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# Cross-linkable and water-soluble phospholipid polymer as artificial extracellular matrix

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**Abstract.** The objective of this study is to prepare an artificial extracellular matrix (ECM) for cell culture by using polymer hydrogels. The polymer used is a cytocompatible water-soluble phospholipid polymer: poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-n-butyl methacrylate-p-nitrophenyloxycarbonyl poly(ethylene oxide) methacrylate (MEONP)] (PMBN). The hydrogels are prepared using a cross-linking reaction between PMBN and diamine compounds, which can easily react to the MEONP moiety under mild conditions. The most favorable diamine is the bis(3-aminopropyl) poly(ethylene oxide) (APEO). The effects of cross-linking density and the chemical structure of cross-linking molecules on the mechanical properties of the hydrogel are evaluated. The storage modulus of the hydrogel is tailored by tuning the PMBN concentration and the MEONP/amino group ratio. The porous structure of the hydrogel networks depends not only on these parameters but also on the reaction temperature. We prepare a hydrogel with 40-50 $\mu$m diameter pores and more than 90 wt% swelling. The permeation of proteins through the hydrogel increases dramatically with an increase in pore size. To induce cell adhesion, the cell-attaching oligopeptide, RGDS, is immobilized onto the hydrogel using MEONP residue. Bovine pulmonary artery endothelial cells (BPAECs) are cultured on the hydrogel matrix and are able to migrate into the artificial matrix. Hence, the RGDS-modified PMBN hydrogel matrix with cross-linked APEO functions as an artificial ECM for growing cells for applications in tissue engineering.

**Keywords:** phospholipid polymer; cytotoxicity; artificial extracellular matrix; hydrogel; cell culture

## 1. Introduction

Polymeric matrices for cell culture are of interest in the fields of tissue engineering and regenerative medicine (Garg and Goyal 2014, Place *et al.* 2009, Stock and Mayer 2001). Generally, aliphatic polyester derivatives are used for this purpose; however, more functionalized polymers are required for advanced cell engineering applications. Cytocompatibility is one of the most important properties required for this purpose.

A novel cell culture matrix should fulfill two criteria: cytocompatibility and cell-specific adhesive properties. Natural extracellular matrix (ECM) is composed of hydrophilic matrix

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proteins and cell adhesive components (Brizzi *et al.* 2012, Gattazzo *et al.* 2014). Thus, polymer hydrogels are one of the candidates for this purpose (Jia and Kiick 2009, Lutolf and Hubbell 2005, Rosso *et al.* 2004, Rowley *et al.* 1999, Sandvig *et al.* 2014, Silva and Mooney 2004). We have been investigated the biomedical application of bioinspired phospholipid polymers, including 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers (Ishihara *et al*. 1990, Ueda *et al*. 1992, Ishihara and Fukazawa 2014). The MPC polymers have excellent blood and tissue compatibility due to extremely reduced levels of protein adsorption at the surface (Iwasaki and Ishihara 2012, Ishihara *et al.* 1991, Ishihara *et al.* 1992, Ishihara *et al.* 1998, Lewis *et al.* 2000). Additionally, when cells are attached to the MPC polymers, they prevent an unfavorable immune response. The excellent properties of the MPC polymers enabled us to design a porous 3-dimensional (3D) scaffold using the water-soluble MPC polymer cross-linked with polymeric chains (Kimura *et al.* 2005, Kiritoshi and Ishihara 2004, Konno and Ishihara 2007). Moreover, the bioactive molecule, such as cell adhesive oligopeptide sequence was conjugated to the polymer to function as cell capturing ligands for controlling cell functions. Herein, poly(MPC-co-n-butyl methacrylate (BMA)-co-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)) (PMBN) was synthesized (Konno *et al.* 2004, Nishizawa *et al.* 2011). Since the MEONP units can react with amino groups under mild conditions, they can be used to cross-link and immobilize the cell-capturing oligopeptide sequences. To achieve a PMBN hydrogel structure with large pores for the insertion of cells and the permeation of the contents of the cell culture medium, the chemical structure and density of the cross-linking points were explored. In this communication, the preparation and the cell culture properties of our artificial ECM have been reported.

