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H. 知的財産権の出願・登録状況

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Prevention of Tissue Adhesion by a Spontaneously Formed Phospholipid Polymer Hydrogel

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Abstract. We investigated phospholipid polymer hydrogels containing Fe^{3+} ions (PMA/PMB/Fe hydrogel) for their use as antiadhesive materials in the healing tissues. These hydrogels were prepared from the aqueous solutions of poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-methacrylic acid) (PMA) and poly(MPC-*co*-*n*-butyl methacrylate) (PMB). The PMA/PMB hydrogel is formed by the intermolecular interactions between PMA and PMB, and it reversibly dissociates under physiological conditions. The addition of Fe^{3+} ions could control the gelation time and the dissociation time. Mechanical properties such as the gelation time and viscoelastic properties can be controlled by the $FeCl_3$ concentration. With regard to biocompatibility, no evidence of inflammation was observed *in vivo*. Therefore, the PMA/PMB/Fe hydrogel has a potential to be used as an antiadhesive material.

Introduction

Adhesion of tissues such as a tendon and intestines after an injury or surgery is a type of inflammatory reaction. It can cause difficulty in movement or pain, thereby decreasing the quality of life of a patient [1]. Although some efforts have been invested in developing antiadhesive materials for tissues, no effective material has yet been put to practical use. The existing antiadhesive materials serve as a physical barrier to prevent contact of the healing tissue with the surrounding normal tissues. However, these materials have some drawbacks [2]: (1) The permeability of bioactive molecules such as cytokines is so low that healing is delayed. (2) Certain degree of tissue adhesion may occur after an operation for the removal of non-biodegradable materials and during adsorption of biodegradable materials.

Therefore, we propose a spontaneously formed phospholipid polymer hydrogel as a novel anti-adhesive material. This hydrogel can be prepared under physiological conditions simply by mixing the aqueous solutions containing poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-methacrylic acid) (PMA) and poly(MPC-*co*-*n*-butyl methacrylate) (PMB) (Fig. 1) [3]. The hydrogel is formed by molecular interactions such as hydrogen bonding and hydrophobic interactions, and it demonstrates physical properties that correspond to the polymer structure [4,5]. Thus, it can be dissociated by changing the surrounding conditions, namely, pH, ionic strength, temperature, etc. The *in vivo* injection test did not show toxicity of the constitutive polymers—PMA and PMB. Since the PMA/PMB hydrogel has more than 95 wt% aqueous medium, it is expected to be (1) porous to allow the permeation of humoral factors, (2) biocompatible in order to prevent an inflammatory reaction, and (3) biodegradable so that a special procedure for its removal is not required after the tissues heal. The PMA/PMB hydrogel is dissociated in a large amount of aqueous medium within a few hours, and it is expected to dissociate *in vivo* within a relatively short time. Biodegradability is an advantageous property for its medical use because it can control the release of content as the degradation and eliminate the need of surgery for its removal. However, since the dissociation time of the PMA/PMB hydrogel is short, its long-term application to tissues as an antiadhesive material

is not possible. Thus, we introduced another crosslinking mechanism, that is, ionic crosslinking between counter-cation and carboxylate anion in the PMA/PMB hydrogel for achieving stabilization. Although NaCl and $CaCl_2$ did not show the expected stabilization effect, $FeCl_3$ improved the stability of the PMA/PMB hydrogel in a large amount of aqueous medium [3].

In this study, we investigated the PMA/PMB hydrogel containing $FeCl_3$ for their use as an antiadhesive material in tissues. We examined the stabilization of the PMA/PMB hydrogel by $FeCl_3$ *in vitro* and *in vivo*. We also evaluated the performance of the PMA/PMB hydrogel as an antiadhesive material *in vivo*.

Materials and Methods

Materials. The phospholipid polymers, PMA (Mn = 2.7×10^5, Mw = 8.4×10^5, and MPC mole fraction = 0.3) and PMB (Mn = 1.1×10^5, Mw = 8.6×10^5, and MPC mole fraction = 0.8), were prepared from the corresponding monomer by radical polymerization [Figure 1][6]. For this study, these polymers were supplied by the NOF Corporation (Tokyo, Japan) as 5 wt% aqueous solutions. Iron (III) hexahydrate ($FeCl_3$) was purchased from Kanto Chemical Co.

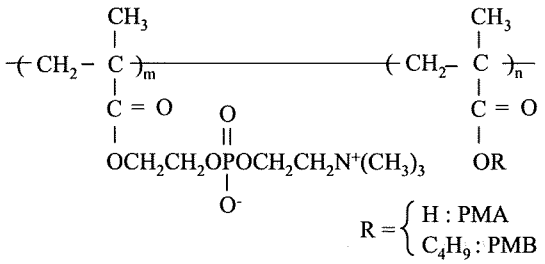

Fig. 1. Structure of PMA and PMB.

Hydrogel Preparation. Equal volumes of 5 wt% PMA and PMB aqueous solutions were taken in a microtubing and vigorously stirred for 10 s. After 10–20 s, the mixture of these MPC polymer solutions was spontaneously transformed into a hydrogel state. A hydrogel containing $FeCl_3$ (PMA/PMB/Fe hydrogel) was prepared by using PMB containing $FeCl_3$. The final concentration of $FeCl_3$ in a hydrogel is expressed by the number following the PMA/PMB/Fe hydrogel, for example, PMA/PMB/Fe hydrogel-71 implies a PMA/PMB hydrogel containing 71 mM of $FeCl_3$.

Stability of the PMA/PMB/Fe Hydrogel *in vitro and in vivo*. One gram of PMA/PMB/Fe hydrogel was put in a nylon mesh bag and immersed in 100 mL of phosphate buffered saline (PBS; 0.15 M, pH 7.1). The mesh bag was weighed at specific time intervals, and the weight of the remaining hydrogel was determined. For an *in vivo* test, a diffusion chamber (pore size, 0.3 μm) containing the PMA/PMB hydrogel or PMA/PMB/Fe hydrogel-71 was implanted subcutaneously into a mouse. After 3 weeks, the chamber was removed, and the hydrogel was observed by SEM.

