Controlled adhesion of human lymphocytes on electrically charged polymer surface having phosphorylcholine moiety

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Abstract

The human lymphocytes were interacted with polymer surfaces whose surface potential was controlled by the formation of a polyelectrolyte complex (PIC) having a phosphorylcholine moiety. 3-(Methacryloyloxypropyl)-trimethyl ammonium iodide as the cationic unit or potassium 3-methacryloyloxypropyl sulfonate as the anionic unit was copolymerized with 2-methacryloyloxyethyl phosphorylcholine (MPC) and \(n\)-butyl methacrylate. PIC was made at the solid–liquid interface, that is, an aqueous solution containing an anionic polymer with different concentrations was contacted with a cationic polymer coated polymer membrane. The formation process of PIC was followed using a quartz crystal microbalance, and the PIC surfaces were analyzed by \(\zeta\)-potential and X-ray photoelectron spectroscopy. The surface potential on the PIC was controllable from \(+20\) to \(-16\) mV, which increased in the amount of adsorbed anionic copolymer as the \(\zeta\)-potential decreased toward the negative charge. The PIC surface in contact with human lymphocyte for 5 h was observed using a scanning electron microscopy and the density of the adherent human lymphocyte was determined by the lactate dehydrogenase method. The lymphocyte adhesion on the surface was gradually reduced with an increase in the negative value of the \(\zeta\)-potential. The morphological change in the adherent lymphocytes was not observed on the polymer surfaces with MPC units. The adherent lymphocytes were not activated on the PIC surface. The lymphocyte adhesion with reduced activation could be controlled by changing the surface potential on the polymer with the MPC unit.

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1. Introduction

The biocompatibility of material surfaces is heavily dependent upon their physical and biological interactions [1]. By controlling these interactions makes possible to produce new biofunctional devices. Of particular interest is biomimetic surfaces involving the immobilization of bioactive peptides or protein architectures, designed to induce specific cell and tissue responses. On the other hand, different parameters such as hydrophobicity and hydrophilicity, surface charge, roughness, and free energy, affect protein adsorption and thereby subsequent cellular adhesion. The definition of general rules is not straightforward, and the observed cellular behaviors probably depend not only on a single parameter but also on a complex combination of different factors. Substrate properties somehow modulate the ability of adsorbed species to interact with cells.

The copolymers composed of 2-methacryloyloxyethyl phosphorylcholine (MPC) and \(n\)-butyl methacrylate (BMA) have shown a nonthrombogenicity, that is, suppression of platelet adhesion, activation, and aggregation when the copolymers come in contact with human whole blood even in the absence of an anticoagulant [2–6]. The amount of adsorbed proteins and the number of adherent blood cells decreased with an increase in the MPC units in the polymer chains. Based on the CPK model, the MPC polymer possibly had a structure with a biomembrane-like surface [7]. The polyelectrolyte complex (PIC) composed of anionic and cationic charged MPC copolymers induced the selective adhesion of platelets but did not activate the adherent platelets even when the PIC having a phosphorylcholine moiety was contacted with whole blood [3]. These findings
induced us to develop a new polymeric material for a cell separation system.

The prime function of the immune system is to exclude a foreign virus or cells by human lymphocytes, that is, to understand the function of human lymphocytes adhered to biomaterials is very important.

On the PIC surfaces composed of anionic and cationic polyelectrolytes, electrostatic interactions with cells can be controlled. Kataoka et al. reported the reduced thrombogenicity of an anionic PIC composed of a polystyrene derivative having sulfonate groups and that with quaternary ammonium groups [8,9]. They also found the separation of a subpopulation of lymphocytes based on the ionic characteristics of a graft copolymer with a polyamine side chain.

Recently, the most common methods for the preparation of ultrathin films have been the alternate adsorption method based on the electrostatic interactions of oppositely charged polyelectrolyte layers [10–14]. An alternate adsorption can be performed under mild conditions and is applicable to variously shaped surfaces. However, in general, the introduction of an electrical charge in a polymer induced considerable cell activation and protein denaturation due to a strong interaction between the polymer and the blood components. In addition, the relation between the distribution of the electrical charge and cell adhesion is still unclear. The concept of PIC surface is to form ionic bond on the material surface. PIC surface controlling surface potential, charge density and surface distribution is constructed at changing the amount of adsorbed anionic polymer.

