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Efficient differentiation of stem cells encapsulated in a cytocompatible phospholipid polymer hydrogel with tunable physical properties

Haruka Oda [a], Tomohiro Konno [b], Kazuhiko Ishihara [a, b, *]

[a] Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[b] Department of Bioengineering, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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ABSTRACT

A large number of lineage-committed progenitor cells are required for advanced regenerative medicine based on cell engineering. Due to their ability to differentiate into multiple cells lines, multipotent stem cells have emerged as a vital source for generating transplantable cells for use in regenerative medicine. Increment in differentiation efficiency of the mesenchymal stem cell was obtained by using hydrogel to adjust the proliferation cycle of encapsulated cells to signal sensitive phase. Three dimensional (3-D) polymer networks composed of poly(2-methacyloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-vinylphenylboronic acid (VPBA)) (PMBV) and poly(vinyl alcohol) (PVA) were prepared as a hydrogel. The proliferation of cells encapsulated in the PMBV/PVA hydrogel was highly sensitive to the storage modulus (G′) of the hydrogel. That is, when the G′ value of the hydrogel was higher than 1.0 kPa, the cell proliferation was ceased and the proliferation cycle of cells was converged to G1 phase, whereas when the G′ value was below 1.0 kPa, cell proliferation proceeded. By changing the G′ value of hydrogels under encapsulation the cells, proliferation cycle of encapsulated mesenchymal stem cells was regulated to G1 phase and thus signal sensitivity were increased. 3-D polymer networks as hydrogels with tunable physical properties can be effectively used to control proliferation and lineage-restricted differentiation of stem cells.

1. Introduction

Regenerative medicine based on cell and tissue engineering has been the subject of extensive study in recent years, owing to the ability of cells generated *in vitro* to integrate into host tissue and restore lost function. Stem cells are one of the most promising sources for transplantable cells that can be induced to differentiate into desired cell lineages [1–4].

Diverse environmental cues, including chemical [5–7] and physical signals [8], direct the differentiation of stem cells into specific lineages. Differentiation occurs during cell turnover, and signal sensitivity is known to be associated with the G1 proliferative phase of the cell cycle [9,10]. Therefore, cells are often grown to confluence to induce contact inhibition and arrest proliferation at the G1 phase or are chemically treated to maintain the G1 phase before differentiation is induced.

During the cell fate determination process, stem cells sense and react to physical properties of their microenvironment. Accordingly, some cell fates are reached only in three-dimensional (3-D) cell cultures [11,12]. To date, in spite of extensive research efforts to control stem cell differentiation, the efficiency achieved in lineage-restricted differentiation is often poor. In addition to limited yields, inefficient differentiation leads to the need for exhaustive isolation and purification steps for cells. In order to apply cells for preparation of tissue, large numbers of cells with similar properties are needed. Efficiency in differentiation does not only mean the number of signals to induce differentiation, but also the number and ratio of cells required to react simultaneously to chemical and physical signals in order to obtain the needed quantity of differentiated cells.

We set out to prepare 3-D hydrogel environments capable of arresting cells at the most sensitive state at the time of signal inducement to improve stem cell differentiation efficiency by chemical cues. Oxygen and protein permeability of the polymer hydrogel is critical to culturing cells in 3-D conditions. Water-soluble polymers containing both 2-methacryloyloxyethyl phosphorylcholine (MPC) units and *p*-vinylphenylboronic acid (PMBV)

* Corresponding author. Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.
E-mail address: ishihara@mpc.t.u-tokyo.ac.jp (K. Ishihara).

