Cytocompatible and reversible phospholipid polymer hydrogels for encapsulation to provide unified quality cells

Haruka Oda1*, Tomohiro Konno2 and Kazuhiko Ishihara1,2
1Department of Materials Engineering, 2Department of Bioengineering, School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo113-8656, Japan
Fax: +81-3-5841-8647, e-mail: oda@mpc.t.u-tokyo.ac.jp

As the field of regenerative medicine based on stem cell engineering starts to play the important role in the new generation of bioengineering, the cells are starting to be treated as one of the materials to be controlled and optimized rather than those to be observed. Temporal and spatial encapsulation of the cells by the hydrogels are getting an attention as a novel way to handle cells in the three dimensional condition. In this study, cytocompatible and reversible phospholipid polymer hydrogels were prepared by mixing the aqueous solutions of poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate-co-p-vinylphenylboronic acid) (PMBV) and poly(vinyl alcohol) (PVA). The murine pluripotent stem cell line C3H10T1/2 is known to suppress its proliferation when encapsulated in the PMBV/PVA hydrogel with storage modulus of 1.2 kPa. The cell cycle of the encapsulated cells were unified to G1 phase in cell proliferation cycle after 1 day of encapsulation. The differentiation of C3H10T1/2 to osteogenic cells by bone morphogenetic protein 2 (BMP-2) was evaluated by polymerase chain reaction method after induction of BMP-2 signal for 3 days. The cells encapsulated in the PMBV/PVA hydrogel showed 1.7-fold increase of early-stage osteoblast gene expression with well-defined quality will make substantial contribution to the field of regenerative medicine.

Key words: phospholipid polymer, cytocompatibility, stem cell differentiation, polymer hydrogel

1. INTRODUCTION

Regenerative medicine based on cell and tissue engineering has been the subject of extensive studies in the recent years owing to the ability of cells generated in vitro to integrate into the host tissue and restore lost functions. Stem cells are one of the most promising candidates for generating transplantable cells such as chondrocytes [1,2].

Diverse environmental cues, including chemical [3,4] and physical signals [5], direct the differentiation of stem cells into specific lineages. The differentiation process occurs during the cell turnover, and signal sensitivity is known to be associated with G1 phase of the cell proliferation cycle [6,7]. Therefore, cells are often grown to a confluent state to induce contact inhibition and cell proliferation cycle arrest at G1 phase before differentiation is induced. Cells are synchronized to G1 phase and allowed to re-proliferate at the same time to achieve efficient differentiation.

In order to achieve higher differentiation efficiency, cells should uniformly be present in G1 phase at the time of differentiation signal induction. Our approach to resolve this problem was to make changes to the cellular environment. The cellular environment is created by polymer hydrogel, achieved by mixing two polymer solutions, poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate-co-p-vinylphenylboronic acid) (PMBV) and poly(vinyl alcohol) (PVA). The hydrogel is equally functioning to every cell encapsulated inside. The cells respond to the physical characteristics of their environment; therefore, we tried to control their proliferation cycle by forming a hydrogel such that its physical properties could be manipulated. When the hydrogel is prepared by using 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers, the physical property of the hydrogel could be traced to its cross-linking density owing to its bioinert property [8]. The cross-linking density of the polymer hydrogel could be controlled by its chemical composition and polymer density. The cross-linkings are formed by the chemical bond between phenylboronic acid of PMBV and diols of PVA. The chemical bond could be replaced with other diols with higher binding constant, such as sugar molecules (Fig.1). This enables the dissociation of hydrogel, which makes it possible to recollect the encapsulated cells from the hydrogel.
2. MATERIALS AND METHODS

2.1 Materials

MPC synthesized by a modified version of a previously reported procedure was purchased from NOF Co., Ltd. (Tokyo, Japan) [9]. n-Butyl methacrylate (BMA) was purchased from Nakalai Tesque Inc. (Kyoto, Japan), and p-vinylphenylboronic acid (VPBA) was obtained from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). These chemicals were used without further purification. Poly(vinyl alcohol) (PVA) (with a polymerization degree of 1000, completely hydrolyzed) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Other organic chemicals and solvents used in experiments were commercially available extra-pure grade. Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS), as well as other products used for cell culture, were purchased from Invitrogen Corporation (NY, USA).