## 2. Experimental methods

### *2.1 Materials*

MPC was purchased from NOF Co. Ltd. (Tokyo, Japan), which has been synthesized by a previously reported method and purified by recrystallization from acetonitrile (Ishihara *et al*. 1990). ω-Methacryloyl poly(ethylene glycol) (MEOOH) and bis(3-aminopropyl)-poly(ethylene oxide) (APEO, weight-averaged molecular weight [Mw=$1.5\times10^3$]) were purchased from Sigma-Aldrich. APEOs with various Mw values (Mw=$3.4\times10^3$, $5.0\times10^3$, $1.0\times10^4$, $2.0\times10^4$, $3.0\times10^4$) were kindly supplied by NOF. Poly(L-lysine) (Mw=$3.8\times10^4$), ε-poly(L-lysine) (Mw=$3.9\times10^4$), and 4-nitrophenyl chlorocarbamate were purchased from Sigma-Aldrich. Diisopropylamine and BMA were obtained from Wako Pure Chemical Co. (Osaka, Japan) and used without further purification. Other reagents and solvents were extra-pure grade reagents.

### *2.2 Preparation of MEONP*

MEONP was synthesized by a previously described method (Nishizawa *et al.* 2011). Briefly, 0.050 mol of *p*-nitrophenyl chloroformate and 90 mL chloroform were placed in a stirred, four-necked round-bottomed flask, immersed in a methanol bath, and a mixture of equimolar amounts of MEOOH and diisopropylamine was added dropwise over 1 h. The temperature of the reaction mixture was maintained at -30℃ for 5 h. After the reaction, the precipitate was filtered off, and the solvent in the filtrate was evaporated until half of the volume remained. Diethyl ether (30 mL) was added to the solution to precipitate the diisopropylamine hydrochloride. The precipitate was

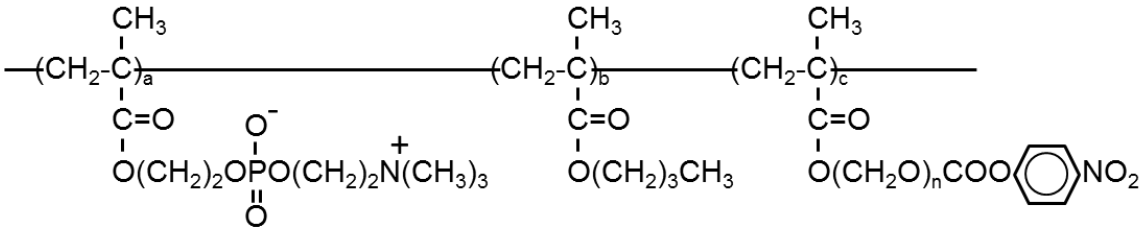

Fig. 1 Chemical structure of PMBN

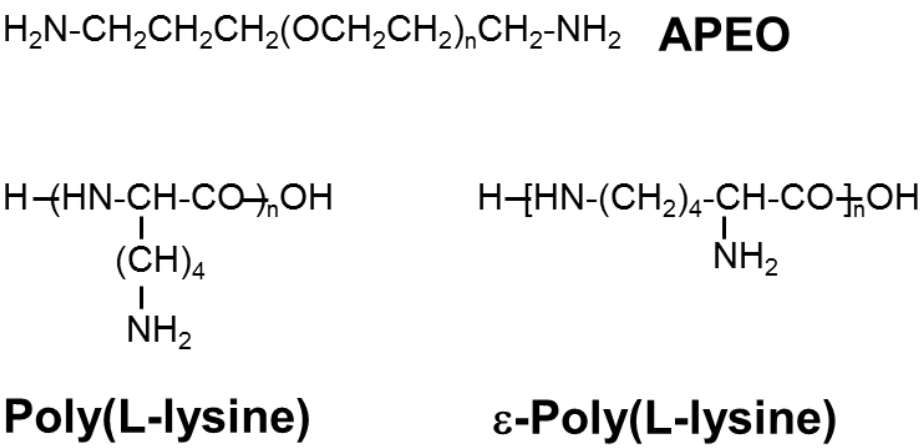


Fig. 2 Chemical structure of polymeric amine compounds as cross-linking agents

removed by filtration and filtrate evaporation was used to completely remove the solvent. The MEONP obtained was analyzed by 1H-NMR (JEOL AL300, Tokyo, Japan) and Fourier-transform infrared spectroscopy (FTIR; FT/IR-610; Jasco Co. Ltd., Tokyo, Japan).

