Micropatterning of Phospholipid Hydrogel Layer on the Substrate for Controlled Cell Immobilization

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Abstract. Cell patterning plays an important role in the field of the tissue engineering owing to the influence of surrounding environment like cell-cell interaction or cell-matrix interaction. To construct a good tissue by the cell assembly, three-dimensional (3D) culturing of the cells should be examined. We focused on this, 3D patterning hydrogel layer for cell immobilization on the substrate was prepared, by photo reactive polymer and spontaneously cross-linking polymer system. We found that when the water-soluble poly (2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-vinylphenylboronic acid (VPBA)) (PMBV) and poly (vinyl alcohol) (PVA) mixed together in cell culture medium; they form hydrogel under mild conditions from these solutions. The mechanical properties of the PMBV/PVA hydrogel were controllable and adjusted by the change in the concentration and mixing ratio of two polymers. Cells can be immobilized by encapsulation with the PMBV/PVA hydrogel matrix with no adverse effects on the cell functions. Also, no significant interactions occurred between polymer matrix and cells. We utilized the photoreaction of PVA at the substrate to make basement of the micropatterned layer, then, by reacting to the PMBV containing cells to form a 3D cell laden micropatterned layer. As the results, the PMBV/PVA hydrogel layer was fabricated following the PVA pattern, and cells were immobilized in the hydrogel on substrate in desired hydrogel pattern. These results will give a new glimpse towards cells patterning, tissue patterning, and tissue engineering.

1. Introduction

Many researches have been carried out regarding the patterning of cells and constructing cell assembly in the field of tissue engineering [1]. The characteristics of the cells are greatly influenced by the environment surrounding such as the cell-cell interactions or the cell-extracellular matrix interactions (like the humoral factors and the surrounding mechanical stress, and etc.) For example, the extracellular matrix (ECM) secreted from cells with different cell-fate can lure surrounding cells step into the same cell fate with the cells secreted ECM. In other words, the controlling of the cells also will control the distribution of released chemical substances and the ECM, which have influence on surrounding cells. Therefore, cell patterning on an artificial substrate is significant in the controlling of cell proliferation, cell differentiation, and etc. However, the current cell patterning basically still stays in two-dimensional direction (2D). In this research, we focused on the fabrication of cell patterning in three-dimensional (3D) direction.

Hydrogels are examined as a matrix for cell immobilization. It is due to higher swelling in aqueous medium, suitable physical properties as the ECM, and good permeability of the solute even providing the network structure. Thus, the hydrogels are expected for using in the field of 3D cell culture. There are numerous kinds of the hydrogels on the market for 3D cell culture system, with different gelation process, like UV irradiation, temperature variation, or the change of the moisture content. However,
these processes induce some effects on laden cells. We have found new-type of hydrogel formation by the reaction between poly (2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-vinylphenylboronic acid (VPBA)) (PMBV) and poly (vinyl alcohol) (PVA) under room temperature, normal pressure, neutral pH and even in cell culture medium [2]. Also, the immobilization of the cells is succeeded with the PMBV/PVA hydrogels [3-5]. In this research, to fabricate a micro-patterned hydrogel layer on the substrate, we designed a double-layered structure of hydrogels using photoreactive PVA as the base layer. By utilizing the reaction with photoreactive PVA with poly(ethylene terephthalate) (PET) substrate, the PVA chains chemically bound on the PET with desired pattern, which the same as the photomask when performed the UV irradiation. After remove the unreacted PVA, the PMBV could react with the PVA layer and form hydrogel in desired area. During PMBV reaction with PVA layer, cells could be entrapped in the polymer matrix and immobilization has done.

Figure 1. Micropatterning process using the PMBV/PVA hydrogel

The conventional micropatterning cells aims at the further research of cell-cell/cell-ECM interaction through the eyes in 2D, but this time, the hydrogel circumstances provide a 3D angle of view, and offering more possibilities to tissue regeneration.

