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Multilayered phospholipid polymer hydrogels for releasing cell growth factors

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Abstract. Polymer multilayered hydrogels were prepared on a titanium alloy (*Ti*) substrate using a layer-by-layer (LBL) process to load a cell growth factor. Two water-soluble polymers were used to fabricate the multilayered hydrogels, a phospholipid polymer with both *N*, *N*-dimethylaminoethyl methacrylate (DMAEMA) units and 4-vinylphenylboronic acid (VPBA) units [poly(MPC-co-DMAEMA-co-VPBA) (PMDV)], and the polysaccharide alginate (ALG). PMDV interacted with ALG through a selective reaction between the VPBA units in PMDV and the hydroxyl groups in ALG and through electrostatic interactions between the DMAEMA units in PMDA and the anionic carboxyl groups in ALG. First, the *Ti* substrate was covered with photoreactive poly vinyl alcohol, and then the *Ti* alloy was alternately immersed in the respective polymer solutions to form the PMDV/ALG multilayered hydrogels. In this multilayered hydrogel, vascular endothelial growth factor (VEGF) was introduced in different layers during the LbL process under mild conditions. Release of VEGF from the multilayered hydrogels was dependent on the location; however, release continued for 2 weeks. Endothelial cells adhered to the hydrogel and proliferated, and these corresponded to the VEGF release profile from the hydrogel. We concluded that multilayered hydrogels composed of PMDV and ALG could be loaded with cell growth factors that have high activity and can control cell functions. Therefore, this system provides a cell function controllable substrate based on the controlled release of biologically active proteins.

Keywords: multilayered hydrogels; phospholipid polymer; proliferation; growth factor protein

1. Introduction

Recent technological progress in cell production and cell function control has opened the field of regenerative medicine based on cell and tissue engineering. It is well known that three components are needed for successful regenerative medicine, the cells themselves, a scaffold to construct an artificial extracellular matrix, and bioactive molecules to control cell function. Controlled release of bioactive molecules in situ has important applications in regenerative

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medicine. The dose and spatiotemporal release of bioactive molecules at the site of injury, which trigger healing and regeneration, are crucial to achieve a successful outcome.

Vascular endothelial growth factor (VEGF) is a potent angiogenic signal transduction molecule. It can regulate angiogenesis, which is the growth of new capillary blood vessels, by signaling endothelial cells. It also plays a role in the stimulation of endothelial cell proliferation, migration, and differentiation into new blood vessels (Patel and Mikos 2004). Delivery of excess amounts of VEGF is known to cause adverse effects, including hemorrhage, enhancement of tumor growth, and blocking the extracellular VEGF signal through negative feedback control (Gu *et al.* 2004 and Matsusaki *et al.* 2007). However, it is difficult to apply VEGF directly because of its sensitivity to denaturation and degradation by external environmental factors such as pH, organic solvents, sonication, and enzymes. Therefore, it is necessary to control the amount released, the rate of release, and the delivery site for effective tissue regeneration (Itoh *et al.* 2008).

In our previous study (Konno and Ishihara 2007), we obtained a spontaneously formed hydrogel system composed of two water-soluble polymers, a phospholipid polymer with 2-methacryloyloxyethyl phosphorylcholine (MPC) units and 4-vinylphenyl boronic acid (VPBA) units (PMDV) and poly vinyl alcohol (PVA). The MPC polymer can bind to PVA through selective chemical bonding between the VPBA unit in PMDV and the hydroxyl groups in PVA. Therefore, cross-linking points are generated. We immobilized cells or bioactive molecules in the hydrogel (Aikawa *et al.* 2012, 2013, Oda *et al.* 2013 and Xu *et al.* 2010a,b) and we fabricated multilayered hydrogels on the substrate by using the LbL method (Choi *et al.* 2008, 2009). However, when we used this system to immobilize bioactive proteins, we observed much stronger interaction between the polymer components in the hydrogel than needed. Therefore, we suspected that there were additional electrostatic interactions between the two polymers in the hydrogel system. These interactions may stabilize the bioactive protein in the hydrogel layers and control its release.