### *2.3 Preparation of MPC polymers*

The MPC polymer used in this study for making the ECM, a water-soluble amphiphilic PMBN, was prepared by conventional radical polymerization of MPC, BMA, and MEONP in the molar ratio of 0.40/0.40/0.20, respectively, using 2,2′-azobisisobutyronitrile (AIBN) as an initiator (Konno *et al.* 2004, Nishizawa *et al.* 2011). The concentrations of the total monomer and AIBN were adjusted at 1.0 mol/L and 10 mmol/L, respectively. The monomer solution was transferred into glass tubing, and the tubing was sealed. After polymerization at 60℃ for a given period, the reaction mixture was precipitated using a chloroform/diethyl ether (10/90 vol/vol) mixture as a solvent. The polymer precipitated was filtered off, and then the remaining solvents were removed completely by a freeze-drying method.

The chemical structure and molar fractions of each unit in PMBN were determined using $^{1}$H-NMR and FTIR. The molecular weight of the PNBN was determined by gel-permeation chromatography (Jasco System, Tokyo, Japan) with the Ohpak SB-804 column (Shodex, Tokyo, Japan) in a water/methanol (30:70 vol/vol) mixture as a solvent and with poly(ethylene glycol)s as

standard samples. Fig. 1 shows the chemical structure of PMBN. The amount of MEONP units in the polymer was calculated from the amount of 4-nitrophenoxy ions released from the polymer by hydrolysis of the PMBN with 0.10 mol/L NaOH aqueous solution.

### *2.4 Preparation of MPC polymer hydrogels cross-linked with diamine compounds*

Ethylene diamine, poly(L-lysine), ε-poly(L-lysine), and APEO were used as cross-linking reagents of PMBN. A given concentration of PMBN aqueous solution was prepared. In this solution, the amine compound was added and the pH of the solution was adjusted at pH 8.0. The reaction was carried out at 37℃ for 48 h. The chemical structure of the reactant was determined with 1H-NMR, and the viscosity of the solution was measured with an Ostwald-type viscometer at 25℃. In Fig. 2, chemical structures of polymeric amine compounds are shown.

### *2.5 Characterization of the hydrogel prepared from PMBN and bis(3-aminopropyl) poly(ethylene oxide)*

Rheological properties of the hydrogel were evaluated by measuring the dynamic viscoelasticity with a Rheolograph-micro instrument (Toyo-seiki, Tokyo, Japan). The network structure of the hydrogel was observed with a scanning electron microscope (SEM; SM-200, Topcon, Tokyo, Japan) after freeze-drying the hydrogel, and the pore size of the hydrogel was determined.

### *2.6 Immobilization of oligopeptide to the hydrogel*

The tetrapeptide arginine(R)-glycine(G)-aspartic acid(D)-serine(S) (RGDS) was purchased from Sigma-Aldrich and used without further purification. The RGDS was dissolved in a 5 mM aqueous solution of $NaHCO_3$, and the pH of the solution was adjusted to 8.4 by the addition of a dilute NaOH aqueous solution. The PNBN was cross-linked with APEO ($Mw=1.5\times10^3$) at 37℃ for 48 h. In this case, the [$-NH_2$]/[MEONP unit] ratio was adjusted to 0.33 and 0.50. Two milligrams of the PMBN hydrogels was immersed in the RGDS solution (2.0 mg/mL) and stored at 37℃ for 24 h. After immobilization of the RGDS peptide, the hydrogel was immersed in a 0.10 M NaOH solution for hydrolysis of the remaining MEONP units. Then, the hydrogel was dialyzed against pure water for 10 days at 4℃. As a control, glycine was reacted with the hydrogel instead of RGDS under the same conditions. The immobilization was confirmed by FTIR spectroscopy.

### *2.7 Cell culture in the hydrogel*

Both the PMBN hydrogels (with and without RGDS immobilization) were placed in 24-well cell culture dishes, and 1.5 mL of cell culture medium (Eagle MEM) without serum was added to the wells. The hydrogels were equilibrated for 24 h and then sterilized with UV light irradiation. Bovine pulmonary artery endothelium cells (BPAECs) were cultured using a conventional procedure, and cell suspension was prepared with cell culture medium without serum after treatment with trypsin. The cell suspension was added to each well. The number of cells was adjusted to $1.0\times10^4$ cells per well. After the culture period, the hydrogels were removed from the wells, rinsed lightly with cell culture medium, and freeze-dried. The hydrogels were observed with SEM.