Viscoelastic Properties of the Hydrogels. The aqueous solutions of PMA (0.75 mL) and PMB (0.75 mL) were injected slowly into both sides of a vibration blade (1.71 cm^2) in a liquid cell. Immediately after the injection, the blade was set in motion (vibrational amplitude, 200 μm; frequency, 20 Hz) to mix these polymer solutions in the cell in order to enable the PMA/PMB hydrogel formation. Changes in the elastic modulus (G') and the viscous modulus (G") were recorded using a rheometer (Rheolograph-Micro, Toyoseiki, Tokyo, Japan). The gelation time is defined as the time when G' becomes greater than G". The gelation time of the PMA/PMB/Fe hydrogel was also measured by using PMB containing $FeCl_3$ in the same manner. In addition, the viscoelastic properties of the hydrogel were investigated using the rheometer at a predetermined time after the hydrogel was prepared and kept at room temperature.

Results and Discussion

Fig. 2 shows the effect of $FeCl_3$ addition on the stability of the PMA/PMB/Fe hydrogel. The relative weight of PMA/PMB/Fe hydrogel-14 gradually decreased, and it completely dissociated within 6 h after its immersion in PBS. PMA/PMB/Fe hydrogel-28 also dissociated completely within 24 h, although PMA/PMB/Fe hydrogel-39 and PMA/PMB/Fe hydrogel-71 retained almost a constant weight after a slight initial decrease. Because a Fe^{3+} ion theoretically interacts with 3 carboxylate anions, the residual carboxylic acid groups exist in PMA/PMB/Fe hydrogel-14 and PMA/PMB/Fe hydrogel-28. The $FeCl_3$ concentration is adjusted to the theoretical ratio in

PMA/PMB/Fe hydrogel-39. Thus, PMA/PMB/Fe hydrogel-39 and PMA/PMB/Fe hydrogel-71 did not dissociate because of the higher $FeCl_3$ concentration.

The relative weight of PMA/PMB/Fe hydrogel-28 showed a slight initial increase, and then it steadily decreased. This suggests swelling of the hydrogel immediately after its immersion in PBS, followed by its dissociation. When the PMA/PMB/Fe hydrogel is immersed in PBS, swelling of the hydrogel and diffusion of $FeCl_3$ starts. Influx of water can cause ionization of the carboxylic acid groups and lead to electrostatic repulsion between the carboxylate anions. As a result, the polymer concentration and the crosslinking density decreases. Subsequently, the polymer networks collapse, i.e., dissociation of the hydrogel occurs. As seen in the yellowish PBS obtained after the immersion of the hydrogel, diffusion of the Fe^{3+} ions and swelling of PMA/PMB/Fe hydrogel-71 and PMA/PMB/Fe hydrogel-39 is possible. However, even during the swelling process, these hydrogels retained the polymer network and attained equilibrium due to a high density of Fe^{3+} crosslinking.

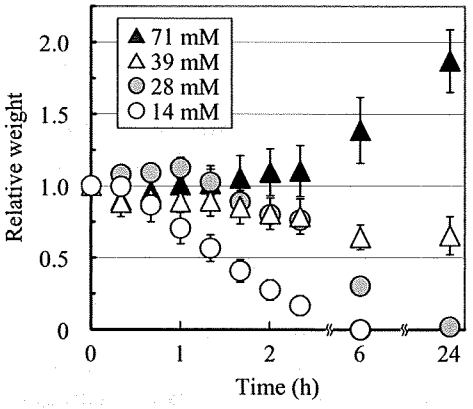

Fig. 2 Change in the weight of PMA/PMB hydrogel containing $FeCl_3$ immersed in PBS.

Although PMA/PMB/Fe hydrogel-71 was implanted subcutaneously, stabilization of the PMA/PMB hydrogel containing Fe^{3+} was observed. That is, while the PMA/PMB hydrogel was dissociating, PMA/PMB/Fe hydrogel-71 remained and maintained the hydrogel state even after 3 weeks. A three-dimensional network structure could be observed under SEM, and the results of viscoelastic measurements also indicated the defining characteristic of a hydrogel, that is, G' > G".

Table 1 shows the gelation time of the PMA/PMB hydrogel and the PMA/PMB/Fe hydrogel. The gelation time was longer for the PMA/PMB hydrogel than for the PMA/PMB/Fe hydrogel. Since the $FeCl_3$ solution has a low pH, ionization of the carboxylic acid groups in PMA can be suppressed by mixing PMA with PMB containing $FeCl_3$. Suppression of the carboxylic acid groups leads to hydrogen bond formation, resulting in the shortening of the gelation time of the PMA/PMB/Fe hydrogel when compared with that of the PMA/PMB hydrogel. Increase in the $FeCl_3$ concentration decreased the gelation time. This is because the pH of PMA/PMB/Fe hydrogel-142 was lower than that of PMA/PMB/Fe hydrogel-71.

Table 1 Gelation time of PMA/PMB hydrogel containing $FeCl_3$

	Gelation time (s)
PMA/PMB hydrogel	1007 ± 137
PMA/PMB/Fe hydrogel-71	605 ± 101
PMA/PMB/Fe hydrogel-142	468 ± 47

Thus, the addition of $FeCl_3$ significantly reduced the gelation time and the dissociation time of the PMA/PMB/Fe hydrogel; moreover, these parameters can be controlled by the $FeCl_3$ concentration.