Alginate (ALG) is an anionic polysaccharide that is widely used due to its gelling properties in aqueous solutions, which are related to interactions between the carboxylic acid moieties and bivalent counter ions (Augst *et al.* 2006 and Rowley and Mooney 2002). As the kinetics of gel formation are very fast and sufficiently strong, several drug and protein delivery applications have been proposed. Many studies have focused on controlled release because VEGF possesses a heparin-binding domain with a high affinity for polyanions such as ALG (Cleland 2001). Moreover, VEGF has a net positive charge at pH 7.4, and can electrostatically interact with negatively charged ALG (Miralem 2001).

The aim of this study is controlled, sustained release of VEGF from PMDV/ALG multilayered hydrogels that were constructed via the LbL process. The hydrogel was constructed using an MPC polymer with cationic moieties and ALG to stabilize VEGF and regulate cell proliferation through VEGF release from the multilayered hydrogel. We used a titanium alloy (*Ti*) substrate as a base material because *Ti* substrates have been used to prepare implantable medical devices in both cardiovascular and orthopedic medicine. Drug release from a cytocompatible surface on the *Ti* substrate may be important to make implantable medical devices.

2. Experimental

2.1 Materials

MPC was obtained from NOF (Tokyo, Japan), which was synthesized using the method

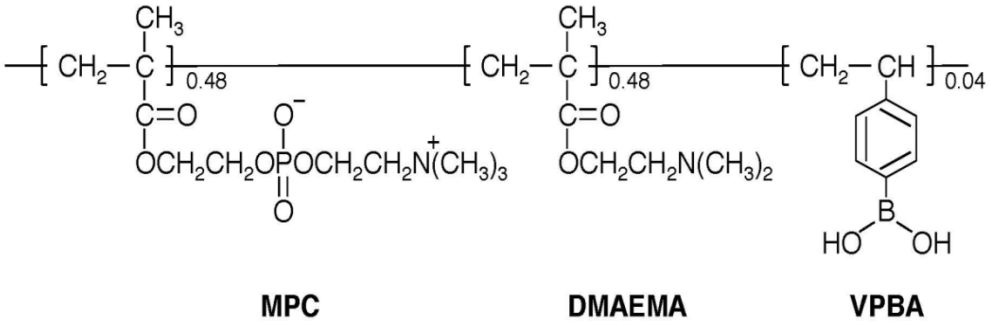

Fig. 1 Chemical structure of poly(MPC-co-DMAEMA-co-VPBA) (PMDV)

reported by Ishihara *et al*. (1990a). The *N*, *N* -dimethylaminoethyl methacrylate (DMAEMA) and VPBA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium alginate and rhVEGF were purchased from Sigma (Japan). The rhVEGF ELISA kit was purchased from Endogen (USA). Octadecyltriethoxysilane (ODS) was purchased from ShinEtsu Chemical Co., Ltd. (Tokyo, Japan). Water-soluble photoreactive PVA containing phenyl azide units (AWP) was purchased from Toyo Gosei Co., Ltd. (Japan). The *Ti* substrate was obtained from DENISPY-Sankin K.K. (Tokyo, Japan). Other reagents and solvents were extra-pure grade and were used without further purification.

2.2 Synthesis of phospholipid polymer

PMDV was synthesized with corresponding monomers by using a previously reported conventional radical MPC polymerization method (Ueda *et al.* 1992). The structure of the copolymer was confirmed with ^{1}H-NMR (α-300; JEOL, Tokyo, Japan) and Fourier-transform infrared spectrometry (FT-IR; FT/IR-615; JASCO, Tokyo, Japan). The molecular weight was determined by gel permeation chromatography (GPC; JASCO). The chemical structure of PMDV is shown in Fig. 1. The unit mole fraction of MPC, DMAEMA, and VPBA was 0.48, 0.48, and 0.04, respectively.

2.3 Fabrication of PMDV/ALG multilayered hydrogels on a Ti substrate

PMDV and ALG were dissolved in PBS at concentrations of 50 mg/mL and 15 mg/mL, respectively. To fabricate multilayered hydrogels, a *Ti* substrate modified with AWP was used. The method used to modify the Ti substrate is as follows [8, 9]. After the *Ti* substrate ($1.0 \times 1.0 \times 0.05$ cm^3) was treated with piranha solution (hydroxyl peroxide:sulfuric acid, 1:3) for 1 h, it was silanized with 10 mM triethoxyoctadecylsilane in toluene at 80℃ for 24 h. The silanized *Ti* substrate was coated with AWP using an aqueous solution (1 wt%), and immobilized by UV-irradiation (135 mW/cm^2) for 40 sec. The multilayered hydrogels were constructed by alternately dipping the *Ti* substrate bonded with AWP in PMDV (50 mg/mL) and ALG (15 mg/mL) solutions for 10 min each, and subsequently rinsing them with PBS for 1 min. Six layers (3 bilayers) terminated with a layer of PMDV were obtained by the LbL method. The structure of the PMDV/ALG multilayered hydrogel on the *Ti* substrate is illustrated in Fig. 2.