## 3. Results and discussion

### *3.1 Preparation of PMBN*

The chemical structure of MEONP was analyzed using FTIR and $^{1}$H-NMR. From the FTIR spectrum, the representative absorbance readings were observed at 1770 $cm^{-1}$ (attributed to ester bonds), 3081 $cm^{-1}$ and 1346 $cm^{-1}$ (attributed to aromatic rings), and 1637 $cm^{-1}$ (attributed to vinyl groups). In addition, the absorbance at 3400 $cm^{-1}$ that is attributed to the hydroxy group in MEOOH disappeared. From the NMR spectrum, signals were observed for 7.4 and 8.3 ppm for aromatic protons, and 5.7 and 6.1 ppm for methacrylate protons. From these results, the structure of the MEONP was confirmed (Nishizawa *et al*. 2011).

Polymerization of MPC, BMA, and MEONP was achieved homogeneously in ethanol. We used various polymerization times ranging from 5 h to 20 h. The chemical structure of the polymer PMBN was determined by $^{1}$H-NMR and gel permeation chromatography. The MPC unit composition was similar to that of the monomer solution. However, MEONP hardly copolymerized in this system, and the composition of MEONP unit in the polymer was about half of that in the feed monomer solution. The molecular weight was about $1.0\times10^{5}$ and did not change in each polymer. The yield of the polymer increased with increase in polymerization times. For optimal polymerization yield and molecular weight distribution, we used a 10 h polymerization time for preparing the hydrogel.

### *3.2 Cross-linking reaction of PMBN with various diamine compounds*

The MEONP unit has *p*-nitrophenyl ester group, which is well-known as an active ester group. The nitro group bound to the benzoic acid can withdraw an electron in the aromatic ring. And the activity of carboxyl group increases. That is, the MEONP units can react with amino compounds and formed amide bond under very mild condition in aqueous medium. In our previous research, the PMBN can be applied as surface modification compound on the polymer substrate to immobilize enzymes, antibodies and DNA molecules (Nishizawa *et al.* 2011). In this research, we used the PMBN as a reactive polymer with diamine compounds for making chemically cross-linked hydrogels.

Ethylene diamine, poly(L-lysine), ε-poly(L-lysine), and APEO were investigated for use as the crosslinking reagent of PMBN. For ethylene diamine and both types of poly(L-lysine), while the viscosity of PMBN increased with reaction time, we did not obtain hydrogels. Since ethylene diamine is a low molecular weight compound, reactivity against MEONP units in the PMBN may be high. However, cross-linking between polymer chains was limited by steric hindrance due to the bulky MPC units. On the other hand, for both types of poly(L-lysine), the reactivity of amino groups was low, because of their polymeric structure. That is, most of the poly(L-lysine) chains took an $\alpha$-helical structure under this reaction condition (Grigsby *et al*. 2002). Hence, the steric hindrance by the poly(L-lysine) chains may suppress cross-linking reactions. The amino groups in the ε-poly(L-lysine) have low basicity due to the carbonyl groups of their neighbors (Hyon *et al.* 2014).

Accordingly, the reactivity of the amino groups to MEONP groups decreased, and the hydrogel could not be obtained. These results suggest that the structure of the diamine compounds for cross-linking the PMBN is an important consideration. Specifically, compounds with two primary amino groups connected with flexible spacer chains would be suitable for this purpose. Thus, we decided

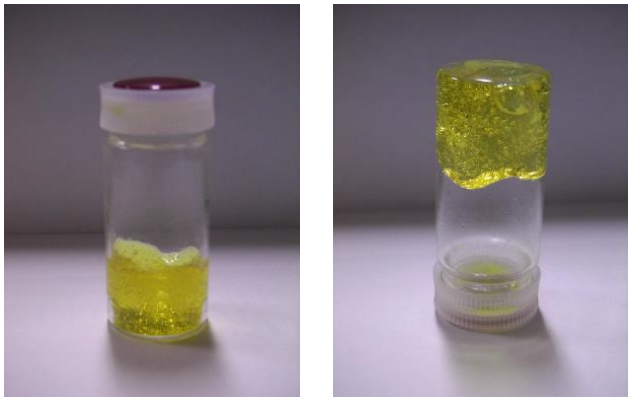

Fig. 3 PMBN hydrogel cross-linked with APEO (Mw=1.5×$10^3$)

to choose a poly(ethylene oxide) diamine such as APEO. Fig. 3 shows a picture of the PMBN hydrogel cross-linked with APEO.