The viscoelastic properties of the PMA/PMB hydrogel and the PMA/PMB/Fe hydrogel are shown in Fig. 3. With regard to the gelation time, agitation efficiency was so high that it took shorter time compared to the preparation method described in the gelation time measurement section. Vigorous stirring by a vortex mixer for 10 s is sufficient to prepare both PMA/PMB and PMA/PMB/Fe hydrogels. The mechanical strength of the PMA/PMB/Fe hydrogel immediately after its preparation (10 s) was so low that it appeared almost sol. Both G' and G" of PMA/PMB/Fe hydrogel-142 were lower than those of PMA/PMB/Fe hydrogel-71. G' and G" of the

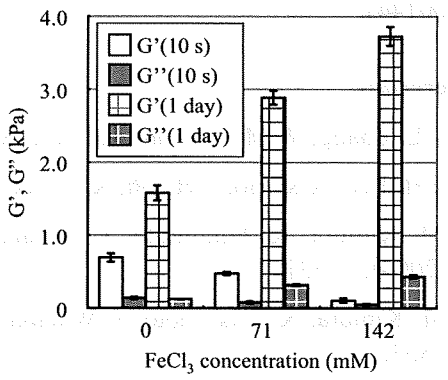

Fig. 3 Change in the elastic modulus (G') and the viscous modulus (G") of PMA/PMB hydrogel containing $FeCl_3$.

PMA/PMB hydrogel were higher than those of the PMA/PMB/Fe hydrogel. One day after the hydrogel preparation, increase in G' and G" was observed in all the hydrogels; G' of the PMA/PMB hydrogel was more than double its value immediately after the hydrogel preparation, while G" remained almost constant. It is noteworthy that G' of PMA/PMB/Fe hydrogel-71 increased 6-fold and that of PMA/PMB/Fe hydrogel-142 increased more than 60-fold after 1 day. G" of PMA/PMB/Fe hydrogel-71 also increased by approximately 4-fold and that of PMA/PMB/Fe hydrogel-142 increased more than 8-fold. Interestingly, among the 3 hydrogels, G' of PMA/PMB/Fe hydrogel-142 was the lowest immediately after the hydrogel preparation, but it was the highest after 1 day; this observation can be explained by the ionic crosslink formation.

During clinical application, the treated tissue will be covered by an antiadhesive agent and then sutured. Thus, the change in the mechanical properties during and after surgery would determine the clinical usefulness of an antiadhesive agent. A point worth noting is that immediately after its preparation, the PMA/PMB/Fe hydrogel is a weak gel; however, it improves its mechanical strength with time. This change in the mechanical properties of the PMA/PMB/Fe hydrogel can enable the hydrogel to attain a specific shape according to the application site and solidify after suturing; these features are a requisite for an antiadhesive material.

Furthermore, implantable antiadhesive materials should also be biocompatible in order to prevent the occurrence of any inflammatory reaction that would result in adhesions. Based on the results of the *in vivo* injection test, we have previously reported that PMA and PMB do not show notable adverse effects [5]. Although $FeCl_3$ is applied to dental materials to facilitate the adhesion of dental prosthesis to the dentin tissue, a report has shown the potential of $FeCl_3$ to cause oxidative stress on cells leading to the development of mutation [7]. The biocompatibility of the PMA/PMB/Fe hydrogel should be closely examined. We have been investigating the antiadhesive property of hydrogels and its effect on healing. No evidence of inflammation was observed in the tissues surrounding the hydrogel. We shall provide detailed reports of the results elsewhere.

Conclusions

A PMA/PMB hydrogel containing $FeCl_3$ (PMA/PMB/Fe hydrogel) shows that the mechanical properties of a hydrogel can be controlled by a combination of hydrogen bonding and ionic crosslinking. Severe inflammatory reaction was not observed in the tissues surrounding the hydrogel. Therefore, it can be concluded that the PMA/PMB/Fe hydrogel satisfies the basic requirements for an antiadhesive material.

Acknowledgement

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Temporal and spatially controllable cell encapsulation using a water-soluble phospholipid polymer with phenylboronic acid moiety

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Abstract

Temporal and spatially controllable cell encapsulation based on a water-soluble phospholipid polymer is reported in this study. Phospholipid polymers, i.e., poly(2-methacryloyloxyethyl phosphorylcholine-*co*-*n*-butyl methacrylate-*co*-*p*-vinylphenylboronic acid) (PMBV), were synthesized. A series of hydrogels was prepared between the water-soluble PMBV and other water-soluble polymers having multi-valent alcoholic groups, such as poly(vinyl alcohol) (PVA). The PMBV/PVA hydrogels were formed not only in water, but also in a cell culture medium, and dissociated by the excess addition of low molecular weight di-valent hydroxyl compounds, such as D-glucose. The PMBV/PVA hydrogel was applied as a cell-container which has three-dimensional matrices for the reversible encapsulation of living cells without any response in it. Uniform cell seeding can be achieved using the hydrogels due to the homogenous gel formation of PMBV and PVA in the cell culture medium. Fibroblast cells were encapsulated in the PMBV/PVA hydrogel and maintained for 1 week. After dissociation of the PMBV/PVA hydrogel, the cells were seeded on conventional tissue culture polystyrene. The cells adhered and proliferated as usual on the plate. That is, the PMBV/PVA hydrogel will be useful as a cell-container, which can maintain the cells without any significant adverse effect on the entrapped cells.

Keywords: Phospholipid polymer; Cell engineering; Polymer complex; Reversible hydrogel; Cytocompatibility; Encapsulation

1. Introduction

Recent cell engineering has progressed toward regenerated medicine and cells. At such a time, cells with high functions should be obtained and treated by significantly developed nano- bio-technology. Polymer matrices for cell culture are very important for realizing these goals.

Three-dimensional (3-D) matrices for cell cultures have been prepared from poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers [1–3]. These classic biodegradable polymers are almost highly hydrophobic. Therefore, these 3-D matrices inhibited the penetration of the cell culture medium [4]. Also, the nutrients did not penetrate into these matrices. As a result, the seeded cells did not grow in the inner portion of the matrices. Several studies have developed 3-D matrix materials to solve these problems. Unfortunately, the resulting cell distribution in the polymer matrix is often not uniform, with most of the cells attached only on the surface.

