.058:0.194 (sodium alginate:NHS:EDC). The mixture was gently stirred for 20 h at room temperature, and the resultant polymer was dialyzed against deionized water for 3 days. During the dialysis period, the existing water was replenished every 6–10 h. The sample was then lyophilized.

2.3. Gelation time

Gelation times of Alg-Ph solutions were determined in calcium-free Krebs–Ringer Hepes buffer (CF-KRH, pH 7.0) at room temperature. Alg-Ph solution was poured into a 48-well plate at 300 μl $well^{-1}$. Subsequently, 100 μl of HRP solutions was poured into each well and stirred at 80 rpm using magnetic stirrer bars (10 mm long, 4 mm diameter). Finally, 100 μl of H_2O_2 solution was poured into each well as stirring continued. Formation of the gel state was signaled when magnetic stirring was hindered and the surface of the solution swelled. The final concentration of Alg-Ph was 2.0% (w/v), and that of HRP was 1 or 10 U ml^{-1}. The final concentration of H_2O_2 was 1 mM, 15 mM or 30 mM for 1.0 U ml^{-1} HRP solution and 15 mM for 10 U ml^{-1} HRP solution.

2.4. Adhesion and proliferation of cells

The Alg-Ph gels for cellular adhesion and proliferation studies were prepared by pouring the mixture of Alg-Ph solution, HRP solution and H_2O_2 solution cooled at 4 °C into a 6-well plate at 1 ml $well^{-1}$. The poured mixture was spread to completely cover the bottom of the wells before gelation and allowed to stand 4 h at 37 °C. The final concentrations of Alg-Ph, HRP and H_2O_2 were the same as those for the gelation time study. CF-KRH was poured into each well at 5 ml $well^{-1}$. After standing for 4 h, the CF-KRH in each well was removed, and the wells were subsequently rinsed three times with cell culture medium to remove residual H_2O_2 and HRP. After removing the medium for rinsing, L929 cells suspended in MEM containing FBS were seeded onto the resultant Alg-Ph gels prepared at 5.0×10^5 or 5.0×10^4 cells $well^{-1}$ to study the adhesion of cells at 4 h after seeding and their growth profiles for 1 week, respectively. After an appropriate incubation period, the cells were recovered by treatment with trypsin to determine the number of adherent cells. The Alg-Ph gels were prepared from 2.0% (w/v) Alg-Ph solution. The final concentrations of HRP and H_2O_2 for obtaining the gels were the same as those used in the gelation time study.

2.5. Mechanical properties

Alg-Ph was dissolved in CF-KRH at 2.0% (w/v). To this solution, 1/10 vol. of CF-KRH containing HRP was added. To this mixture, 1/11 vol. of CF-KRH containing H_2O_2 was added. The final concentration of HRP was 1 U ml^{-1} or 10 U ml^{-1}, and H_2O_2 concentration was 1 mM, 15 mM or 30 mM. Immediately after mixing with a vortex mixer, the solution was poured into a mold (15 mm wide × 15 mm long × 5 mm deep) at 1.0 ml $well^{-1}$. After 12 h of standing at room temperature, gels were collected from the molds. Compression resistance force profiles for the specimens were measured using a Table-Top Material Tester (EZ-Test-500N, Shimadzu, Kyoto, Japan) at a compression rate of 2 mm min^{-1}.

2.6. Harvesting cell sheets

L929 cells were incubated on Alg-Ph gels in 6-well cell culture dishes. To each well containing medium, a medium containing