### *3.3 Physical properties of PMBN hydrogels cross-linked with APEO*

Using APEO (Mw=1.5×$10^3$) as a cross-linking reagent, some factors related to the preparation of the hydrogel with PMBN were evaluated. Fig. 4 shows the PMBN concentration dependence on the elastic modulus (G') and viscous modulus (G'') of PMBN hydrogels cross-linked with APEO. And Fig. 5 shows [-$NH_2$]/[MEONP unit] ratio dependence on G' and G'' of PMBN hydrogel cross-linked with APEO. Both G' and G'' increased with the higher concentrations of PMBN and the [-$NH_2$]/[MEONP unit] ratio.

Hence, reactions occurred between the amino groups and the MEONP units in PMBN. The gelation points are defined by the point where G' > G'' (Mo *et al.* 2000). Thus, the minimum critical concentration of PMBN for gelation was 7.0 wt% when the [-$NH_2$]/[MEONP unit] ratio was 0.50 at 37℃. Additionally, the minimum [-$NH_2$]/[MEONP unit] ratio was 0.29 with a PMBN concentration of 10 wt%. The G' modulated from 0 to 1,500 N/$m^2$. It is corresponding to the range of 10% value of G' of soft tissues. (Walter *et al.* 2011).

We observed the pore structure of the hydrogel after freeze-drying. Fig. 6 shows the SEM pictures of hydrogels prepared with various conditions. Every hydrogel had porous structures. The size of the pore depended on the preparation conditions. For the hydrogel with 10 wt% PMBN and a [-$NH_2$]/[MEONP unit] ratio of 0.50, the pore size distribution was roughly uniform with a diameter of ca. 10 $\mu$m (Fig. 6(a)). The decrease in the [-$NH_2$]/[MEONP unit] ratio resulted in an increase in the average pore size (Fig. 6(b)). When the PMBN concentration was decreased to 8.0wt%, the pores became heterogenic, and the size increased to about 30-50 $\mu$m (Fig. 6(c)). The data suggest that the PMBN concentration may influence the pore size and distribution throughout the hydrogel more strongly than [-$NH_2$]/[MEONP unit].

The temperature during the gelation reaction was also an important factor in determining the pore structure. Fig. 7(a) shows an SEM image of the PMBN hydrogel prepared at 60℃. The mechanical properties of the hydrogel are plotted as a function of PMBN concentration (Fig. 7b). When the cross-linking reaction was carried out at 60℃, the pore sizes were smaller than the sizes obtained at 37℃ with the same concentration of polymer. We observed quick gelation at 60℃.

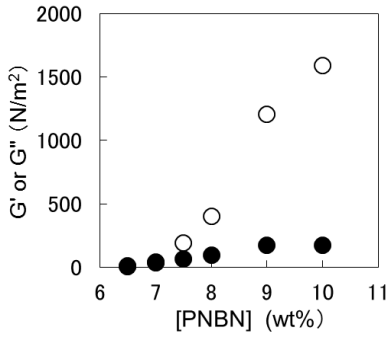


Fig. 4 PMBN concentration dependence on G' and G'' of PMBN hydrogels cross-linked with APEO. The reaction was carried out at 37°C. Open circle: G' elastic modulus; closed circle: G'' loss modulus

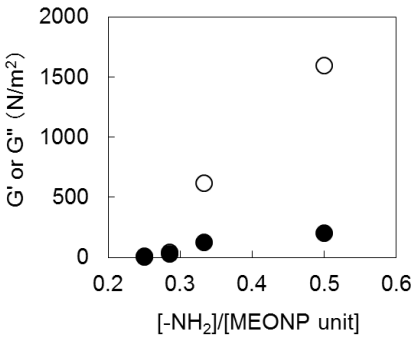


Fig. 5 [-$NH_2$]/[MEONP unit] ratio dependence on G' and G'' of PMBN hydrogel cross-linked with APEO. Reaction was carried out at 37°C. Open circle: G' elastic modulus; closed circle: G'' loss modulus