Based on the properties of the MPC copolymers, we hypothesized that novel types of functional polymer surfaces that show selective adhesion of the cell might be prepared from an electrically charged MPC copolymer. The strategy of this study for preparing electrically controlled phosphorylcholine polymer surfaces is shown in Fig. 1. The anionic and cationic MPC copolymers were synthesized, and the formation of the PIC was followed using a quartz crystal microbalance (QCM). The PIC surfaces with various electrical charges were prepared and human lymphocyte adhesion on the PIC surface was investigated with attention to its surface properties.

2. Materials and method

2.1. Synthesis of MPC copolymers

MPC was synthesized using a previously reported method [15]. BMA was reagent grade and used after vacuum distillation (bp 68.5 °C/32 mm Hg). Potassium 3-methacryloyloxypropyl sulfonate (PMPS) was purchased from Aldrich Chemical (Milwaukee, WI) and used without further purification. 3-Methacryloyloxypropyl trimethyl ammonium iodide (MAPTMAI) was synthesized using a previously reported method [4] and obtained as a white powder (yield 54.5%, mp 164.4 °C). All reagents and solvents were purified by conventional methods.

The desired amounts of MPC, BMA, and PMPS or MAPTMAI were placed in a polymerization tube. 2,2'-Azobisisobutyronitrile (AIBN; Kanto Kagaku, Tokyo, Japan) and ethanol (EtOH) were added to the tube as the polymerization initiator and solvent, respectively. After the tube was sealed, the copolymerization was carried out at 60 °C for 15 h. The reaction mixture was poured into diethyl ether to precipitate the copolymers, and the copolymer was collected and dried in vacuo. The chemical structures of these MPC copolymers were identified by 1H-NMR [4]. The mole fractions of each component in the copolymer were determined by the phosphorus- and elemental analyses of carbon (C), nitrogen (N), sulfur (S), and phosphorus (P). The cationic and anionic MPC copolymers were called PMMB and PMSB, respectively. Fig. 2 and Table 1 show the chemical structures and synthetic results of the copolymers, respectively.

2.2. Preparation of PIC

The poly(ethylene terephthalate) (PET) (20 × 40 mm², 180 µm in thickness, Toli Co., Tokyo, Japan) membrane

![Fig. 1. Schematic representation of an electrically controlled polymer surface prepared with electrostatic adsorption of anionic polymers on cationic polymer substrates.](image-url)
was immersed in an EtOH solution containing the PMMB (0.5 wt%), dried in air at room temperature, and then dried in vacuo. The membrane was immersed in distilled water and stored at room temperature for 2 h to equilibrate the surface. The membrane was transferred to a glass vial containing distilled water (5.6 ml). The desired concentration of PMSB in aqueous solution (0.4 ml) was added to the vial with stirring. The incubation time was adjusted for 5 s at room temperature, rinsed with distilled water, and then dried in vacuo. Various electrically charged surfaces were prepared by changing the PMSB concentration. In this study, we used PMSB aqueous solutions as shown in Table 2.

2.3. Quartz crystal microbalance measurement

The formation of PIC was followed using QCM. A 5 MHz AT-cut quartz crystal with a gold electrode as a resonator purchased from Hokuto Electronics Co., Tokyo, Japan, was used in this study. The resonator was coated with the PMMB using a 0.5 wt% EtOH solution with a spin coater at 3000 rpm for 20 s. The PMMB-coated resonator was dried in vacuo. The resonator was then fixed on the cell. The quantity of the coated copolymer was calculated using Sauerbrey’s equation [16]. For the QCM system, the quantity change per 1 Hz was 2.8 ng. It was assumed that about 810 ng of polymer was coated on the resonator. A 5.6 ml aliquot of distilled water was added in the cell and stored until the resonant frequency was stabilized. Afterwards, the PMSB aqueous solution (0.4 ml) was added to the cell and the resonant frequency change was measured until stabilized at 24 °C.

2.4. Surface analysis

The membrane surface was analyzed by X-ray photoelectron spectroscopy (XPS, ESCA-200, Scienta, Uppsala, Sweden) to determine the phosphorylcholine and sulfonate unit composition on the surface. It was calculated from the ratio of phosphorus and sulfur atoms of the phospholipid and sulfonate unit versus the carbon atoms. The take-off angle of the photoelectron was adjusted at 15° and 90°.