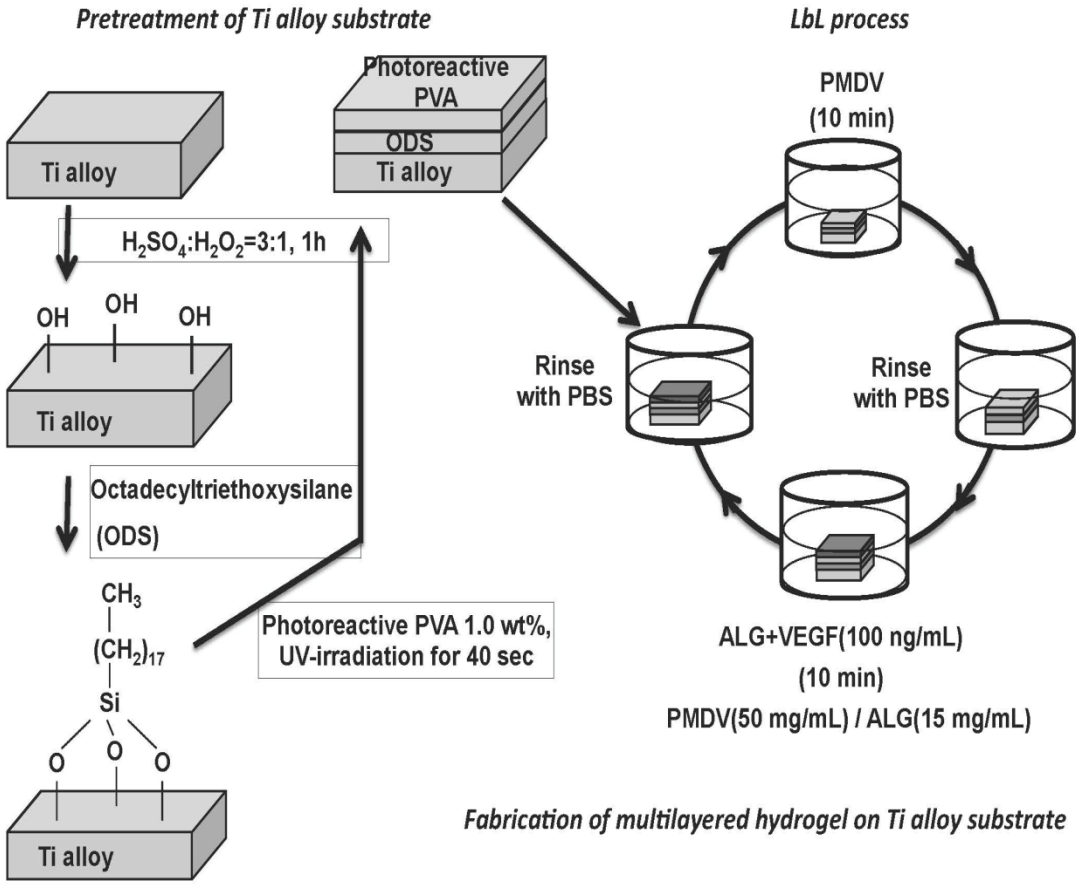

Fig. 2 Preparation of PMDV/ALG multilayered hydrogels on a *Ti* alloy by using the LbL process

2.4 Characterization of PMDV/ALG multilayered hydrogels

To confirm the construction of the multilayered hydrogel, static contact angle and X-ray photoelectron spectroscopy (XPS) (AXIS-His165, Kratos/Shimadzu, Kyoto, Japan) were used with a focused monochromatic Mg Kα X-ray source (1253.6 eV) for excitation. The photoelectron take-off angle was 90 °in the dry state, and the analyzer was operated in constant energy mode for all measurements.

