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Characterization of enzymatically gellable, phenolated linear poly(ethylene glycol) with different molecular weights for encapsulating living cells

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ABSTRACT

Enzymatic hydrogelation has received much attention due to the high biocompatibility and the ease of control of reaction kinetics under physiological conditions. In particular, horseradish peroxidase (HRP)-mediated phenol coupling reaction has great potential for developing in situ hydrogelation systems. Herein, we report the HRP-catalyzed preparation and characterization of hydrogels composed of a terminally bis-phenolated linear poly(ethylene glycol) (PEG-Ph-OH) with different molecular weights (M_ws 3100, 8800, 11,000, 20,000 g/mol). The gelation time of polymer solution can be controlled in the range from few second to few minute, suggestion that the PEG-Ph-OH has a potential as a in situ forming hydrogel. In addition, the physicochemical properties of the hydrogels, such as swelling ratio, mesh size and mechanical property, were controlled by the molecular weight of the PEG-Ph-OH. The results could be attributed to the alteration in the cross-linking density by the variation of molecular weight of the gel precursor. Furthermore, the viability of mammalian cells encapsulated in the PEG-Ph-OH hydrogels was approximately 90%. These results indicate that PEG-Ph-OH has potential for biomedical applications including tissue engineering.

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1. Introduction

Hydrogels are insoluble, three-dimensional, polymeric networks that absorb and retain significant amounts of water. A large number of different types of hydrogels have been studied widely for biomedical applications, such as controlled drug delivery systems and tissue engineering, because of their favorable physical and biological properties such as high water content, good biocompatibility, and modulability to the surrounding environment [1,2]. In situ-forming hydrogels in particular have attracted much attention because of their easy application based on minimal invasive techniques [3–5]. In situ gellable hydrogels can fill up defects in body tissues by injection using syringes for instance. A variety of approaches to in situ gel formation have been studied, with recent notable emergence of enzyme-mediated cross-linking reactions.

Here, we focus on cross-linking reactions involving horseradish peroxidase (HRP). HRP can catalyze the oxidation of phenolic hydroxyl (Ph-OH) moieties using hydrogen peroxide (H_2O_2) as an oxidant, resulting in the formation of polyphenols via aromatic ring coupling (C—C and C—O) between Ph-OH moieties. In recent years, the fabrication of hydrogels using HRP catalysis has been demonstrated through the conjugation of Ph-OH moieties with polysaccharides [6–8], proteins [9,10], and synthetic polymers [11–13]. The major advantage of the HRP system in comparison to other enzymes, such as transglutaminase, is the fast gelation rate [14]. The gelation time of polymer solutions using HRP catalysis can be controlled over a range from a few seconds to a few minutes by tuning the concentrations of the polymer, HRP, and H_2O_2. Thus, hydrogels prepared via HRP-catalyzed reactions have potential as in situ gellable materials.

Poly(ethylene glycol)-based hydrogels have received much attention in the field of drug delivery systems and tissue regeneration because they have highly desirable properties such as good biocompatibility, non-immunogenicity, and resistance to protein adsorption [15,16]. PEG hydrogels can be easily subjected to functionalization treatments, such as cellular adhesion, proliferation, migration, and proteolytic degradation, by incorporating bioactive

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peptides and/or proteins into the polymer network [17–20]. In the previous report, we synthesized linear PEG-based gel precursor (M_w 8800 g/mol) that was modified with tyramine to introduce Ph-OH moieties at both termini, generating PEG-Ph-OH hydrogels. The hydrogels were successfully employed in the preparation of protein–polymer hybrid hydrogels that could capture biotinylated biomolecules by co-cross-linking of PEG-Ph-OH with recombinant streptavidin fused with a peptide tag containing tyrosine residue [13].

In this study, we synthesized PEG-Ph-OH hydrogels of varying molecular weights to control the physicochemical properties of the resulting hydrogels. PEG with molecular weights of 3100, 8800, 11,000, and 20,000 g/mol was used as a polymer backbone and modified with tyramine at both termini. The dependence of the gelation kinetics, swelling ratio, mesh size, and mechanical property of the hydrogels on the PEG-Ph-OH molecular weight was investigated. In addition, cell encapsulation in the PEG-Ph-OH hydrogels was performed to evaluate their use as scaffold for tissue engineering.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG, M_ws 3100, 8800, 11,000, 20,000 g/mol) was provided from NOF Corporation (Tokyo, Japan). Tyramine and *p*-nitrophenyl chloroformate (NPC) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Horseradish peroxidase (HRP) and 30% (w/w) H_2O_2 aqueous solution were purchased from Wako Chemicals (Osaka, Japan). Cellstain-double staining kit was purchased from Dojindo (Kumamoto, Japan). Mouse fibroblast L929 cells (RCB1451) were provided by the Riken Cell Bank (Tsukuba, Japan).

2.2. Synthesis of poly(ethylene glycol)–tyramine conjugates

Poly(ethylene glycol)–tyramine conjugates (PEG-Ph-OH) were prepared as previously reported [13]. Briefly, PEG (M_ws 3100, 8800, 11,000, 20,000 g/mol, 4.0 mmol/OH) and triethylamine (TEA, 20.0 mmol) were dissolved in dichloromethane (DCM). The solution mixture was placed in an ice bath. NPC (20.0 mmol) dissolved in DCM was added dropwise to the mixture solution. The reaction proceeded in an ice bath for 4 h, and then at room temperature for more than 12 h with stirring under a nitrogen atmosphere. PEG activated with *p*-nitrophenyl-carbonated groups (PEG-NPC) was precipitated in excess diethyl ether, and the precipitates were washed and dried under vacuum.