![Chemical structure of MPC copolymers](image)

**Table 1**
Characterization of MPC copolymers

| Abb. | Monomer (X) | Mole fraction [MPC/X/BMA] | Yield (%) | Solubility |
|------|-------------|--------------------------|-----------|-----------|
|      | In feed     | In copolymer*             |           | H₂O       | EtOH      |
| PMMB | MAPTMAI     | 0.20/0.20/0.60            | 77.1      | –         | +         |
| PMSB | PMPS        | 0.45/0.05/0.50            | 88.5      | +         | –         |
| PMB  | –/–/0.80    | 0.21/–/0.79               | 83.5      | –         | +         |

[Monomer] = 1.0 mol/l, [AIBN] = 0.5 mmol/l; polymerization was carried out at 60 °C for 15 h; +: soluble, –: insoluble.

* Determined by phosphorus- and elemental analysis.

**Table 2**
Preparation of PIC between PMMB and PMSB

| Abb. | PMSB concentration (wt%) | Final concentration |
|------|--------------------------|---------------------|
|      | Aqueous solution         |                     |
| PIC 1 | 0.1                      | 6.7 × 10⁻³           |
| PIC 2 | 0.5                      | 3.3 × 10⁻²           |
| PIC 3 | 1                        | 6.7 × 10⁻²           |
| PIC 4 | 10                       | 6.7 × 10⁻¹           |

PMMB was coated on the substrate and PMSB aqueous solutions were used to prepare the PIC at 25 °C.
The phosphorylcholine and sulfonate unit compositions on the film surface are summarized in Table 3. The PET membrane without the MPC copolymer coating was immersed in distilled water and stored at room temperature for 12 h to equilibrate the surface. The $\zeta$-potential of the membrane was measured at 10 mM NaCl phosphate buffered solution (PBS; pH 7.4) using a ELS 8000 (Otsuka Electron Co., Tokyo, Japan).

2.5. Lymphocyte adhesion test

Human lymphocytes were isolated from fresh whole blood anticoagulated with sodium citrate using a centrifugation technique with Ficoll–Paque (Pharmacia) as the isolation medium. To a test tube of whole blood an equal volume of Tris’s buffer solution (pH 7.6) was added. In a 15 ml test tube, 4 ml of the diluted blood was gently layered over 3 ml of Ficoll–Paque and centrifuged at 1500 rpm for 35 min at room temperature. The lymphocyte layer was transferred to another 15 ml test tube, washed with Tris’s buffer solution, and centrifuged at 800 rpm for 10 min at room temperature. The lymphocyte was resuspended in PBS. The concentration of the cells was determined using a hematocytometer (EKDS, Tokyo, Japan) and was found to be $4.0 \times 10^5$ cells/ml. PET membranes coated with the MPC copolymers in a disk shape (14 mm diameter) were placed in the 24-well cell culture plate and secured with a silicone rubber ring. PBS was allowed to stand in the wells for a day to equilibrate the surface. A lymphocyte suspension (1 ml) was poured into each well and incubated for 5 h at 37 °C. After the lymphocyte suspension was removed and rinsed 3 times with PBS, the density of the adherent lymphocytes was determined by the lactate dehydrogenase (LDH) method [17]. From the morphological evaluation of the adherent lymphocytes, the membranes were placed in a saline solution containing 2.5 vol% glutaraldehyde for 2 h at room temperature. The film was sufficiently rinsed with distilled water, dehydrated with alcohol, freeze-dried in vacuo, and sputtered with gold. The surface of the membrane was observed using a scanning electron microscope (SEM, JSM-5400, JEOL, Tokyo, Japan).

3. Results

The characterizations of MPC copolymers are summarized in Table 1. The composition of each monomer unit in the copolymer was good agreement with their fraction in the feed. The MPC copolymer used in this study was dissolved in EtOH, but the cationic PMMB and neutral PMB were not dissolved in water.

