Change in cell adhesion property on cytocompatible interface using phospholipid polymer grafted with poly(D,L-lactic acid) segment for tissue engineering

Junji Watanabe, Kazuhiko Ishihara*

Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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Abstract

Tissue engineering is a multi-disciplinary science that utilizes basic principles from materials engineering and molecular biology to reconstruct tissues from polymer matrices and cellular components. Artificial skins were well known as one of the concrete examples. Technological innovation of the tissue engineering must be contributed to improve quality of life. From the viewpoint, design of cytocompatible materials for tissue engineering would be the most important candidate to reconstruct tissue. 2-Methacryloyloxyethyl phosphorylcholine (MPC), n-butyl methacrylate, and polylactic acid (PLA) macromonomer were polymerized for the preparation of cytocompatible interface. The polymer may involve following novel properties: (i) cytocompatibility by phospholipid groups, and (ii) enhancement of cell adhesion by PLA segment. The results of X-ray photoelectron spectroscopy showed the MPC unit and PLA segment on the membrane, which was prepared by dip coating. The surface mobility by contacting water was estimated with static contact angle measurement. The contact angle by water decreased after contact with water due to the chain rearrangement of hydrophilic MPC unit. Fibroblast cells adhesion and protein adsorption on the membranes were studied. The number of cell adhesion and cell proliferation on the membrane was well correlated with each other. Furthermore, the number of cell adhesion was proportional to the PLA macromonomer (MaPLA) composition. The adherent cell morphology showed round shape, because of the existence of MPC unit. However, the cell morphology would be spread after the cell proliferation. These findings suggest that the change in the polymer composition by combination of MPC and MaPLA could regulate the number of cell adhesion and the morphology.

1. Introduction

Tissue engineering consists of a multi-disciplinary science, including fundamental principles from materials engineering and molecular biology. Langer et al. reported a leading work on tissue engineering; cells, bioactive molecules, and polymer matrices are required to regenerate tissue [1]. Recent biological achievements regarding cell culture using bioactive molecules are promising techniques to regenerate tissue. Therefore, materials for tissue engineering play a great important role towards the development of biomaterials. As for the biomaterials on tissue engineering, biodegradable polymers have been used, for example, synthetic polymers such as poly(α-hydroxy acid) and also natural polymers such as hyaluronan and collagen. The synthetic and natural polymers were molding for the membrane, plate, sponge-form, and hydrogel. The conventionally using polymers involve excellent molding process, however, no guarantee regarding the cytocompatibility exists on the polymers because the conventional polymers are not properly designed for tissue engineering. From this, preparation of cytocompatible polymer material is quite important on further development for tissue engineering.

Cytocompatibility on the biomaterials was generally evaluated using lactate dehydrogenase (LDH) assay [2] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [3]. The LDH assay provides the information regarding cellular membrane damage by the release of the cytosolic enzyme, reflecting short incubation time with biomaterials. On the other hand, MTT assay shows detrimental intracellular effects on mitochondria and
metabolic activity. The obtained information was limited after suffering damage from the biomaterials. Anderson et al. systematically evaluated the cytokine production from macrophages using cytotoxic activity and enzyme-linked immunosorbent assay (ELISA) [4]. Though the ELISA technique was detectable, the information on contacting cell on the biomaterials, however, sensitivity was major and dominant problems due to the detection of quite low amount of secreted proteins. Kishida et al. proposed a novel methodology for the cytocompatibility, the evaluation of one of the well-known cytokines, interleukin-1β (IL-1β) mRNA expression from the cells contacting with the biomaterials [5]. The expression of IL-1β indicated inflammatory response in the cells.