Spontaneously gelation polymers can provide the desired size and shape for the seeded and encapsulated cells. Moreover, it will be useful that the cross-linking network can be dissociated by the addition of chemical or physical stimulations. Based on these requirements, recoverable hydrogels have been prepared by mixing two kinds of polymer aqueous solutions.

First, cross-linking of the polymer chains under physiological conditions should be necessary for this purpose in

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order to avoid any reduced activity of the entrapped cells and biomolecules. The cytocompatiblity and nutrient permeability of the hydrogel are important factors for the entrapped cells.

To obtain a cytocompatibility and nutrient permeability of the hydrogels, we focused on the cell membrane structure. We have reported that the 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers containing a phospholipid polar group in the side chain have an excellent biocompatibility due to inhibiting the non-specific interaction with biomolecules including serum proteins, platelets, and cells [5–7]. Also, the MPC polymer hydrogel membranes have a high gas permeability [8] and solute permeability [9]. We succeeded in the spontaneously gelation of water-soluble MPC polymers from their aqueous solutions by hydrogen bonding without any physical treatments [10–13]. The MPC polymer hydrogel prepared from an aqueous solution containing 5 wt% poly[MPC-*co*-*n*-butyl methacrylate (BMA)] and poly(MPC-*co*-methacrylic acid) could be dissociated by a change in pH. In this hydrogel, the internal pH of the hydrogel was too low (pH < 4) to entrap the cells. It is necessary to provide more compatible with the biological system, when the hydrogel is applied to the cell entrapment matrices.

In this study, we prepared a new reversible hydrogel system composed of MPC polymers which can encapsulate the cells and proteins by mild treatment with a high viability and activity. As the cross-linking mechanism between the MPC polymers in an aqueous medium, we modified the specific reaction between boronic acid and multi-valent alcoholic compounds.

The boronic acid in a tetrahedral anionic structure produces stable complexes with the alcohol compounds including PVA, glucose, sorbitol, etc. [14–26]. If the boronic acid moieties are introduced into the polymer chain and the polymer is reacted with PVA, the hydrogel may be formed in an aqueous medium. The hydrogel is reversibly dissociated by the addition of low-molecular-weight compounds such as D-glucose. This reaction mechanism is well known as the glucose concentration responsive hydrogel for application as glucose sensors [15–22]. The hydrogels containing the boronic acid moiety have attracted attention in the affinity chromatography of biological agents [23–26]. We propose a new cell maintenance system called the "cell-container" based on this hydrogel. This system will be useful for maintaining the cells with a high activity and allow their specific functions after being released from the hydrogel accompanied by dissociation of the hydrogel.

To achieve these objectives, we synthesized a cytocompatible water-soluble MPC polymer containing *p*-vinylphenylboronic acid (VPBA) units, that is, poly[MPC-*co*-BMA-*co*-VPBA] (PMBV). The characterization of the PMBV, formation and properties of the PMBV/PVA hydrogel, and behavior of the fibroblast cells in the hydrogel are reported. Finally, we demonstrate the usefulness of the PMBV/PVA hydrogel as the cell-container.

2. Materials and methods

2.1. Materials

MPC was synthesized by a previously reported method and used after recrystallization from acetonitrile [27]. BMA was purchased from Nakalai Tesque Co., Ltd. (Kyoto, Japan) and purified by distillation under a 30 mmHg reduced pressure/b.p. fraction of 60 °C. VPBA was kindly provided by Osaka Organic Chemical Industry, Ltd. (Osaka, Japan) and used without further purification. PVA (polymerization degree 1500, saponification value 86–90 mol%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The other organic reagents and solvents were commercially available reagents of extra-pure grade and were used without further purification. The cell culture medium Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), and other substances for the cell cultures were purchased from Invitrogen Corporation, Grand Island, NY, USA.

2.2. Synthesis and characterization of the phospholipid polymer

The PMBV was synthesized by a conventional radical polymerization technique using α,α′-azoisobutyronitrile (AIBN) as the initiator. The synthesis procedure is briefly described as follows: the desired amount of MPC, BMA and VPBA were placed in a glass ampoule, AIBN was dissolved in the mixture (1.0 mmol/L), and the mixture was then diluted with ethanol to a 1 mol/L monomer concentration. Argon gas was bubbled into the solution for 5 min to eliminate the oxygen, and then the glass ampoule was sealed. The polymerization was performed at 60 °C for a specific time. After cooling the glass ampoule, the contents were poured into a large amount of a mixture of diethyl ether and chloroform (8/2 by volume) to eliminate any remaining monomer and precipitate the polymer. The precipitate was filtered off using a glass-filter and dried in vacuo. The chemical structure of the PMBV was confirmed by ^{1}H NMR (α-300, JEOL Co., Ltd., Tokyo, Japan) and FT-IR (FT-IR 615, Jasco Co., Ltd., Tokyo, Japan) measurements. The composition of each component in the PMBV was determined by the ^{1}H NMR measurement. The molecular weight of the polymers was measured by gel permeation chromatography (GPC, JASCO Co., Ltd., Tokyo, Japan). The mixture of methanol and water (7/3 by volume) containing 10 mmol/L of lithium bromide was used as an eluent for the GPC measurement, and well-defined poly(ethylene oxide) (PEO) was used as the standard samples for the calibration curve.

2.3. Preparation and basic characterization of the hydrogel

To confirm the gelation between the water-soluble PMBV and PVA, various compositions of these polymer solutions were prepared. The inner morphology of the PMBV gels was observed using a scanning electron microsope (SEM, SM-200, Topcon Co., Ltd., Tokyo, Japan). For the SEM observation, the PMBV/PVA hydrogels were prepared using distilled water, and the lyophilized samples were observed.