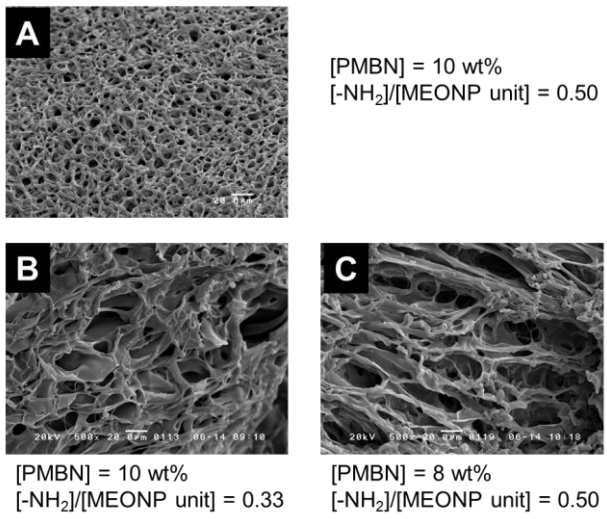


Fig. 6 SEM pictures of PMBN hydrogel cross-linked with APEO (Mw=$1.5\times10^3$). Cross-linking reaction was carried out at 37℃. a: [PMBN]=10 wt%, [-$NH_2$]/[MEONP unit]=0.50; b: [PMBN]=10 wt%, [-$NH_2$]/[MEONP unit]=0.33; c: [PMBN]=8 wt%, [-$NH_2$]/[MEONP unit]=0.50

Specifically, it took 48 h to prepare hydrogels at 37℃ but only 30 min at 60℃. This was due to the conformation of PMBN. In our previous study, we reported that the MPC polymer with hydrophobic moieties forms a polymer aggregate in an aqueous medium (Ishihara *et al.* 1999a, Konno *et al.* 2003). However, when the temperature is raised, there is a change in the structure of the polymer. Since the hydrophobic interaction between MEONP groups in the aqueous medium weakened when the temperature was above 60℃, most MEONP groups were located in the outer region of the polymer aggregate. The amino groups in APEO attached more easily at 60℃, and gelation proceeded. We obtained NMR spectra of the PMBN solution in $D_2O$ at various temperatures (data not shown). The signal intensity of *p*-nitrophenyl groups in the NEONP units and the trimethyl ammonium groups in the MPC units increased with increase in temperature. This phenomenon suggests that the PMBN aggregate structure dissociated when the temperature was raised. As shown in Fig. 7(b), the minimum critical concentration of PMBN for gelation was 5.5 wt%. Compared to gelation at 37℃, as seen in Fig. 4, the minimum concentration decreased here.

Fig. 8 summarizes the effects of several parameters on pore size, as determined by SEM, during the preparation of the PMBN hydrogel. The pore size of the hydrogel was strongly influenced by the concentration of PMBN (Fig. 8(a)), the ratio of amino groups in the cross-linker, and active ester groups in the polymer (Fig. 8(b)). The pore size varied between 5 $\mu$m and 50 $\mu$m.

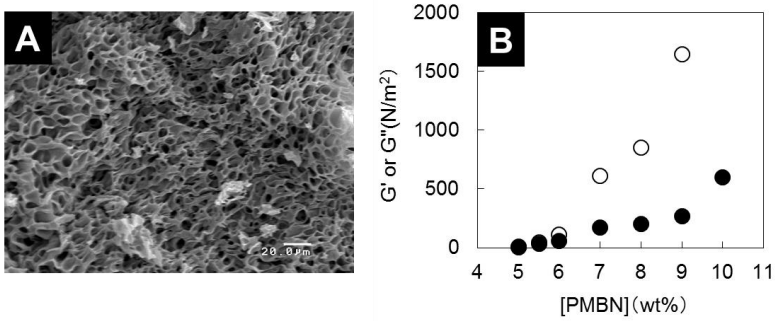


Fig. 7 (a) SEM pictures of PMBN hydrogel cross-linked with APEO (Mw=$1.5\times10^3$) [PMBN]=8 wt% [-$NH_2$]/[MEONP unit]=0.50, and (b) mechanical properties of the PMBN hydrogel with various concentrations of PMBN and [-$NH_2$]/[MEONP unit]=0.50. The cross-linking reaction was carried out at 60℃. Open circle: G' elastic modulus; closed circle: G" loss modulus