2. Experimental

2.1. Synthesis of PMBV
The water soluble cytocompatible polymer, the PMBV (Fig. 2) was synthesized by a conventional radical polymerization. In short, the initiator $a, a'$-azoisobutyronitrile, and the monomer MPC, BMA, and VPBA were dissolved in ethanol to make 0.5 mol/L solution under room temperature. The polymerization was carried out in a sealed glass test tube under argon gas atmosphere at 65 °C for 6 hours. The polymer formed was collected after the purification of re-precipitation by washing the polymer with ether/chloroform (80/20, v/v) mixture. Filtered off the precipitated polymer, dried under reduced pressure over one night. Dissolved the polymer again into water, and dialysis the solution against distilled water for 2 days using 3.5 kDa-dialysis tubing. After that, freeze-dry the aqueous solution for 2 days to have the PMBV. Using the same procedure, small amount of fluorescence monomer (methacrylate with rhodamine group in the side chain) was added in the polymerization system to obtain fluorescence labeled PMBV (PMBVr). The chemical structure and the monomer unit compositions of the PMBV were confirmed by $^1$H-NMR in C$_2$D$_2$OD. The molecular weight of the PMBV was measured by gel permeation chromatography (GPC) using 70% aqueous methanol containing 10 mmol/L of the lithium bromide and 1.0 mg/mL of the D-sorbitol. The calibration curve was measured by standard samples of poly (ethylene oxide) s with different molecular weights.
2.2. Preparation of PMBV/PVA hydrogels
The hydrogel was obtained as the steps below: the PMBV was dissolved in water to make 5 wt% solutions. The PVA (polymerization degree was 1,000) was dissolved in water at 90 °C and finally the concentration of the PVA was adjusted 5 wt%. The concentration of PMBV and PVA in the solution was controlled from 1.0 wt% to 5.0 wt%. Also, just sufficiently mixing two solutions until complete forming hydrogel with desired mixing ratio varied from 90 vol% of the PMBV solution to 10 vol% of the PMBV solution.

2.3. Rheological measurement of PMBV/PVA hydrogels
The creep meter was utilized for the measurement of the rheological properties of the PMBV/PVA matrix. The whole test was lasted for 120 sec. with 2.0 N load cells. During the first 60 sec, 0.10 N was loaded on hydrogel and the next 60 sec. was for the hydrogel recovery. The step and the speed of the program were set as 0.010 mm and 0.50 mm/sec., respectively. The diameter of the contact sensor was 8.0 mm. The 24-well dish was used as the replacement of the cylindrical container with diameter 16 mm. Total volume of PMBV matrix was more than 1 mL with desired blend ratio of PMBV and PVA solution [3].

2.4. Preparation of PVA micropatterned layer on the substrate
At first, the surface of the PET substrate was treated with the photoreactive PVA solution. The concentration of the polymer was determined the viscosity of the solution. Therefore, the concentration of the PVA in initial solution was regulated from 0.25 wt% to 2.0 wt%. After the solution evaporated, put a mask on the substrate surface during the UV irradiation. After that, rinse the substrate for 3 times until a clear pattern could be observed. Store the treated substrates in desiccator over one night.

2.5. PMBV/PVA hydrogel formation with micropatterned layer
In order to have a distinct micropatterned layer of the hydrogel, the PMBVr was used instead of the PMBV. The PMBVr was dissolved in DMEM (with the addition of 10% of the FBS and 1% of the penicillin) and meet the concentration at 5 wt%. PMBVr solution was applied on the PVA-treated substrate, which was prepared using the 1.0 wt% PVA solution, for 10 min, then remove the residual solution and wash the substrate with DMEM for one time.

2.6. Cell immobilization in PMBV/PVA hydrogel matrix
Murine fibroblast L929 cells were used in this research with routine culture process: cells cultured in DMEM (including 1 % of the penicillin and 10 % of the FBS) at 37 °C under 5.0 vol% of the CO2 atmosphere. The PMBVr was dissolved in DMEM (w/o penicillin or FBS), and sterilized using porous filter with 0.45 μm in diameter. After the sterilization, add FBS and penicillin. After trypsinization-collecting of the cells, cells with desired cell number was suspended with PMBVr solution before gelation. After put the cell laden PMBVr solution on PVA-treated substrate for 10 min, then remove the residual solution and wash the substrate with DMEM for one time. The morphology of immobilized cells was observed by a phase contrast microscope.
3. Results and Discussion

3.1. Characterization of PMBV
The PMBV was synthesized successfully with mole fraction of MPC, BMA, and VPBA in polymer as 0.72, 0.11, and 0.17, respectively. The molecular weight of PMBV is about $1.5 \times 10^4$. From the results, the fraction of the PMBV is close to the desired ratio with high molecular weight.

