Glycoprotein recognition for cell capturing on artificial lectin surface

Aya Saito 1,3,5, Tomohiro Konno 2,3,4, Hiroki Ikake 5, Kimio Kurita 5, and Kazuhiko Ishihara 1,2,3,4

1) Department of Materials Engineering, 2) Department of Bioengineering, and 3) Center for NanoBio Integration, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
4) Japan Science and Technology Agency-CREST, 5 Sanban-cho, Chiyoda-ku, Tokyo 102-0075, Japan
5) Department of Materials and Applied Chemistry, Graduate School of Science and Technology, Nihon University, 1-8-14, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8308, Japan
Fax: +81-3-5841-0595, e-mail: saito@mpc.t.u-tokyo.ac.jp

We prepared a new biointerface for obtaining specific recognition of glycoproteins having polysaccharide chains to capture corresponding cells by a phospholipid polymer having phenylboronic acid moiety (PMBV), so-called artificial lectin. The PMBV was coated on the surfaces of Au substrate and tissue culture polystyrene. The affinity of the PMBV toward the glycoproteins and its effects on cell adhesion were evaluated. X-ray photoelectron spectroscopic analysis revealed that the PMBV existed on both substrates. Although adsorption of protein without polysaccharide chain was suppressed, glycoprotein as fibronectin (FN) adsorbed effectively, which was confirmed by surface plasmon resonance. Kinetic parameters calculated by SPR measurements showed high affinity between PMBV and FN, 1.4×10^5 (M^-1). Cell adhesion and growth behavior on PMBV was observed using L-929 fibroblast cells. It was demonstrated that cells proliferated well on PMBV surface and maintained their sphere morphology. Also, the activity of cells was kept in higher level on the PMBV surface compared with conventional surface.

Key words: phospholipid polymer, phenylboronic acid, molecular recognition, cell capturing, glycoprotein

1. INTRODUCTION

The systems using adhesive cell play increasingly important and indispensable roles in the wide fields of bioengineering, such as drug discovery, biosensors, and tissue engineering [1, 2]. An important thing of assaying or culturing cells is to reflect natural conditions of cells in vivo. Reflecting natural conditions would allow for minimizing difference of effect between in vivo and in vitro (i.e., efficacy of a drug). This would lead to effective drug discovery and cell therapy. Incidentally, the materials having an ability to bind biomolecules via a specific molecular recognition received widespread attention as the materials for the control of function of cells. The approaches that immobilizing of biomolecules onto material surfaces for the control of function of cells have been employed in the past. Immobilization of Arg–Gly–Asp (RGD) peptide is one of the most common methods. This sequence binds to integrin receptors on cell surfaces, inducing cell spreading and intracellular signaling, hence mimicking cell-to-extracellular matrix interactions [3–5]. In addition, surfaces that immobilized of various proteins have been reported for any purpose. These proteins include collagen, fibronectin (FN), vitronectin, and laminin. However, problems that decrease of activity of biomolecules immobilized onto surfaces and inflammatory response of cultured cells are remained. These two problems are undesirable for reflect of natural cells in vivo conditions. In this study, we proposed a new biointerface to capture cells. This biointerface have affinity with glycoproteins, which play the important roles in cell adhesion, proliferation, and differentiation as the antenna molecules of cell membrane [6, 7]. First, we designed and synthesized a phospholipid polymer (PMBV) composed of 2-methacryloyloxyethyl phosphorylcholine (MPC), n-butyl methacrylate (BMA), and 4-vinylphenylboronic acid units (VPBA). The MPC has a phosphorylcholine (PC) group, which is a polar group also found on living cell membranes. Hence, the surfaces coated with the MPC polymer inhibit a nonspecific protein adsorption, and perform better than most other types of polymeric materials with respect to bio/blood compatibility [8–12]. In addition, the MPC polymers have the ability to inhibit a conformational change and denaturation of proteins [13]. They also could effectively reduce the inflammatory reaction of surrounding cells and tissues [14]. That is, conventional MPC polymers, such as poly(MPC-co-BMA) (PMB), did not have affinity for specific biomolecules. To obtain affinity to a polysaccharide in the glycoproteins, the VPBA units were introduced in the MPC polymer. The VPBA units could recognize and form the covalent bond with 1,2- and 1,3-diois units in polysaccharide chain of glycoproteins. The proteins called lectin exist in the cell membrane, and it recognize the polysaccharide chain. The lectins control the cell functions. PMBV surface have the structure to imitate such lectin, so is an artificial lectin having ability of recognition of the polysaccharide chain. This covalent bonding is reversible and in equilibrium state of boronate esters [15]. Herein we report the PMBV surfaces which have two characteristic moieties, i.e. "inhibiting the nonspecific protein adsorption" of MPC units and "having high affinity with glycoproteins" of VPBA units. And the PMBV surface is aimed to capture cells through adhesive glycoproteins binding selectively onto the surface.
2. EXPERIMENTS
2.1 Synthesis of polymers
The PMBV and PMB were synthesized by a conventional radical polymerization using 2,2'-azobisisobutyronitrile (AIBN) as an initiator. The MPC was obtained from NOF Corporation (Tokyo, Japan); it was synthesized using a method developed by Ishihara. The BMA was purchased from Nagakura Tesque, Inc. (Kyoto, Japan) and VPBA was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). PMBV is the polymer that mole fractions of MPC, BMA, and VPBA in feed are 0.25, 0.55, 0.20, respectively. The mole fraction of MPC in the PMB is 0.30. The PMBV and PMB were obtained as a white powder. The ratio of monomer unit composition was determined by $^1$H-NMR (JEOL JNM-NR30, Tokyo, Japan). The molecular weight was evaluated by gel-permeation chromatography (GPC) with OHpak SB-804 HQ column (Shodex, Tokyo, Japan). Fig. 2 and Table I show the chemical structures and synthetic results of the PMBV.