2.2 Synthesis and characterization of PMBV

Poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate-co-p-vinylphenylboronic acid) (PMBV) was synthesized according to a previously reported procedure by a convenient radical polymerization [10]. The monomers, MPC, BMA, and VPBA were dissolved in ethanol at mole fractions of 0.60, 0.24, and 0.16, respectively. The total monomer concentration in solution was 1.0 mol/L. t-Butyl peroxycetanoate (50 mmol/L) was used as an initiator. Solutions were purged with argon for 15 min to eliminate oxygen. Polymerization was performed at 60°C for 3 h. After the polymerization, the reaction mixture was poured into large amount of a solution containing diethyl ether and chloroform (40:10 [v/v]) to eliminate any remaining monomers and allow precipitation of the polymer. The precipitate was dissolved in water and dialyzed using a Spectra/Por® dialysis membrane (MWCO: 3500; Funakoshi Co., Ltd., Tokyo, Japan) for three days. The aqueous polymer solution was freeze-dried to obtain PMBV in the form of a white powder. The composition of each monomer unit of PMBV was determined by 1H-NMR (α-300; JEOL Co., Ltd., Tokyo Japan). The molecular weight of the polymers was measured by gel permeation chromatography (Jasco Co., Ltd., Tokyo, Japan). The molecular weight distribution of the polymer was calculated as 1.9.

2.3 Preparation and swelling of PMBV/PVA hydrogel

The PMBV/PVA hydrogel was prepared according to a previously reported procedure [10] by using PMBV (5.0 wt%) and PVA (2.5 wt%) dissolved in DMEM. PMBV and PVA polymers were dissolved in DMEM supplemented with FBS (10 wt%) at a concentration of 5.0 wt% and 2.5 wt%, respectively. The two solutions were mixed at a ratio of 60/40 (v/v) by repeated pipetting until the gelation was visually confirmed. The PMBV/PVA hydrogel was soaked in the excess amount of DMEM for an optimal time to allow the hydrogel to swell. The storage modulus of the hydrogels were measured before and after the swelling to see the relationship between the volume increase and the storage modulus.

2.4 Rheological measurement of PMBV/PVA hydrogel

The rheological properties of the PMBV/PVA hydrogels were measured using a creep meter (RE2-3300S; Yamaden Co., Ltd., Tokyo, Japan), following previously reported methods [10]. A total volume of 15 mL of each PMBV/PVA hydrogel was prepared in a cylindrical container of 50 mm in diameter. A stress of 0.01 N was applied to the PMBV/PVA hydrogels for 60 sec by using a plunger with diameter of 30 mm. The storage modulus of the PMBV/PVA hydrogels was calculated on the basis of the creep during the 60 sec of load and the subsequent 60 sec of recovery. The rheological properties of PMBV/PVA hydrogel encapsulated cells were measured as described earlier.

We used a six-parameter model [11] to calculate the viscoelastic modulus automatically by fitting the creep chart to equation. The elastic part of its modulus is referred to as the storage modulus (G’) of the hydrogel.

2.5 Cell culture in PMBV/PVA hydrogel

Murine mesenchymal stem cells (C3H10T1/2) were used as model cells. The C3H10T1/2 cells were routinely cultured in DMEM containing 10% FBS at 37 °C and 5.0% CO2. After trypsinization, cells were resuspended in DMEM containing 5.0 wt% PMBV. This suspension was mixed with a 2.5 wt% solution of PVA in DMEM. For every PMBV/PVA hydrogel formed, the cell density was maintained at 5.0 × 105 cells/mL. At this density, the cells were dispersed with sufficient distance from each other (~126 μm). The cells encapsulated in PMBV/PVA hydrogel were cultured for three days in an incubator. The PMBV/PVA hydrogel was then gently pipetted with 0.3 M D-sorbitol in PBS solution to dissociate the hydrogel.