The hydrogel was formed by mixing together of PMBV and PVA solution under atmosphere. The polymer concentration and mixing ration were influenced the formation of the PMBV/PVA hydrogels. The PMBV/PVA hydrogel was obtained successfully above 3.0 wt% in the concentrations of the both polymers. Also, small amount of rhodamine units incorporated in the PMBV did not significant effect for this gelation of the polymers. When the blending composition ratio of PMBV against the PVA was higher than 30 %, the PMBV/PVA hydrogel could obtain successfully. Therefore, when fabricating a hydrogel on substrate with PVA layer, excess amount of the PMBV was expected for making hydrogel layer.

3.2. Storage modulus of the PMBV/PVA hydrogel
The blending composition of PMBV aqueous solution to the PVA solution during the gelation process was examined from 0 % to 100 %. The results are shown in Fig. 3. The storage modulus of the PMBV/PVA hydrogel showed an obvious curvilinear relation with the change of the blending composition ratio. The cell immobilization in the PMBV/PVA hydrogel was succeeding with 1.0 kPa of the storage modulus hydrogels [3]. Thus, it was determined that the hydrogels with 5.0 wt% of the PMBV, and the blending composition ratio from 50 % to 90 % is possible for cell immobilization.

![Figure 3](image.png)

Figure 3. The storage modulus of PMBV/PVA hydrogel with different blending composition ratio of PMBV

3.3. Micropatterning of PVA on substrate
The photoreactive PVA was diluted with water and the final concentration was adjusted in the range from 0.25 wt% to 2.0 wt%. After coating, the substrate was masked using photomask and performed UV irradiation. The micropatterned layer was observed by microscopic as shown in Fig. 4. In the case of 0.25 wt% PVA solution coating, just a shallow pattern was fabricated. With 0.50 wt% of the PVA solution, the pattern was fabricated with clear edge. And in the group with 1.0 wt% of the PVA solution, there are weak winkles heap up on the edge, and with higher concentration like 2.0 wt%, the winkles get obvious. These are due to the accumulation of excess PVA on the edge. Finally, the 0.50 wt% of the PVA solution was chose as the suitable concentration for the base layer of hydrogel formation.
Figure 4. The morphology of the micropatterned PVA layer on substrate prepared by photoreaction. The concentration of initial PVA solutions is indicated in the picture. The scale bar means 100 μm.

3.4. Micropattern of PMBV/PVA hydrogel layer on substrate
After the PVA patterning, the patterning of the hydrogel was verified. By utilizing the spontaneous gelation of PVA with PMBV, the PVA-treated substrate was immersed in PMBVr solution. After the gelation, remove the residual solution. The formation of the hydrogel was verified by fluorescence of the PMBVr. The morphology of the hydrogel is shown in Fig. 5. From the results, a transparent hydrogel layer was fabricated on the substrate with the same micropattern as photomask. By the fluorescence microscope, it could be observed the fluorescence based on the rhodamine group in the PMBVr. From these observations, the formation of the micropatterned PMBV/PVA hydrogel layer was confirmed.

Figure 5. The morphology of the micropatterned layer of the hydrogel on substrate surface. Left side is phase-contrast microscopic image and right side is fluorescence microscopic image. The base PVA micropatterned layer was prepared by using 1.0 wt% solution. The scale bar means 100 μm.

3.5. Cell immobilization in micro-patterned PMBV/PVA hydrogel
Next, the cell immobilization was performed in the micropatterned PMBV/PVA hydrogel layer. The cells were suspended in the PMBVr solution with DMEM, and then, performed the gelation between cell laden PMBVr with micropatterned PVA layer on the substrate. The microscopic images are shown in Fig. 6. From the results, most of the cells were immobilized in the PMBV/PVA hydrogel on the substrate surface.
Figure 6. Cells immobilized in the micropatterned PMBV/PVA hydrogel layer. Left side is phase-contrast microscopic image and right side is fluorescence microscopic image. The scale bar means 50 μm.

4. Conclusion
Cell immobilization with micropatterned on substrate surface was performed successfully by a simple procedure under mild condition using photoreaction and molecular complication of two water-soluble polymer systems. Although it is necessary to understand the activity and functionality of the cells immobilized in the hydrogel layer, we think that these results can bring a new glimpse towards cell patterning and tissue engineering. The research is undergoing in the laboratory.

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