To estimate the spontaneous gelation time, a dynamic viscoelasticity measurement was performed using a rheometer (Rheolograph-Micro, Toyoseiki, Tokyo, Japan). Equal amounts (0.75 mL) of PMBV (5.0 wt%) and PVA (2.5 wt%) aqueous solutions were slowly injected into both sides of a vibration blade (surface area of the brade was 5 cm^2). Just after injection, these polymer solutions were mixed by automatic vibration (20 Hz). Changes in the storage modulus (G') and the loss modulus (G'') were recorded. The gelation time was estimated as the time when the G' value becomes greater than G'' [28].

2.4. Cell entrapment in the PMBV/PVA hydrogel

The mouse fibroblast cell line, L929, was used as the model cells. The L929 cells were routinely cultured in DMEM containing 10% FBS at 37 °C in a 5% CO_2 atmosphere. After trypsinization, the cell-density was adjusted to 5×10^3 cells/mL by a culture medium containing 5 wt% of

PMBV. The L929 suspension in the PMBV solution was mixed with 2.5 wt% of the PVA aqueous solution. The mixture was pipetted and gently shaken until the gel formation was visually confirmed. The shape and morphology of the encapsulated L929 cells were observed using a phase contrast microscope (BX60, OLYMPUS Co., Ltd., Tokyo, Japan). After 1 week, the PMBV/PVA hydrogel was dissociated by the addition of an excess amount of D-glucose, and the recovered L929 cells were then plated on the tissue culture polystyrene (TCPS, Asahi Technoglass Corp., Chiba, Japan).

2.5. Cell proliferation test

Measurement of the cell proliferation rate in the PMBV/PVA hydrogel was then performed. The L929 cells were resuspended in DMEM containing 10% FBS, and adjusted to 4.0×10^5 cells/mL. The cell suspension (500 μL) was mixed with an equal amount of 10% PMBV solution dissolved by DMEM. The PMBV solution containing the L929 cells was mixed with an equal amount of 2.5% PVA solution diluted by DMEM. The mixture was slowly pipetted and mixed in the 24-well multiplate until the gelation was confirmed. The PMBV/PVA hydrogel containing the L929 cells were prepared using this procedure. Likewise, the cells were seeded on the 24-well plate without the PVA solution (only 5% PMBV), PMBV solution (only 2.5% PVA), and without any polymers (only medium). After 3 days, the increase in the cell number under the various conditions was counted by a hemocytometer.

3. Results and discussion

3.1. Preparation of PMBV and formation of PMBV/PVA hydrogel

Water-soluble PMBV could be synthesized by a conventional radical polymerization. The polymerization homogenously proceeded in ethanol. The chemical structure and synthetic results of the PMBV are summarized in Fig. 1 and Table 1. The chemical structure of the formed polymer was determined by ^{1}H NMR. Fig. 2 shows the ^{1}H-NMR spectrum of PMBV. The NMR peaks completely assigned each component of the PMBV. The solubility in water of the PMBV depended on the MPC composition in the polymers. For example, when the monomer unit compositions of MPC, BMA, VPBA were 0.4, 0.5, 0.1, respectively, the obtained polymer did not dissolve in water.

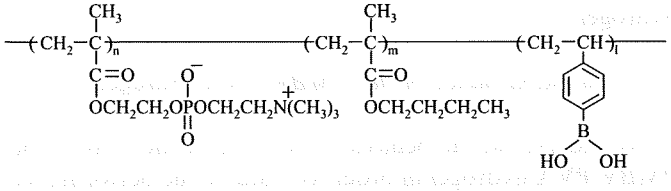

Fig. 1. Chemical structure of poly(MPC-*co*-BMA-*co*-VPBA) (PMBV).

The water-soluble PMBV was used to make a hydrogel with multi-valent hydroxyl group compounds such as PVA. When the polymer aqueous solutions contained PMBV and PVA, a hydrogel was formed within a short period after gentle shaking at room temperature. Fig. 3 shows a schematic representation of the covalently cross-linking mechanism between PMBV and PVA. The gelation mechanism was based on the covalent cross-linking between the phenylboronic acid moiety of PMBV and the hydroxyl groups of PVA. There are some reports about this phenomenon [29]. It is well known that the boronic acid is a protecting group for 1,2-diol compounds including saccharides in an organic synthesis [30]. The hydrogel formation between the phenylboronic acid moiety bearing a polymer and PVA has been investigated to develop a novel glucose-responsible drug delivery system [31–34]. That is, the hydrogel from PMBV and PVA is also reversibly dissociated by the addition of glucose into the system. Before mixing, both aqueous polymer solutions were low viscosity liquids. After mixing, the viscosity

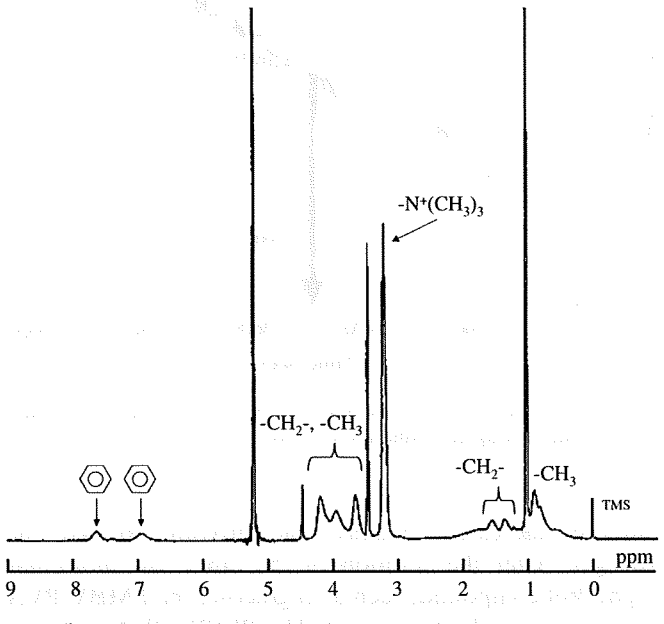

Fig. 2. ^{1}H NMR spectrum of water-soluble PMBV containing 60 mol% MPC unit.