2.6 Differentiation of encapsulated cells

Differentiation of hydrogel encapsulated C3H10T1/2 cells to chondrocytes was accomplished by stimulating the cells with bone morphogenetic protein-2 (BMP-2). Typically, C3H10T1/2 cells were cultured at a density of 5.0 × 105 cells/mL for one day in PMBV/PVA hydrogels of storage modulus of 1.2 kPa. Thereafter, cells were maintained for three days in chondrocyte differentiation medium consisting of DMEM supplemented with a final concentration of 100 ng/mL BMP-2. The amount of the medium was set to half the volume of the initial PMBV/PVA hydrogel to change the storage modulus from 1.2 kPa to 0.7 kPa. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was treated with DNase I (Invitrogen Corp.,
Extracted RNA was reverse transcribed using a Thermoscript RT-PCR system (Invitrogen Corp., Carlsbad, CA, USA). cDNA synthesis was performed according to manufacturer’s instructions. Polymerase chain reaction (PCR) were performed in 20 μL of a mixture containing 1 μL of cDNA, 1 μM each of forward and reverse primers. The PCR cycle profile was, 95°C for 10 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The PCR primers used in this study correspond to the following three genes: collagen type 1 alpha1, early-stage osteoblasts (Col1a1), bone gamma carboxyglutamate protein 1, late-stage osteoblasts (Bglap1), and eukaryotic translation elongation factor 1 gamma, housekeeping gene (Eef1g). The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide for visualization and imaging. Relative expression levels of various genes were estimated from the intensity of bands stained by ethidium bromide. Eef1g was used as the control.

3. RESULTS AND DISCUSSIONS
3.1 Storage modulus control of PMBV/PVA hydrogel

Fig. 3 shows the storage modulus of PMBV/PVA hydrogel in relation to its increased volume by swelling. The storage modulus of the hydrogel is related to its cross-linking density. The cross-linking in PMBV/PVA hydrogel is formed through chemical bonding, which does not dissociate in the medium. When the medium was added to the hydrogel, the number of cross-linking remains to be the same while the volume of the hydrogel increases by swelling. This results in the decrease of cross-linking density. The increased volume shows a linear relationship with storage modulus decrease, which matches with the theory.

![Fig. 3. The storage modulus of PMBV/PVA hydrogel in relation to its increased volume (original volume=1.0, original storage modulus = 1.2 kPa)](image)

Fig. 4 shows the stability of the polymer network during and after the swelling. The medium was added after one day of incubation. The storage modulus decreases to 0.7 kPa after the addition of the medium, and the same storage modulus was kept for three days of incubation. Also the loss modulus is below the storage modulus throughout this process, indicating the formation of three-dimensional networks.

![Fig. 4. The storage modulus (●) and the loss modulus (○) of PMBV/PVA hydrogel during the 4-day incubation.](image)

3.2 Stem Cell proliferation control through PMBV/PVA hydrogel

Fig. 5 shows the number of cells encapsulated in the PMBV/PVA hydrogel during 4-days incubation. The cells were not added during the incubation, so the increase of numbers is result of cell proliferation. Fig. 6 shows the fraction of cells in G1 phase during the same time period. The number of cells does not show much increase for the first day of incubation inside the PMBV/PVA hydrogel with storage modulus of 1.2 kPa. The fraction of cells in G1 phase became more than 90%. The G1 phase, or growth 1 phase, cells grow in terms of the protein and mRNA production getting ready for mitosis. The increase of cells in G1 phase matches with the suppression of proliferation in the first 1 day. The cells are reported to show proliferation in the PMBV/PVA hydrogel with storage modulus of 0.5 kPa to 1.0 kPa. After incubating the cells in 1.2 kPa hydrogel for 1 day, the storage modulus of the hydrogel was lowered to 0.7 kPa. The cells start to proliferate and doubled its number after three days culture in 0.7 kPa hydrogel. The percentage of the cells in G1 phase decreased as the cells start to proliferate. This result confirms the restart of cell proliferation.
hydrogel could be reduced by swelling the hydrogel while stem cells were encapsulated inside. Thus, the stem cell proliferation was controlled by changing the storage modulus of the hydrogel. The cell proliferation cycle converges to G1 phase, when the proliferation was suppressed. The differentiation efficiency of the stem cell encapsulated were increased by the induction of BMP-2 with the controlled proliferation. This is hypothesis that the differentiation efficiency of the whole group of cells increased because they were all arrested to G1 phase, also known as the growth phase in preparation for the mitosis. Stem cell differentiation using PMBV/PVA hydrogel as its environment will enable easy and mass-productive differentiation with high efficiency.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas “Nanomedicine Molecular Science” from Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

(Received February 28, 2014; Accepted May 19, 2014)