2.2 Characterization of polymer surface
The polymer surface was prepared by two methods; spin coating method and dip coating method. Spin-coated surface was prepared for SPR measurements. The Au substrate was purchased from Moritex, Inc (Tokyo, Japan). The PMBV and PMB were dissolved in ethanol (EtOH) under stirring to prepare 0.2 wt% solution. The Au substrates were cleaned in EtOH with ultrasonic agitation. The plates were spin coated at 1000 rpm for 1 min. Then chips were dried under vacuum overnight, and obtained polymer-coated the Au substrate. Dip-coated surface was prepared for use in cell culturing. PMBV and PMB solutions were made up to 0.5 wt% concentrations in ethanol, and were dropped on the surface of tissue culture polystyrene (TCPS). After dried under room temperature, repetition these process, obtained polymer-coated cell culture surface. The surfaces of polymer-coated on Au and TCPS were analyzed by X-ray photoelectron spectroscopy (XPS) with a photoelectron take-off angle of 45° (Kratos/Shimadzu, Kyoto, Japan). To prevent charge-up, an electron gun to neutralize was used.

2.3 Evaluation of kinetics parameter for binding of FN to PMBV by SPR

The interaction analysis between PMBV and glycoprotein was carried out by SPR instrument (SPR-670M, Moritex). The running buffer, phosphate buffered saline (PBS, pH 7.1), was flowed on polymer-coated the Au substrate at 15 L/min. The FN (Wako, Osaka, Japan) was used as glycoprotein for evaluation of specific adsorption. Bovine serum albumin (BSA: Sigma-Aldrich, St. Louis, USA) was used for protein non-specific adsorption because BSA does not have polysaccharide chains in the molecule. All measurements were done at 25 °C. The FN (0.5 mg/mL) and BSA (4.5 mg/mL) solutions diluted with running buffer were injected into the SPR instrument, and contacted with polymer-coated the Au substrate for 4 min. Then, the plates were rinsed with PBS immediately. Kinetics parameters for binding ($k_a$) and detaching ($k_d$) of FN were determined from these SPR results. Also, affinity constant $K_a = (k_a / k_d)$ was calculated.

2.4 Cell adhesion on the polymer surfaces
Mouse fibroblast cells (L-929) were purchased from RIKEN cell bank (Saitama, Japan). The L-929 cells were seeded onto 96-well culturing plates coated with the polymers at a concentration of $5.0 \times 10^5$ cells/well. The cell-culturing medium was Dulbecco’s Modified Eagle Medium low glucose (D-MEM, Gibco, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco). The cells were incubated for 24h at 37 °C in a 5% CO$_2$ atmosphere. After rinse with PBS, the adherent cells were observed by phase-contrast microscope (IX71S1F-2, Olympus Optical Co. LTD., Tokyo, Japan).

2.5 Evaluation of velocity of cell migration
The observation of cell migration was carried out by cultured cell monitoring system (CCM-M1.4, Astec Co., Fukuoka, Japan). The L-929 cells were seeded onto 35 mm culturing dishes coated with and without polymers at a concentration of $5.0 \times 10^5$ cells/well. The cells were incubated and observed for 20 h at 37 °C in a 5% CO$_2$ atmosphere. The velocity of cell migration was analyzed using the public domain software for image analysis, ImageJ (NIH, Bethesda, USA; http://rsb.info.nih.gov/ij/).

2.6 Cell proliferation assay
Cell proliferation was assessed by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-di sulphonylphenyl)-2H-tetrazolium, monosodium salt] assay. L-929 cells were seeded onto 96-well culturing plates (PMBV-coated and bare TCPS) at a concentration of $5.0 \times 10^3$ cells/well. Cells were incubated for 3 h, 24 h, and 48 h at 37 °C. After incubation, 10 L WST-8 reagent was added to the wells and incubated for 3h at 37 °C. And then the absorbance at 450 nm was measured with the Appliskan multimode microplate reader (Thermo Scientific). The number of cells in each well was calculated by absorbance and standard curve.