2.5 Cell culture experiments

Human epidermal carcinoma A431 cells were purchased from RIKEN (Saitama, Japan), and cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco, USA) in the presence of 10% heat-inactivated fetal bovine serum (FBS; Gibco) at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cells were grown to 70% confluence, trypsinized with 0.25% trypsin-EDTA, and plated in 6-well plates.

To examine the cytocompatibility of the polymers used in this study, cell viability was determined after incubation in the presence of these polymers. A431 cells were seeded in 96-well plates at a density of 5.0×10^3 cells/well and incubated for 24 h to attach the cells. The polymer was added at concentrations ranging from 10 mg/mL to 10^{-3} mg/mL, and the cells and polymer were incubated for 24 h. Cell viability was determined using a commercially available WST-8 assay kit according to the manufacturer's instructions (Dojindo Chemical Corporation, Japan). We used a representative anionic detergent, sodium dodecyl sulfate (SDS), as a control for this evaluation.

2.6 Control of cell proliferation by VEGF release

VEGF was loaded in the multilayered hydrogels prepared on a *Ti* substrate ($1.0 \times 1.0 \times 0.05$ cm^3). First, VEGF was dissolved in the ALG solution at a concentration of 100 ng/mL. To fabricate a multilayered hydrogel composed of PMDV and ALG, the ALG/VEGF solution was applied to the substrate to load the VEFG in a specific layer. Here, we prepared two types of VEGF-containing multilayered hydrogels, a "bottom-type," which contained VEGF in the first ALG layer (closet to the substrate) and a "top-type," which contained VEGF in the third ALG layer. For the bottom-type, the PVA modified-Ti substrate was immersed in PMDV solution for 10 min at room temperature, and then rinsed with PBS. It was subsequently exposed to ALG for 10 min at room temperature, and then rinsed with distilled water. These procedures were repeated sequentially to make a bottom-type VEFG-loaded multilayered hydrogel, used ALG without VEGF in subsequent immersions. For the top-type, all of the procedures were the same as those for the bottom-type, except that VEGF dissolved in AGL was only used for the last AGL layer.

Multilayered hydrogels were immersed in 1.0 mL aliquots of release medium (0.1 wt% bovine serum albumin (BSA) and 0.05 wt% sodium azide (NaN_3) in PBS, pH 7.1). Activity of the released VEGF was determined by an enzyme-linked immunosorbent assay (ELISA). The ELISA measured the concentration of active VEGF, that is, VEGF that maintained binding to the polyclonal antibody. Plates precoated with polyclonal antibody were used to capture VEGF in each sample. After washing, a biotinylated monoclonal antibody specific for VEGF was added, which bound to the immobilized VEGF captured during the first incubation. Biotinylated VEGF specifically bound to streptavidin-peroxidase, which allowed for chromogenic detection at 450 nm upon the addition of substrate (tetramethylbenzidine [TMB] solution). The absorbance of the samples and standards were measured at 450 nm using a microplate reader. In addition, the total amount of VEGF in the multilayered hydrogel was determined by using the same method after the hydrogel was dissociated by the addition of glucose.

The activity of VEGF released from the multilayered hydrogel was determined using a cell proliferation assay. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). HUVECs were maintained in EGM-2 medium (CC-3162; Cambrex Bio Science Walkersville, Inc.) containing 2% FBS and growth factors, and were used at the third or fourth passage. Briefly, HUVECs were seeded in 6-well tissue culture plates at a density of 1.0×10^4 cells/well, and a WST-8 assay was used to quantify viable cells. Cell proliferation was determined at different time points over a period of five days. The relative number of cells was calculated based on the number of cells adhered to the culture plate (as the control) at day -1. More than six samples were evaluated for each hydrogel system. The data were compared by Student's *t*-test, and *p* values less than 0.05 were considered significant.

3. Results and discussion

3.1 PMDV/ALG multilayered hydrogels formed on a Ti substrate

To maintain growth factors at the site of implantation for sustained release over long periods of time, a hydrogel was designed with a biocompatible phospholipid polymer containing MPC units. It is well known that MPC polymers have a surface that resists protein adsorption and cell adhesion (Ishihara *et al.* 1990b, 1992, 1998 and Lewis *et al.* 2000, 2001). They are also useful for making hydrogels, including spontaneously formed hydrogels and thermally reversible hydrogels (Ishihara *et al.* 1990a, Morisaku *et al.* 2008, Kimura *et al.* 2005, Nam *et al.* 2002, Li *et al.* 2005 and Sammon *et al*. 2006).