Table 1
Synthetic results of PMBV

Abbreviation	In feed (mole fraction) MPC/BMA/VPBA	In copolymer (mole fraction) MPC/BMA/VPBA	Polymerization time (h)	Yield (%)	Solubility in water	Mw (10^{-4})	Mw/Mn
PMBV	0.6/0.3/0.1	0.6/0.3/0.1	6	71	+	5.4	2.6

[Monomer] = 1.0 mol/L. [AIBN] = 1 mmol/L. Polymerization temperature = 60 °C.
Solubility was determined by 1.0 mg/mL each polymer sample and described as soluble (+) and insoluble (−).

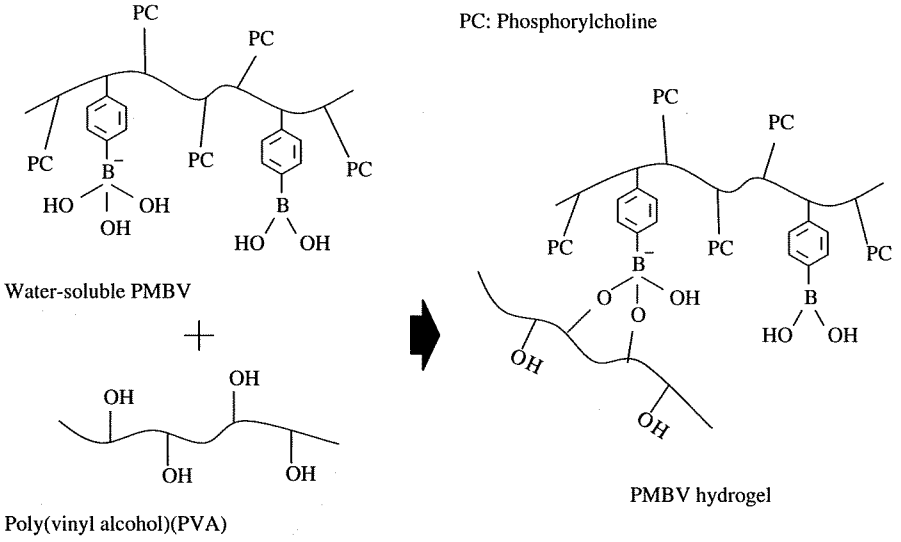

Fig. 3. Schematic representation of cross-linking mechanism between phenylboronic acid moiety in the PMBV and hydroxyl group in the PVA.

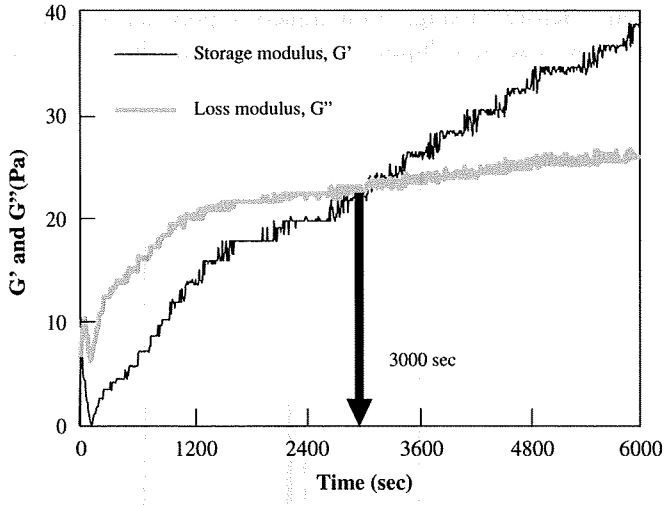

Fig. 4. Change in the storage modulus (G', black line) and loss modulus (G'', gray line) during gel formation process.

gradually increased. Finally, the mixture formed the hydrogel. After the addition of the other low molecular weight diol compound, such as D-glucose, the PMBV/PVA hydrogel gradually dissociated. The PMBV/PVA hydrogel has a reversibility between hydrogel formation and dissociation. The dynamic viscoelasticity measurement of the mixed solution was performed. Fig. 4 shows the typical result when the 5 wt% PMBV and 2.5 wt% PVA mixture were applied. The gelation was based on the diffusion of both polymer chains in the mixed solution. Therefore, the gelation times were relatively longer than that of the gentle shaking under these experiment conditions. It was obviously confirmed that the cross point between the storage modulus (G′) and loss modulus (G″) is at 3000 s. This result indicated that the PMBV and PVA mixture gradually formed the cross-linking network, and the mixture finally produced a hydrogel structure. The cross point between G′ and G″ was confirmed for the 2.5 wt% PMBV and 2.5 wt% PVA mixture. The composition ranges of PMBV and PVA for making a hydrogel are summarized in Table 2. Based on these results, it was revealed that the gelation depended on the polymer concentration. The PMBV/PVA hydrogel was realized even in the PBS and cell culture medium. It was considered that this gelation mechanism did not affect the ionic strength in the saline and medium.

Fig. 5 shows SEM images of the cross section of the PMBV/PVA hydrogel after lyophilization. Every hydrogel had a porous structure, because the water content of the hydrogel was above 93%. The pore size was almost 1 μm under the lyophilized condition. The higher water content and micropores in the hydrogel provide permeation of the bioactive molecules including proteins and hormones. This is a very good characteristic for culturing cells inside the hydrogel.