PEG-NPC (1.67 mmol) was dissolved in *N*,*N*-dimethylformamide (DMF). Tyramine (16.7 mmol) dissolved in DMF was added to the PEG-NPC solution, then stirred overnight under a nitrogen atmosphere at room temperature. The product was precipitated in excess diethyl ether. The precipitate was dissolved in DCM and the solution was filtered to remove unreacted tyramine. The filtrate was concentrated under vacuum to afford PEG-Ph-OH as a white powder. The degree of substitution (DS) was determined by ^{1}H NMR (Bruker AV 300M spectrometer) by comparing the integrals of signals at δ 3.49 for PEG and δ 6.95 for Ph-OH. DSs of PEG-Ph-OHs were approximately 95% (M_w = 3100), 92% (8800), 94% (11,000) and 92% (20,000), respectively.

2.3. Gelation test and gelation time measurement of PEG-Ph-OH solution

PEG-Ph-OH hydrogel (500 μL) was prepared in a glass vessel (diameter: 10 mm) at room temperature. Each PEG-Ph-OH sample was dissolved in phosphate-buffered saline (PBS) (pH 7.4) at 3.33% (w/v). PBS solutions of HRP (100 μL, 5 units/mL) and H_2O_2 (100 μL, 50 mM) were added to the polymer solution (300 μL) with gentle pipetting. The final concentrations of PEG-Ph-OH, HRP, and H_2O_2 were 2% (w/v), 1 unit/mL, and 10 mM, respectively.

The gelation time of the PEG-Ph-OH aqueous solution was determined according to previous reports [8–10]. Each PEG-Ph-OH sample was dissolved in PBS (pH 7.4). The polymer solution was poured into a 48-well plate at 100 μL/well. Subsequently, 50 μL of HRP in PBS was poured into each well and the mixtures were stirred at 200 rpm using magnetic stirrer bars (length, 7 mm; width, 3 mm). Finally, 50 μL of H_2O_2 was poured into the well under stirring at room temperature. Dependence of the PEG-Ph-OH molecular weight on gelation time was evaluated at constant concentrations of PEG-Ph-OH, HRP, and H_2O_2. In addition, the effect of polymer and HRP concentrations on gelation time was evaluated. The final concentration of H_2O_2 was set at 10 mM. Gel formation was deemed to have occurred when motion of the magnetic stirrer bar was hindered and the surface of the mixture solution swelled.

2.4. Rheological experiment

Viscoelastic properties of the PEG-Ph-OH hydrogels were evaluated by rheological measurements on a MCR302 rheometer (Anton Paar, Graz, Austria) using a cone plate (diameter, 25 mm; 2.003°) in the oscillatory mode. First, 200 μL of PEG-Ph-OH-HRP mixture solution was placed on the rheometer stage. Subsequently, 100 μL of H_2O_2 was placed on the stage and the sample was immediately mixed by gentle pipetting. The upper plate was immediately lowered and measurement was started. The frequency and strain were set at 0.1 Hz and 0.1%, respectively. The measurement was allowed to proceed until the storage modulus (G') reached an equilibrium value. The final concentrations of PEG-Ph-OH, HRP, and H_2O_2 were 2 and 5% (w/v), 1 unit/mL, and 10 mM, respectively.

2.5. Equilibrium swelling ratio (Q_M), mesh size (ξ) and gel content of the PEG-Ph-OH hydrogels

Following hydrogelation, the PEG-Ph-OH hydrogels (final concentrations of PEG-Ph-OH, HRP and H_2O_2 were 2 or 5% (w/v), 1 unit/mL and 10 mM, respectively) were shaped into disks (diameter, ~1.5 cm; thickness, ~3 mm) and incubated in 10 mL PBS (pH 7.4) at 37 °C for 4 days to reach equilibrium. The mass of the hydrogels after swelling (M_S) was measured. The hydrogels were then dried in an oven and their dry mass (M_D) was measured.

The equilibrium swelling ratio (Q_M) was calculated after excluding the effect of the weight of solutes in PBS according to Eq. (1):

$$Q_M = \frac{M_S}{M_D} \quad (1)$$

Q_M was used further to calculate the volume swelling ratio (Q_V), Eq. (2):

$$Q_V = 1 + \frac{\rho_P}{\rho_S}(Q_M - 1) \quad (2)$$

where ρ_P and ρ_S are the densities of dry PEG (1.12 g/cm^3) and solvent (1 g/cm^3), respectively. The Flory–Rehner equation was used to determine the mesh size (ξ) of the PEG-Ph-OH hydrogels [21–23]. First, the molecular weight between the cross-links ($\bar{M}_C$) was calculated using Eq. (3), as follows:

$$\frac{1}{\bar{M}_C} = \frac{2}{\bar{M}_n} - \frac{(\bar{\upsilon}/V_1)(\ln(1-\nu_2) + \nu_2 + x_1\nu_2^2)}{\nu_2^{1/3} - (\nu_2/2)} \quad (3)$$

where $\bar{M}_n$ is the number-average molecular weight of uncross-linked PEG (Table 1). $\bar{\upsilon}$ is the specific volume of the polymer (ρ_S/ρ_P) and V_1 is the molecular weight of solvent (water: 18 cm^3/mol). ν_2 is the volume fraction of the polymer at equilibrium swelling (ν_2 is the

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