In the MPC polymer, VPBA units and DMAEMA units were introduced to associate with units in ALG. As we expect that the VPBA unit will react with the *cis*-diol in ALG to form reversible covalent bonds (Lorand and Edwards 1959) and that the DMAEMA makes electrostatically interacts with the carboxylic acid of ALG, the mixture of PMDV and ALG was expected to form a self-assembled hydrogel. Moreover, the LbL process aids VEGF release for localized and prolonged delivery, and this method has been studied for drug delivery system (van den Beucken *et al.* 2006, Yamauchi *et al.* 2006, Decher *et al.* 1992, Tang *et al.* 2006). The cell growth control factor VEGF was introduced in the multilayered hydrogel by the LbL procedure. The hydrogel layers formed spontaneously under physiological conditions (25 ℃–37 ℃, pH 7.4) by mixing two kinds of water-soluble polymers, PMDV and ALG. During this procedure, VEGF could be introduced in a suitable layer of the multilayered hydrogel. The process is illustrated in Fig. 2.

When the VEGF was contained in the outermost layer of the multilayered hydrogel, it was called “top-type,” whereas, when the VEGF was contained in the first ALG layer closet to the substrate, it was called “bottom-type.”

Copolymerization of MPC, DMAEMA, and VPBA proceeded well, and a polymer containing these monomer units was obtained. In addition, the obtained PMDV was water soluble. Spectral data from ^{1}H-NMR and FT-IR revealed the chemical structure of the obtained PMDV (Fig. 1).