2.7 Cell viability assay
Cell viability was assessed by lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Wako). L-929 cells were seeded onto 96 well culturing plates (PMBV-coated and bare TCPS) at a concentration of $5.0 \times 10^3$ cells/well. Cells were incubated for 3 h, 24 h, and 48 h at 37 °C.
After incubation, 50 L of supernatant medium of each well was transferred into wells of a reaction-plate and added coloring reagent. After 45 min at room temperature, 50 µl of stop solution was applied. And then the absorbance at 560 nm was measured. The cell viability per well was calculated.

3. RESULTS AND DISCUSSION

3.1 Characterization of polymers and surface treated with these polymers

The synthetic results of the polymers used in this study are summarized in Table I. The compositions of each monomer unit in the copolymers were in good agreement with their compositions in the feed. These polymers were not water-soluble, but soluble in ethanol. From XPS measurements, the peaks of the N1s (402 eV), P 2p (133 eV), and B 1s (190 eV) were observed on the PMBV-coated Au and TCPS substrate. These are attributed to the trimethyl ammonium group (402 eV), phosphate ester group (133 eV), and phenylboronic acid group (190 eV), respectively. And also, Au4f spectra were disappeared on PMBV-coated Au substrate. These results supposed the existence of MPC and VPBA moieties of PMBV-coated on substrate.

3.2 Affinity of FN to PMBV

The binding affinities toward two bio molecules, BSA and FN, were assessed using SPR. BSA was used as a typical protein to evaluate nonspecific adsorption. FN was used as a glycoprotein. FN has been reported to play the role in multiple cellular processes, including cell-cell and cell-ECM adhesion, cell migration, differentiation. The result of kinetic analysis of SPR measurement toward FN was shown in Table II. Kinetic parameters calculated by SPR measurements showed high affinity between PMBV and FN, \(1.4 \times 10^5\) M\(^{-1}\). This is a quite high affinity forward FN as compared to PMB. Fig. 3 shows the profile of SPR measurements injected FN and BSA onto each surface. While injecting BSA onto PMBV surface was increased SPR angle, this angle shift was decreased completely by rinse with PBS. These results indicate that PMBV surface was bind with FN, and nonspecific adsorption of BSA was completely inhibited. That is, PMBV have specific affinity toward glycoprotein, FN.

3.3 Adhesion and migration of L-929 cells on PMBV surface

FN and laminin, major glycoprotein play important roles in the processes such as cell adhesion and migration. Fig. 4 shows the cell monitoring system images of the L-929 cells cultured on surfaces of PMBV (A), PMB (B) and TCPS (C) at 20 h, respectively. Cells on the PMB surface did not adhere, at the same time, but they aggregated each other. This is due to the PC groups in the MPC unit suppress protein adsorption. On the surface of TCPS, cells adhered significantly and they extended. These adhesions were attributed to the
non-specific adsorption of proteins. On the other hand, cells of PMBV held sphere morphology without extending. This adhesion can be explained that the bonding of adhesive glycoprotein of extracellular matrix (ECM) inhibiting non-specific adsorption of other biomolecules. To evaluate the strength of interaction between cell and surface, the cell migration velocities were calculated from cell trajectories using the software for image analysis, ImageJ (Table III). L-929 cells on TCPS surface migrated lately. This phenomenon is suggested that there is strong interaction between cell and surface. In contrast, PMBV surface migrated slightly faster than cells on TCPS. Therefore, it was supposed that PMBV surface inhibited non-specific interaction with surface, and captured cells.

Table III. Velocity of cell migration on each surface.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Velocity (μm/h)</th>
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<tbody>
<tr>
<td>TCPS</td>
<td>39</td>
</tr>
<tr>
<td>PMBV</td>
<td>105</td>
</tr>
<tr>
<td>PM</td>
<td>22</td>
</tr>
</tbody>
</table>

3.4 Cell proliferation assay and cell viability assay
The result of cell proliferation assay using WST-8 kit is shown in Fig. 5A. PMBV induced cell-growth gradually, compared with the cells on TCPS. Therefore, it is suspected that holding of globular form does not give a serious influence in cell growth.

![Fig. 5A: Proliferation assay results.](image)

![Fig. 5B: Cell viability assay results.](image)

**Fig. 5B** shows the results of cell viability assay using LDH assay kit. Interestingly, despite the cells on TCPS was decreased cell viability with the passage of time, cells on PMBV was hold high viability, almost 100%. It was confirmed that surface of PMBV did not induce cellular membrane damages.

By using this polymer surfaces, we are going to control the differentiation of the stem cells.

4. CONCLUSION
We prepared a new biointerface for specific recognition of glycoproteins and for the purpose of cell capturing by a phospholipid polymer bearing phenylboronic acid moiety (PMBV). The PMBV surface bound specifically to adhesive glycoprotein, FN. An affinity constant of PMBV and FN is about 250 times that of phospholipid polymer without phenylboronic acid moiety. The cells cultured on PMBV surface proliferated in keeping globular form without extending or damaging. From these results, we conclude that the PMBV provides cytocompatible and recognizable interface for controlling cell functions.

REFERENCES

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