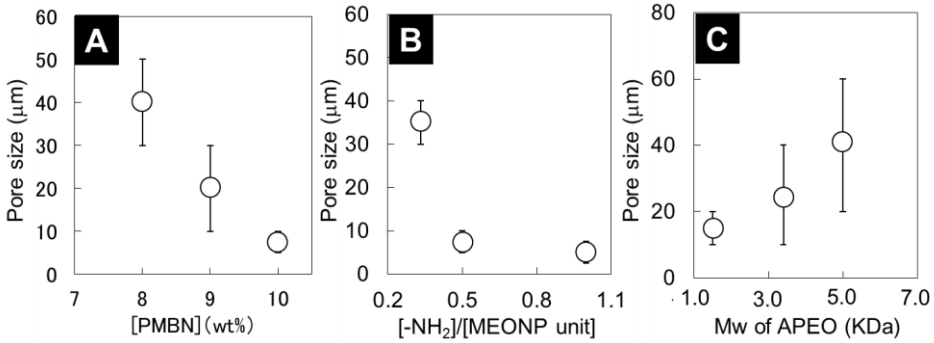


Fig. 8 Parameters affecting the pore size of the PMBN hydrogel cross-linked with APEO. Gelation was carried out at 37℃. Pore size of the PMBN hydrogels depended on [PNBN] (a), [-$NH_2$/MEONP unit] (b), and Mw of APEO (c)

The effects of the molecular weight of APEO on pore structure were evaluated using APEO with Mw values ranging from $1.5\times10^3$ to $3.0\times10^4$. Lower molecular weights, including Mw=$1.5\times10^3$, $3.4\times10^3$, and $5.0\times10^3$, resulted in gelation. However, higher molecular weight APEO (>$1.0\times10^4$) did not induce gelation even when the concentrations of the $NH_2$ groups were adjusted to match that of APEO. The data suggest that the viscosity of the reaction increased with increase in the molecular weight of APEO, and the mobility of the polymer chains was suppressed, resulting in low reactivity and no gelation. In Fig. 8(c), the dependence of Mw on the pore size in the hydrogel is shown. The pore size increased with an increase in the Mw of APEO. The larger pore sizes averaged 40-60 $\mu$m diameter using an APEO Mw of $5.0\times10^3$. Thus, we could control the pore structure by changing the Mw of APEO.

### *3.4 Immobilization of the biological active oligopeptide and cell culture in the hydrogel*

To evaluate the performance of the PMBN hydrogel as an artificial ECM, oligopeptide molecules were immobilized onto the polymer networks. We used the RGDS peptide as one of the model bioactive molecules, which is a well-known cell adhesive peptide (Makgoba *et al.* 1992, Sambu *et al.* 2011). After the cross-linking reaction, MEONP groups remained in the hydrogel. The amino group in RGDS could react with the MEONP group. FTIR spectroscopy was performed on the gels before and after immobilization of the peptide (data not shown). The specific absorbance of the arginine and aspartic acid residues was observed. For the control hydrogel, the same immobilization procedure was used with glycine in place of RGDS.

Fig. 9 demonstrates representative results of cell adhesion on the PMBN hydrogels. We used the PMBN hydrogel obtained from the solution with APEO (Mw=$1.5\times10^3$), [PMBN]=8 wt% and [-$NH_2$]/[MEONP unit]=0.50. As shown in Fig. 6 and Fig. 8, the pore size of the hydrogel strongly depended on the concentration of polymer and cross-linking reagents. This hydrogel had pores with around 30-50 $\mu$m in diameter (see Fig. 6(c)). The hydrogel with smaller pore (around 5 $\mu$m in diameter) could not allow penetrating of cells into hydrogels. This was due to physically disturbing by the polymer networks. When we prepared PMBN hydrogel with lesser cross-linking to make much larger pore, the mechanical properties of the hydrogel became low to handle as cell culturing matrix. By considering these points, we used the polymer hydrogel matrix with 30-50 $\mu$m-pores.

For the glycine-immobilized hydrogel, cells were not observed (Fig. 9(a)). In contrast, the immobilization of RGDS induced cell adhesion, as shown in Fig. 9(b). Cells plated on the hydrogel could traverse into the hydrogel matrix and adhere along the polymer networks via the attachment to the RGDS peptide. However, the adhesive density of cells was not high, and the adherent cells were spherical under these culture conditions.