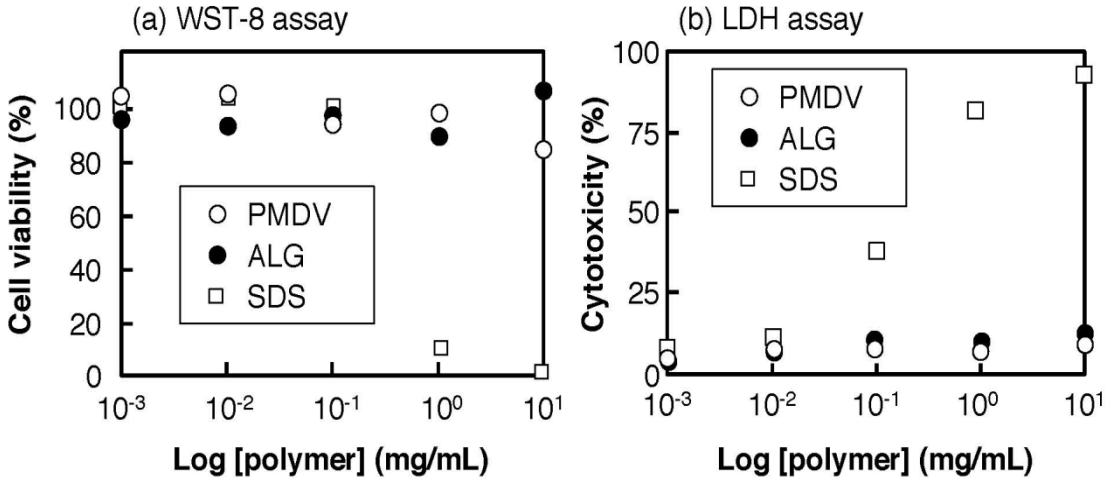

Fig. 3 Cytotoxicity of PMBV and ALG compared to that of SDS

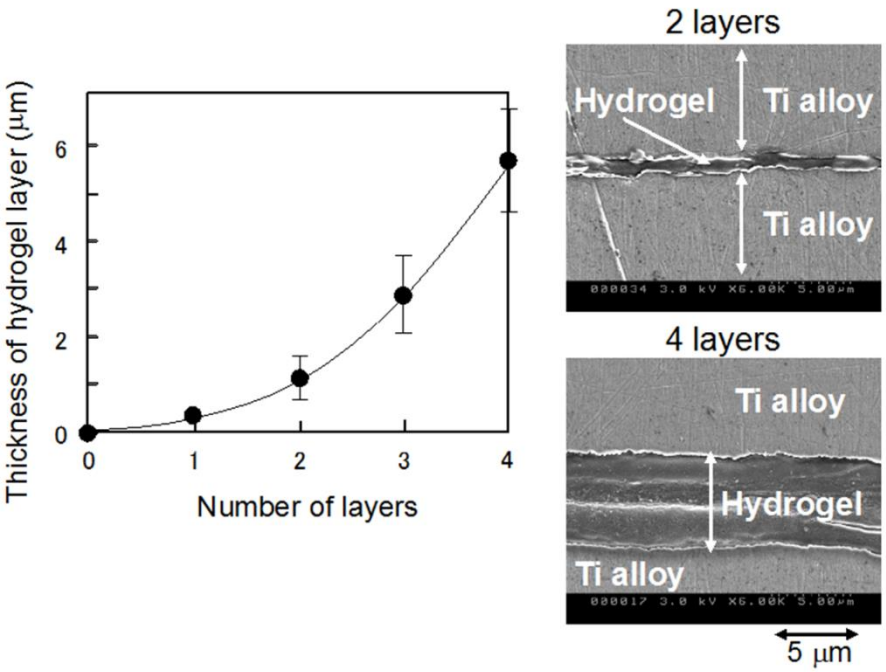

Fig. 4 The thickness of PMDV/ALG multilayered hydrogels prepared on a *Ti* alloy

We examined the cytocompatibility of these polymers against A431 cells. Fig. 3 shows the concentration dependence of the hydrogel on cell viability and cytotoxicity when these polymers were added to the culture medium. Cytotoxicity was observed at high concentrations of a representative anionic detergent, SDS. In contrast, when both polymers in the multilayered hydrogel were added to the cell culture system, they did not influence the viability of the cells, and no cytotoxicity was observed. Therefore, PMDV and ALG were cytocompatible.

Fig. 4 shows the thickness of the PMDV/ALG multilayered hydrogel constructed via the LbL method. PMDV was expected to provide a positive charge from the DMAEMA unit and a *cis*-diol binding site from the VPBA unit, whereas ALG has a negative charge and a *cis*-diol. In addition, ALG is known to have high affinity against VEGF because VEGF is positively charged at pH 7.0, opposed to negatively charged ALG (Gu *et al.* 2004). As the number of layers increased, the thickness of the layers increased from 0.5m to 6m. Based on the results of XPS measurements, the chemical composition of the outmost layer, that is, the PMDV/ALG ratio, can be evaluated from the ratio of phosphorus atoms attributed to the MPC units and the carbon atom (P/C value). After the PMDV immersion process, the surface P/C value increased, whereas after an ALG layer was added, it decreased about one-half. This result corresponded well with how the surface was altered by PMDV and ALG during the LbL process.

3.2 VEGF loading of and release from the PMDV/ALG hydrogels

We loaded VEGF in one of the ALG layers during fabrication of the PMDV/ALG multilayered hydrogel. At that time, the hydrogels were selected as “top-type” and bottom-type” as shown in

Fig. 5. The total amount of VEGF was approximately 1.20 ng in both cases. The VEGF release profiles from the multilayered hydrogels are also shown in Fig. 5. Regardless of the VEGF-loading type, an initial burst release was observed on 1 day, which was 25% of the initial amount loaded in the top-type and 12% of that loaded in the bottom-type. After that, slower release was observed over 14 days. The initial release from the bottom-type hydrogel was lower than that from the top-type. Then, VEGF release continued at an almost constant rate. The location of the VEGF-loading layer was not significant during this period. Assuming that the VEGF diffused throughout the multilayered hydrogel, the initial burst was due to the accumulation of VEGF near the surface layer of the hydrogel. Only one PMDB layer regulated the release of VEGF from top-type hydrogel. In contrast, five polymer layers regulated release from the bottom-type hydrogel. That is, the diffusion of VEGF was not a dominant factor during the period. We also investigated the dissociation of multilayered hydrogel under physiological conditions (pH 7.1 in PBS containing 0.1 wt% BSA and 0.05 wt% NaN_3 at 37℃). Both PMDV and ALG are water-soluble polymers, and a hydrogel composed of these polymers gradually dissociated after one day of immersion. Polymer dissociation appeared to be increasing, and the effect of dissociation may be a dominant factor regulating VEGF release. On day four, almost 60% of the initial amount of polymer was dissociated. The rate of dissociation of the two types of hydrogels was nearly identical. Accompanying this phenomenon, approximately 36% and 25% of the initial amount immobilized in the hydrogel was released from the top-type and bottom-type hydrogels, respectively. However, the release rate of VEGF was nearly the same. Consequently, as a release mechanism, it was thought diffusion due to the difference in VEGF concentration throughout the layers and polymer dissociation may influence VEGF release. Dissociation of the polymer hydrogel may be a dominant factor controlling VEGF release compared to diffusion, owing to the difference in concentration.