Among the numerous kinds of polymer materials, a series of phospholipid polymer, poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)), would be good candidates for the cytocompatibility. Originally, the MPC was strongly inspired by cellular membrane structure, which was assembled phospholipid molecules [6], and then synthetic route was developed in high purity [7]. The most advantageous characteristics of the phospholipid polymer showed excellent blood and biocompatibility in contact with biological components in vivo and in vitro [8,9]. Furthermore, the phospholipid polymer prevented the cells on the biomaterials from inducing the inflammatory response, estimation by IL-1β mRNA expression [10,11]. Taking these characteristics into account, the phospholipid polymer are promising cytocompatible materials for tissue engineering. However, the number of cell adhesion via adsorbed protein layer was quite low, because the phospholipid polymer surface suppressed protein adsorption. Then, the following cell adhesion would not happen. Therefore, cytocompatible interface with cell adhesion property was designed; a novel macromonomer with poly(D,L-lactic acid) (PLA) short segment was used for the cytocompatible interface [12].

In this paper, the cytocompatible polymers were systematically prepared by changing monomer composition in feed, and the obtained polymers were coated on the cell culture substrates. The number of cell adhesion was discussed in relation to the surface properties.

2. Experimental

2.1. Materials

D,L-lactide was kindly supplied by Musashino Chemical Laboratory (Tokyo, Japan) and was recrystallized from ethyl acetate. n-Butyl methacrylate (BMA, Wako Pure Chemical Co., Ltd, Osaka, Japan) and 2-isocyanate ethyl methacrylate (IEMA, Showa Denko Co., Tokyo, Japan) were distilled at reduced pressure, and the following fractions were used (BMA: 50 °C/20 mm Hg, IEMA: 60 °C/2.5 mm Hg). n-Dodecanol, stannous octoate (Sn-(oct)₂), and dibutyltin dilaurate (DBTL) were purchased from Wako Pure Chemical Co., Ltd and used without further purification. MPC was synthesized and purified by a method previously reported [7]. Other reagents were commercially available and used without further purification.

2.2. Preparation of phospholipid polymer membranes

The preparation of phospholipid polymer was already reported in our previous paper [12], the synthetic outline was briefly described. PLA macromonomer (MaPLA, Fig. 1(a)) was synthesized, following two steps:
After treatment with 0.25% trypsin, the cell density was adjusted to 6 × 10^3 cells/ml and the cells were then seeded on the membranes. After 6 h, 1 day, 2 and 5 days, the number of adherent cells were treated with 0.1 wt% SDS solution, and then evaluated by LDH assay kit (Wako Pure Chemical Co., Ltd). The cell morphology was observed by phase contrast microscope (IX71, OLYMPUS, Tokyo, Japan).

2.3. Characterization of surface properties

The surface properties were characterized in terms of elemental analysis and molecular mobility. X-ray photoelectron spectroscopy (XPS, AXIS-HSi, Shimadzu/KRA-TOS, Kyoto, Japan) with MgKα was carried out. The analyzer was placed perpendicular to the surface of the membrane. Static contact angle by water was measured using an automatic contact angle meter apparatus (CA-W, Kyowa Interface Science Co., Ltd, Saitama, Japan) at 25 °C. The drop of pure water was introduced on the membrane using a micro syringe.

2.4. Protein adsorption and cell culture on the membranes

Protein adsorption was evaluated using bovine serum albumin (BSA, A-8022, SIGMA, MO, USA), bovine gamma globulin (ByG, G-5009, SIGMA), and bovine plasma fibrinogen (BPF, F-8630, SIGMA). The concentrations of the proteins were adjusted to 4.5, 1, and 0.3 mg/ml, respectively. The membranes were firstly equilibrated by phosphate buffer saline (PBS) for overnight, and were incubated in each protein solution (pH 7.4) for 3 h. After rinsing with PBS, the adsorbed proteins were removed by 1 wt% of n-sodium dodecyl sulfate (SDS). The recovered proteins from the membranes were evaluated using Micro BCA kit (Pierce, No. 23235, IL, USA).