The formation of the PIC was followed by the adsorption behavior of PMSB at various concentrations (Table 2) on the QCM system with a PMMB coated quartz electrode. In this step, the QCM signals were saturated at 1.5 h regardless of the PMSB concentration in the feed (data not shown). Fig. 3 shows the relationship between the amount of PMSB adsorbed on the PMMB coated membrane and the $\zeta$-potential of the surface. The $\zeta$-potential of the PMMB surface was $+20$ mV and it decreased with an increase in the PMSB adsorption amount. The $\zeta$-potential of the copolymer-coated film depended on the composition of

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**Table 3**

| Abb. | S/C ($\times 10^{-2}$) | P/C ($\times 10^{-2}$) | $\zeta$-potential (mV) |
|------|------------------------|------------------------|-----------------------|
| PET  | --                     | --                     | $-49.8 \pm 12$        |
| PMMB | --                     | 3.6                    | $19.9 \pm 1.6$        |
| PIC 1| 1.4                    | 3.4                    | $15.8 \pm 5.0$        |
| PIC 2| 1.3                    | 3.2                    | $4.7 \pm 2.7$         |
| PIC 3| 1.8                    | 3.9                    | $-9.6 \pm 3.8$        |
| PIC 4| 2.3                    | 4.1                    | $-15.5 \pm 3.8$       |
| PMSB | --                     | 5.1                    | $34.5 \pm 4.7$        |
| PMB  | --                     | 3.0                    | $0.0 \pm 5.0$         |

* Take-off angle of photoelectron in XPS analysis.
each monomer unit in the copolymer. We could control the surface electrical charge between $\pm 20$ and $-16$ mV by changing the amount of PMSB adsorbed on the surface.

The surface compositions of polymers are summarized in Table 3. XPS was used to verify the presence of the phosphorylcholine and sulfonate moieties at the surface. On the PMMB surface, a signal attributed to atomic sulfur was not observed. Measuring at a $90^\circ$ takeoff angle on the PIC surfaces, the sulfur peak was not observed, but it was observed at a $15^\circ$ takeoff angle. The highest S/C atomic ratio was noticed for PIC 4, which was the highest concentration of PMSB solution applied to form the PIC. The results of the XPS analysis were in good agreement with that of the $\zeta$-potential measurement.

Fig. 4 shows the density of adherent lymphocytes on polymer surfaces. Many adherent lymphocytes on the PET and PMMB surfaces were observed. On the other hand, the lymphocyte adhesion was reduced on the PMSB surface. On the PIC 1 surface having a positive surface potential ($\zeta$-potential, $+19.9$), many adherent lymphocytes were observed. There is no significant difference in the number of adherent lymphocytes on PIC 2, PIC 3, and PIC 4. The PMB surface having a nonionic surface potential ($\zeta$-potential, 0) inhibited the adhesion of lymphocytes.

Fig. 5 shows SEM pictures of the polymer surfaces after contact with the lymphocyte suspension for 5 h. Many lymphocytes were adhered on the PET and PMMB surfaces. The adherent lymphocytes on the PET surface were strongly deformed, but less on the PMMB surface. On the PIC 1 surface, many adherent lymphocytes with a spherical shape were observed. The pseudopot formation of lymphocytes adhered on PIC 3 and PIC 4 was completely suppressed.
Based on the SEM observations, the number of adherent lymphocytes on PIC 4 was remarkably lower than that on the other PICs.

4. Discussion

As shown in Fig. 3, an increased amount of adsorbed PMSB induced a decrease in the \( \xi \)-potential toward a negative charge. Alternate layer-by-layer adsorption has been developed as a new technique for the preparation of ultrathin films from polyelectrolytes, which is based on charge neutralization and resaturation between polyanions and polycations \([10–14]\). Richert has observed a sequential charge neutralization and resaturation between polyanions ultrathin films from polyelectrolytes, which is based on been developed as a new technique for the preparation of PIC formation polymer surfaces with both phosphorylcholine and charged groups in the PIC played an important role in maintaining the shape of the adherent lymphocytes as spheroidal. Thus, the PIC composed of charged MPC copolymers provides an excellent surface for the adhesion of lymphocytes and will be used for new cell engineering devices.

5. Conclusion

The polymer surface with both phosphorylcholine and charged groups could be prepared by the formation of the PIC between the cationic and anionic MPC copolymers. The surface charge was varied to the control lymphocyte adhesion. The number of adherent lymphocytes decreased when the surface charge became negative. The phosphorylcholine groups in the PIC played an important role in maintaining the shape of the adherent lymphocytes as spheroidal. Thus, the PIC composed of charged MPC copolymers provides an excellent surface for the adhesion of lymphocytes and will be used for new cell engineering devices.

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