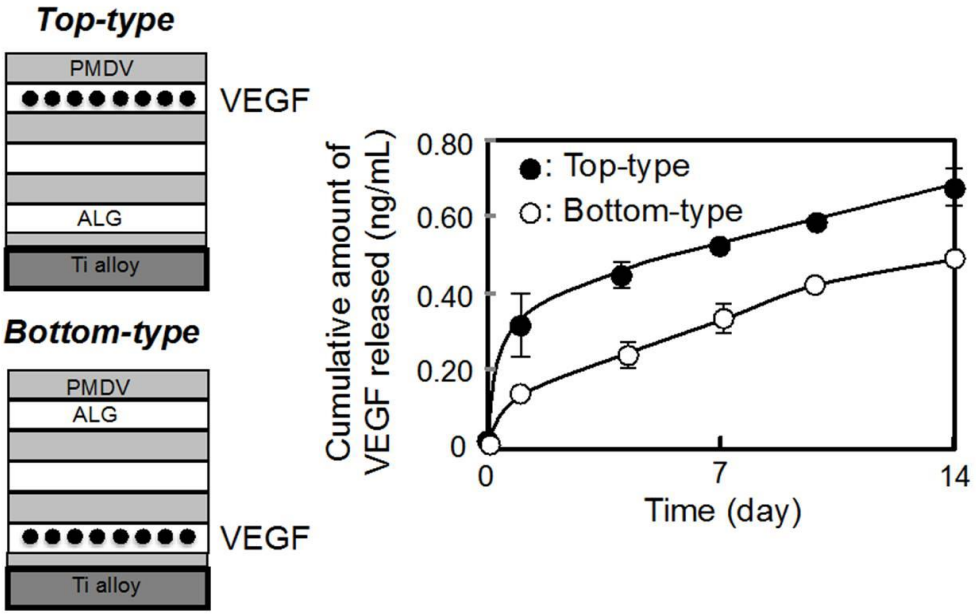

Fig. 5 Release of VEGF form PMDV/ALG multilayered hydrogels

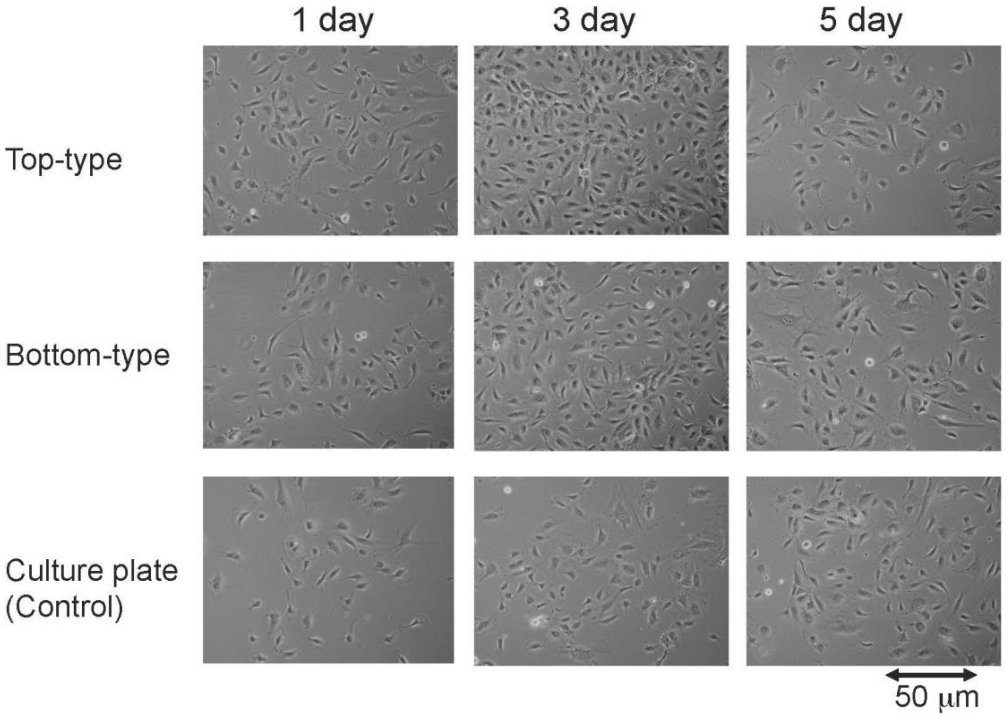

Fig. 6 Morphology and proliferation of HUVECs on VEGF-loaded PMDV/ALG multilayered hydrogels

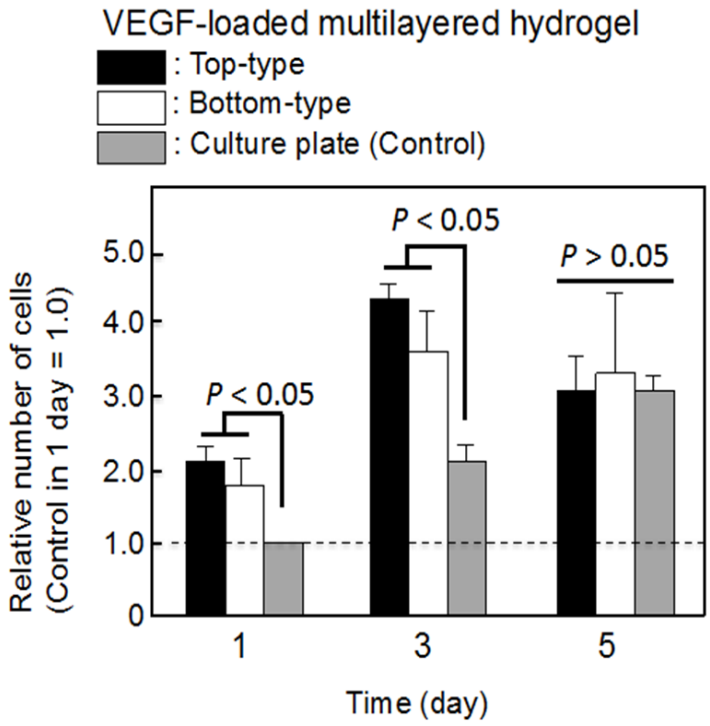

Fig. 7 Relative number of proliferated cells on VEGF-loaded PMDV/ALG multilayered hydrogels

3.3 Control of cell proliferation on the hydrogel

PMDV layer (Data not shown); this is likely due to the nature of MPC polymers (Ishihara *et al.* 1999, Choi *et al.* 2008 and Saito *et al.* 2010). Therefore, we used a conventional cell culture plate as a control for the cell adhesion experiment. On the conventional cell culture plate, the number of cells gradually increased as the culture period increased. On days 1 and 3, a greater number of cells was observed on the hydrogels loaded with VEGF than on the control plate. The number of cells on the top-type VEGF-loaded hydrogel was significantly greater than the number on the bottom-type VEGF-loaded hydrogel on day 3. On subsequent days, cell proliferation was reduced; therefore, although we did not observed any direct evidence of contact, we thought that cell proliferation might have been impeded by contact inhibition. There was no difference in HUVEC proliferation between the two VEGF-loaded hydrogels on day 5. Based on these results, we concluded that the concentration of VEGF released from the hydrogel is sufficient to stimulate HUVEC proliferation.

4. Conclusions

To regulate cell proliferation on a substrate, we constructed VEGF-loaded multilayered hydrogels using water-soluble PMDV and ALG via the LbL method. Spontaneous formation of the hydrogels occurred due to the bonding between phenylboronic acid units and *cis*-diol. In addition, electrostatic interactions between the ternary amino group in the DMAEMA unit in PMDV and the carboxylate anion in ALG may also aid hydrogel formation. These polymers showed good cytocompatibility. The VEGF-loaded PMDV/ALG multilayered hydrogels released VEGF for two weeks, and the VEGF released from the hydrogels controlled cell proliferation. Finally, we conclude that this system could provide cell function control on implantable medical devices, and we expect it to be widely applied in the fields of cell engineering and tissue engineering.

Acknowledgments

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