3.2. Cell maintenance in the PMBV/PVA hydrogel

To understand the behavior and state of the cells in the PMBV/PVA hydrogel in order to evaluate the performance of the PMBV/PVA hydrogel as the cell-container, the mouse fibroblast (L929) cells were cultured (encapsulated) in the PMBV/PVA hydrogel. A schematic illustration of the cell culture procedure is shown in Fig. 6. The PMBV was dissolved using DMEM containing 10% FBS. We have observed that the MPC polymers suppressed the nonspecific interaction with not only the various serum proteins, but also the cells [35]. Among these, the poly(MPC-*co*-BMA) could effectively suppress the L929 cell adhesion compared to that on TCPS due to the reduced cell adhesive protein adsorption. Indeed, the L929 cells adhered and proliferated as usual in the

Table 2
Gelation between PMBV and PVA solutions

PVA (wt%)	PMBV (wt%)				
	5.0	2.5	1.2	0.60	0.30
5.0	○	○	×	×	×
2.5	○	○	×	×	×
1.2	○	×	×	×	×
0.60	○	×	×	×	×
0.30	×	×	×	×	×

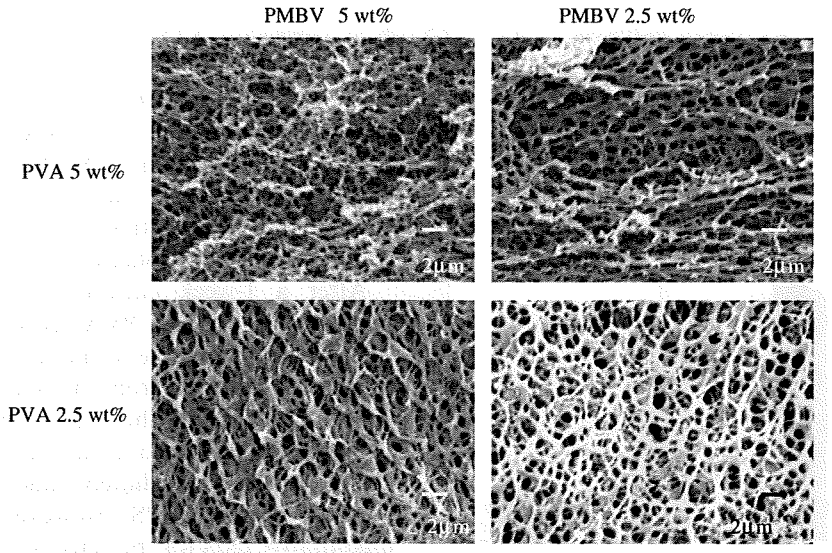

Fig. 5. SEM images of inner structure of PMBV gel (after lyophilization).

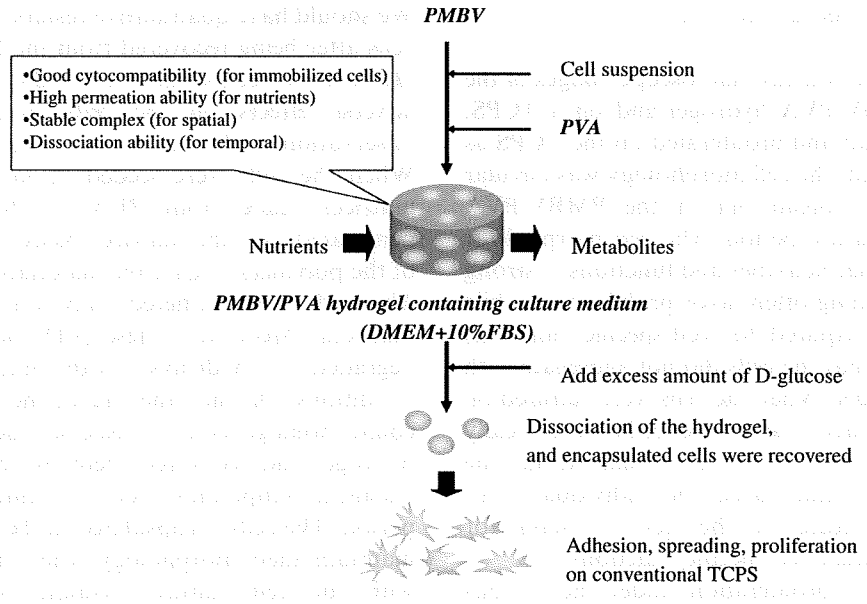

Fig. 6. Schematic illustration of cell culture (encapsulation) method used in this study.

5 wt% PMBV medium solution on the TCPS. The L929 cells were suspended in the 5 wt% PMBV medium solution. The L929 in the PMBV solution was mixed with the 5 wt% PVA aqueous solution. The gelation was confirmed after gentle shaking for 10 s at room temperature.

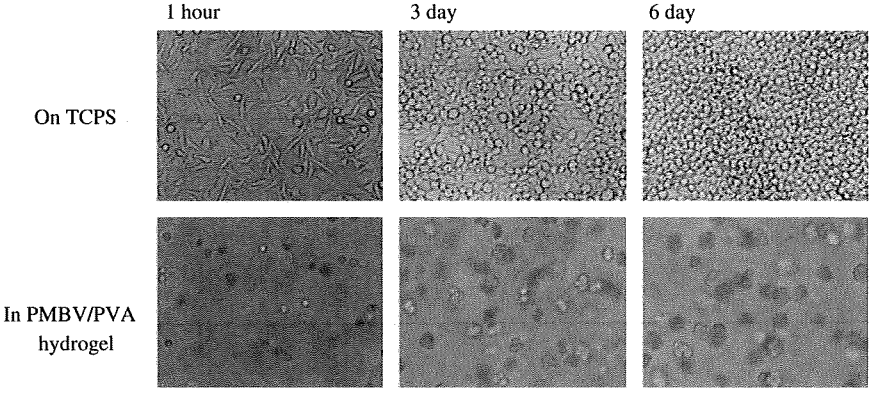

Fig. 7. Phase contrast microscope images of L929 cells on conventional TCPS and in the PMBV gel.