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units are reported to form gels just by mixing with poly(vinyl alcohol) (PVA) in an aqueous medium at physiological pH and room temperature [13,14]. Such PMBV/PVA hydrogels are known to have good oxygen permeability [15]. In addition, the ability of MPC polymers to suppress protein adsorption extends to 3-D conditions, enabling the PMBV/PVA hydrogels to be a suitable environment for 3-D cell culture without inducing any chemical reactions with cells or other biomolecules [16]. As many signals have been discovered to induce cell differentiation, it is important that the PMBV/PVA hydrogel can transport the desired chemical signals without damaging the chemical structures Our approach is to control the physical properties of the cellular environment to arrest cells at a signal-sensitive phase without any chemical treatment. In order to achieve higher differentiation efficiency, cells should be uniformly arrested in the G1 phase [9,10,17,18]. The cells may respond to the physical characteristics of the surrounding hydrogel matrix; therefore, we tried to control proliferation by forming a hydrogel whose physical properties could be regulated. The PMBV/PVA hydrogel acts as an extracellular matrix and can be assumed to contribute similar functional properties to every encapsulated cell. When the hydrogel is prepared using MPC polymer, the influence of physical properties of the hydrogel can be separated from its chemical properties, because it is bioinert [16]. Thus, the physical property of the hydrogel can be controlled through the density of cross-linking. The cross-linking density of the polymer hydrogel, in turn, can be controlled by its chemical composition and polymer density.

We have reported that encapsulation of cells within PMBV/PVA hydrogels can be achieved during the gelation process by simply mixing PVA solution with a cell suspension in PMBV solution. Cells are reported to have been cultured in the hydrogel for more than three days [13,14]. Additionally, cells encapsulated in the PMBV/PVA hydrogels can be retrieved by adding a low-molecular-weight sugar compound that causes dissociation of the hydrogel due to a specific exchange reaction between the PVA and compound [19]. Importantly, the physical properties of the hydrogel equate to the encapsulated cellular microenvironment, and they can be controlled through polymer composition. Producing the PMBV/PVA hydrogel with a high density of VPBA units yields a high storage modulus (G′). Cells encapsulated in the PMBV/PVA hydrogel are known to have different proliferation rates depending on the G′ value of the hydrogel [20]. Specifically, 1.0 kPa was a critical G′ value for optimal proliferation of murine mesenchymal stem cells (C3H10T1/2). Cells encapsulated in a PMBV/PVA hydrogel with G′ values higher than 1.0 kPa were inhibited in their proliferation and converged at the G1 phase in 24 h, while cells encapsulated in a hydrogel with G′ values between 0.50 kPa and 1.0 kPa showed a slower rate of proliferation.

In this study, we confirmed that the PMBV/PVA hydrogel system can be used as a cytocompatible and reversible 3-D cell culture matrix, which can enhance the signal sensitivity of encapsulated cells and efficiently induce differentiation of stem cells. We encapsulated C3H10T1/2 cells in PMBV/PVA hydrogels to control cell proliferation and, consequently, sensitivity to signals. The G′ value of the hydrogels after cell immobilization was altered continuously by changes in the degree of swelling. We observed cell responses to this alternation. Finally, differentiation of cells was induced inside the hydrogels by addition of specific proteins as chemical signals.

2. Materials and methods

2.1. Materials

MPC was purchased from NOF Co., Ltd., (Tokyo, Japan), which was synthesized by a modified version of a previously reported procedure [21]. *n*-Butyl methacrylate (BMA) was purchased from Nacalai Tesque Inc. (Kyoto, Japan), and VPBA was obtained from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). PVA (with a polymerization degree of 1,000, completely hydrolyzed) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Bone morphogenetic protein-2 (BMP-2) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Other organic chemicals and solvents used in experiments were commercially available extra-pure grade. These chemicals were used without further purification. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific Inc. (MA, USA).