It is well known that MPC polymers can inhibit cell adhesion and activation due to suppression of the adsorption and the deformation of cell adhesive proteins from the culture medium (Ishihara *et al.* 1999b, Seo *et al.* 2009, Fukazawa and Ishihara 2009, Byambaa *et al.* 2012). In our previous study, we found that the cell adhesion to the MPC polymer surface in a cell suspension of culture medium with fetal serum is strongly dependent on the composition of the MPC units. When the MPC unit composition was above 10 unit mol%, the number of adherent cells decreased significantly. In addition, when the MPC unit composition was 30 unit mol %, hardly any adherent cells were observed on the surface. This trend correlated to the amount of fibronectin adsorbed on the surface. The properties of the MPC polymer were still maintained in 3D polymer networks constructed with the MPC moiety. While cell adhesion on the glycine-immobilized polymer networks did not occur, the RGDS peptide played an important role in adhering the cells. It may be

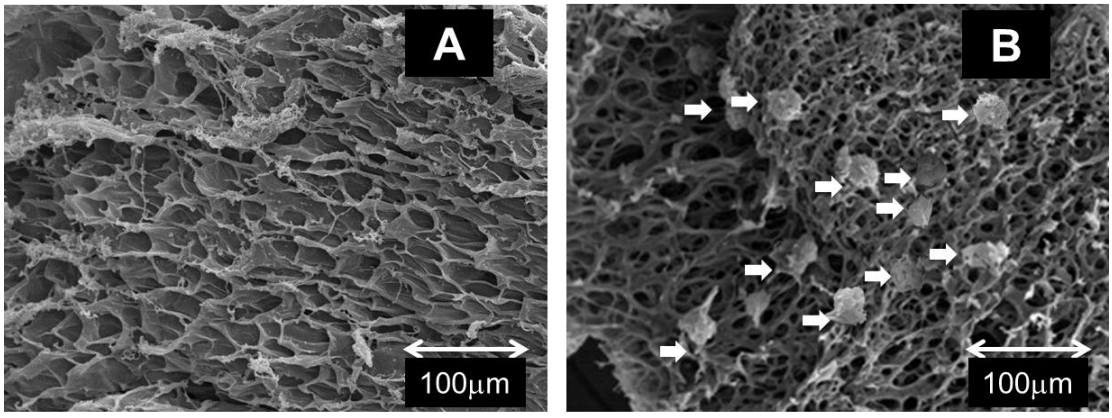


Fig. 9 SEM images of the PMBN hydrogel immobilized with glycine (a) and with the RGDS peptide (b) after culturing hydrogels with BPAEC for 48 h. The arrows represent adherent cells

necessary to increase the density of immobilized RGDS peptides for enhancement of cell adhesion and the construction of tissues inside the hydrogel. It is also critical to evaluate cell function in the hydrogel.

Our recent investigation revealed that the cells encapsulated in the MPC polymer hydrogel are viable and maintain normal function, such as proliferation and differentiation (Aikawa *et al.* 2013, Oda *et al.* 2013, Xu *et al.* 2010). Moreover, the PMBN hydrogel can bind many types of biologically active peptides to control cell function under mild reaction conditions. Several oligopeptides can be reacted simultaneously. Thus, we believe that PMBN hydrogels immobilized with biologically active oligopeptides are potential candidates for the artificial ECM in 3D cell cultures, which regulates cell functions and controls tissue formation.

## 4. Conclusions

In this study, we developed a phospholipid polymer hydrogel matrix using the reactive and water-soluble MPC polymer, PMBN, and we controlled the cross-linking reaction to prepare an artificial ECM. As a cross-linking reagent, polymeric diamine was effective in forming 3D polymer networks. The hydrogels had large pores within the range of 30-50 $\mu$m in diameter, which was sufficiently large enough to allow cells to penetrate. The mechanical properties of the hydrogel matrix were also regulated by the cross-linking reagent. The G' of the hydrogel was comparable to that of soft tissue in a living organism. Cell adhesion was induced by the immobilization of biologically active peptides in the hydrogel. The cell adhesion was strongly dependent on the chemical structure of the biological molecules immobilized onto the hydrogels. From these results, we concluded that the PMBN hydrogel cross-linked with polymeric amine is a promising artificial ECM for cell engineering.

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