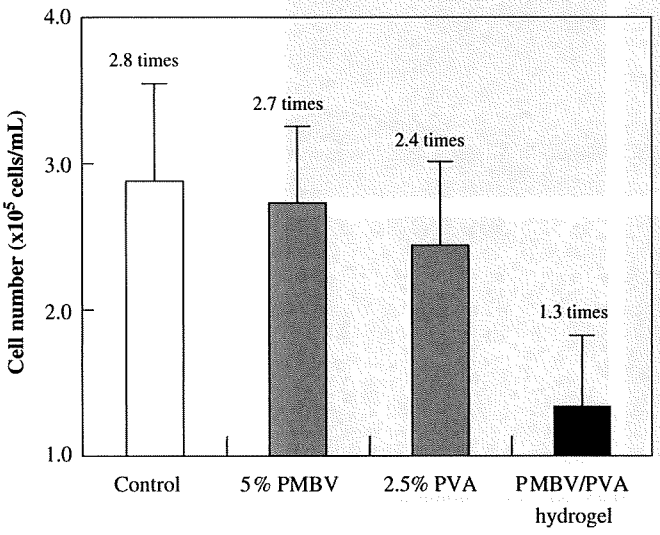

Fig. 8. Change in the cell number under the various conditions after 3 days ($n = 4$). DMEM + 10% FBS was used as the control.

Fig. 7 shows the phase contrast microscope images of the L929 cells in the PMBV/PVA hydrogel and on a TCPS. The cells adhered, spread and proliferated on the TCPS as usual. On the other hand, the cell morphology was circular and the cells did not spread out in the PMBV/PVA hydrogel during the culture period. The cell morphology correlates with the cellular activities and functions; a strong cell adhesion and spreading often favor proliferation while a round cell shape is required for cell-specific functions [36,37]. In noteworthy that the cells did not aggregate with each other in the hydrogel. When the cells were cultured on the MPC polymer surface, the seeded cells were locally aggregated with each other [38]. It was considered that the PMBV/PVA hydrogel immobilized the individual cells. Therefore, the cell culture in the gel is useful for investigating the individual cell-specific functions.

We evaluated the cell proliferation under the various culture conditions. Fig. 8 shows the cell number which was counted by a hemocytometer after dissociation or trypsin treatment ($n = 4$). For the liquid medium with 5% PMBV or 2.5% PVA, the cells were proliferated the same as the control sample (without any polymers). The increase ratio was 2.8, 2.7, and 2.4 times, respectively. Based on this result, both PMBV and PVA did not affect the cell proliferation. For the PMBV/PVA hydrogel, the cells hardly proliferated. The increased ratio was 1.3 times compared to the initial number. The cells were locally proliferated with a spherical shape.

After a 1-week culture without changing the fresh medium, the PMBV/PVA hydrogel was dissociated by the addition of an excess amount of D-glucose, and the cells that were recovered from the hydrogel were seeded on the TCPS. Fig. 9 shows the phase contrast microscope images of the L929 cells recovered and recultured on the TCPS. The L929 cells immediately adhered and proliferated after seeding on the TCPS. However, we do not have any quantitative data for the activity of the L929 cells inside the PMBV/PVA hydrogel at the present time. It is considered that the viability of the L929 cells was maintained even though they were in the PMBV/PVA hydrogel for 1 week. We should have quantitative results on the activation of the cells after being recovered from the PMBV/PVA hydrogel. However, it seems that the PMBV/PVA hydrogel had no adverse effects on the cells from the morphological observation and that is had a good cytocompatibility. When the cells were seeded in/on the conventional 3-D matrices made from PLA or PGA, the cells hardly penetrated into the matrices based on the hydrophobicity of the polymers, even if the materials have a pore structure. The cells were adhered only on the surface of these matrices. Moreover, these 3-D matrices were passively degraded by hydrolysis with time under physiological conditions. On the other hand, the PMBV/PVA hydrogel could homogeneously disperse the cells inside of the hydrogel and recovered them by the addition of natural chemical compounds such as D-glucose in a shorter time period. The cells encapsulated in the PMBV/PVA hydrogel maintain their morphology when they are in suspension with the cell culture medium. When the cells were recovered from the hydrogel, the cells demonstrated good adhesion to the solid substitute and proliferation on the surface.

Based on these results, we considered that the PMBV/PVA hydrogel could be applied as a new tool for cell

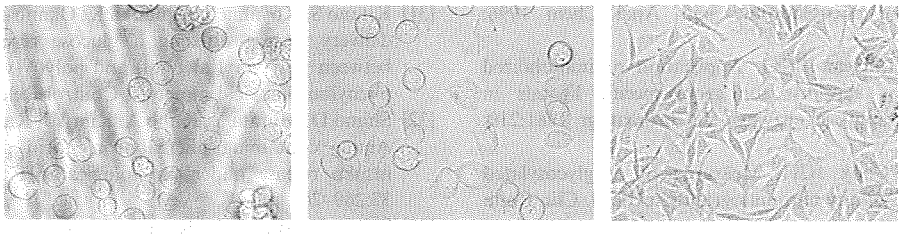

Fig. 9. Phase contrast microscope images of recovered L929 cells after dissociation of the PMBV gel.

engineering, that is, the cell-container. The cell-container provides a mild and suitable environment for cells just like a cell suspension. Treatment of the cells is made much easier by solidification with the PMBV/PVA hydrogel. The cells were also recovered from the hydrogel at any time.

4. Conclusion

We could prepare a phospholipid polymer bearing both MPC and *p*-vinylphenylboronic acid units, PMBV, for preparation of a cytocompatible hydrogel in an aqueous medium without any chemical and physical treatments. The gelation of PMBV spontaneously occurred after gentle mixing with PVA even in the presence of the cell culture medium. This was due to the formation of covalent cross-linking between PMBV and PVA. The cells could be encapsulated and uniformly distributed in the hydrogel. Since the viability of the encapsulated cells was good, the cells were recovered in good condition by the addition of glucose in the medium to dissociate the hydrogel. We concluded that the novel PMBV/PVA hydrogel system might be applied as a cell-container to encapsulate and transport the cells. Also, the hydrogel could be applied as a temporal and spatial controllable cell-containing hydrogel.

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