2.2. Synthesis and characterization of PMBV

Poly(MPC-*co*-BMA-*co*-VPBA) (PMBV) was synthesized according to a previously reported procedure for convenient radical polymerization [13,14,20,22]. For example, the monomers MPC, BMA, and VPBA were dissolved in ethanol at mole fractions of 0.60, 0.20, and 0.20, respectively. The total monomer concentration in solution was 1.0 mol/L. *t*-Butyl peroxyneodecanoate (PB-ND; 50 mmol/L in solution obtained from NOF) was used as an initiator for polymerization. In a glass polymerization reactor, oxygen was purged from solutions with argon gas for 15 min, and then polymerization was performed at 60°C for 3 h. The reaction mixture was poured into diethyl ether and chloroform (40:10 by v/v) to eliminate any remaining monomers and allow precipitation of the polymer. The precipitate was dissolved in water and dialyzed against water using a Spectra/Por® dialysis membrane (MWCO: 3500; Funakoshi Co., Ltd., Tokyo, Japan) for 3 days. The aqueous polymer solution was freeze-dried to obtain PMBV in the form of a white powder. The composition of each monomer unit in the PMBV was determined by ^{1}H NMR (α-300; JEOL Co., Ltd., Tokyo Japan). The monomer unit ratios determined from the integral values were as follows: 3.25 ppm ($-N^+(CH_3)_3$, 9H) for an MPC unit, 1.45–1.63 ppm ($-CH_2-$, 4H) for a BMA unit, and 7.03–7.75 ppm (benzene ring, 4H) for a VPBA unit. The monomer unit fractions of MPC, BMA, and VPBA were 0.60, 0.24, and 0.16. The molecular weight of the polymers was measured by gel permeation chromatography (GPC; Jasco Co., Ltd., Tokyo, Japan). A mixture of methanol and water (70:30 by v/v) containing 10 mmol/L of lithium bromide and 1.0 g/L D-sorbitol was used as the eluant for GPC measurements, whereas polyethylene oxides (TSK standard PEO, Tosoh Co., Tokyo Japan) were used as standard samples for making molecular weight calibration curves. The weight-averaged molecular weight (Mw) of the PMBV was 1.2×10^4, and the molecular weight distribution was calculated as 1.9.

2.3. Preparation and swelling of PMBV/PVA hydrogel

The PMBV/PVA hydrogels were prepared according to a previously reported procedure, with a DMEM solution containing 5.0 wt% PMBV and 2.5 wt% PVA, respectively [20]. DMEM was supplemented with 10 wt% FBS. The two solutions were mixed at a ratio of 60:40 (v/v) or 50:50 (v/v) by repeated pipetting until gelation was visually confirmed to form a PMBV/PVA hydrogel with G′ values of 1.2 kPa and 0.70 kPa, respectively.

In preliminary experiments, we soaked the PMBV/PVA hydrogels in an excess amount of DMEM for an optimal time to allow the hydrogel to swell, and the amount of DMEM was optimized to regulate changes in the swelling volume. The chemical structure and gelation reaction between PMBV and PVA used in this study are shown in Fig. 1.

2.4. Measurement of rheological properties of the PMBV/PVA hydrogel

The rheological properties of the PMBV/PVA hydrogels were measured using a creep meter (RE2-33005B; Yamaden Co., Ltd., Tokyo, Japan), following previously reported methods [20]. The PMBV/PVA hydrogel, with a total volume of 15 cm^3, was prepared in a cylindrical container of 50 mm in diameter. Stress of 10 mN was applied to the PMBV/PVA hydrogels for 60 s using a plunger with a diameter of 30 mm. The G′ value of the PMBV/PVA hydrogels was calculated on the basis of creep during the 60 s of loading and the subsequent 60 s of recovery. The rheological properties of PMBV/PVA hydrogel-encapsulated cells were measured as described earlier. We used a six-parameter model [23] to calculate the viscoelastic modulus automatically by fitting the creep chart to an equation. The elastic part of its modulus is referred to as the G′ value of the hydrogel.

2.5. Cell culture in PMBV/PVA hydrogel

C3H10T1/2 cells were used as model cells in this study. The C3H10T1/2 cells were purchased from RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were routinely cultured in DMEM containing 10% FBS at 37°C in a 5.0% CO_2 atmosphere. After trypsinization, cells were resuspended in a DMEM solution containing 5.0 wt% PMBV. This suspension was mixed with a DMEM solution containing 2.5 wt% PVA. The mixture was repeatedly pipetted until gel formation was visually confirmed. For every PMBV/PVA hydrogel formed, the cell density was maintained at 5.0×10^5 cells/mL. At this density, the cells were dispersed with sufficient distance from each other (~120 μm). The cells encapsulated in the PMBV/PVA hydrogel were cultured for several days in an incubator at 37°C in a 5.0% CO_2 atmosphere. The morphology of the encapsulated C3H10T1/2 cells was observed using a phase contrast microscope (BX60; Olympus Co., Ltd., Tokyo, Japan). The PMBV/PVA hydrogel was gently pipetted with 0.30 M D-sorbitol PBS solution to dissociate the hydrogel after every 24 h for 4 days. Every sample was treated in the