Mouse fibroblast (L-929) cells were purchased from RIKEN cell bank (Saitama, Japan) and were routinely cultured in Eagle’s Minimum Essential Medium (E-MEM, Nissui, Tokyo, Japan), supplemented with 5% calf serum (CS, Gibco, NY, USA) at 37 °C in a 5% CO2 atmosphere. After treatment with 0.25% trypsin, the cell density was adjusted to 6 × 10^5 cells/ml and the cells were then seeded on the membranes. After 6 h, 1 day, 2 and 5 days, the number of adherent cells were treated with 0.1 wt% SDS solution, and then evaluated by LDH assay kit (Wako Pure Chemical Co., Ltd). The cell morphology was observed by phase contrast microscope (IX71, OLYMPUS, Tokyo, Japan).

3. Results and discussion

3.1. Surface properties on membranes

Repeating number of lactic acid unit in the PLA segment was determined ca. 40 using 1H NMR. The polymer composition is summarized in Table 1. The PLA unit was quantitatively incorporated into each phospholipid polymer. The compositions of the MaPLA unit in the polymers were 5, 10, 29, and 53 mol%. Each polymer was abbreviated as PMBLA5, PMBLA10, PMBLA30, and PMBLA50, respectively. On the other hand, MPC unit was incorporated in the range of 10–20 mol%. All of the polymers were dissolved in non-polar solvent such as chloroform.

The polymer membranes were prepared by dip coating onto the PET substrates. Interference fringe was observed on the substrates; therefore, nano-scale polymer coating layer (below 100 nm) was then prepared. The PMBLA involved hydrophilic monomer unit (MPC) and hydrophobic graft segment (PLA). Generally, block- and graft-type copolymer would spontaneously form domain structure. Thus, the possibility of the domain formation would be undeniable. On the XPS measurement, the releasing angle of the photoelectron for each atom was fixed 90°. The detecting depth was about 10 nm, which is thinner than the thickness of the polymer membrane by dip coating. Therefore, obtained results indicated surface properties regarding the coating polymer. Fig. 2 shows XPS results of the membranes after contact with water. In the case of N1s core level spectra of PMBLA5, nitrogen peaks of both urethane bonds and trimethyl ammonium groups were observed at 399 and 402 eV, respectively. Phosphorus peak corresponding to the phosphate ester in PMBLA5 was also found at 133 eV. However, the differences on peak intensity attributed to the MaPLA and MPC units were not clearly observed. In the case of PET substrate, no nitrogen and phosphorus peaks were found as expected. These results indicate that the phospholipid moiety and PLA segment were located onto the surface after contacting water.

Table 1

| Abbreviation | Monomer unit composition (mol%) | Yield (%) |
|--------------|-------------------------------|-----------|
| In feed      | In polymer                    |           |
|              | MPC   | BMA  | MaPLA | MPC   | BMA  | MaPLA |
| PMBLA5       | 5     | 90   | 5     | 10    | 85   | 5     | 40   |
| PMBLA10      | 5     | 85   | 10    | 13    | 77   | 10    | 46   |
| PMBLA30      | 5     | 75   | 20    | 20    | 51   | 29    | 63   |
| PMBLA50      | 5     | 65   | 30    | 16    | 31   | 53    | 53   |

*Preparative condition: [Monomer] = 0.5 mol/l, [AIBN] = 2.5 mmol/l, 60 °C, 24 h.
* Determined by 1H NMR.
measured as shown in Table 2. For example, 88.9° was found on the PMBLA10 before contacting water. After contacting water, the contact angle decreased, and the hysteresis was then ca. 17°. The other PMBLAs were also observed of the hysteresis, 7–10°. This result indicated that spontaneous rearrangement of the hydrophilic–hydrophobic segment on the membrane occurred to reduce surface free energy. It was suggested that MPC unit was located onto the membrane after contacting water. In the case of PLGA coating surface, the contact angle did not change, indicating relatively higher hydrophobic surface.

3.2. Protein adsorption on membranes

Protein adsorption was evaluated using BSA, BγG, and BPF. These proteins are very popular for evaluation of biocompatibility on the surface. The amount of protein adsorption is summarized in Fig. 3. In the case of PMBLA5, all kinds of protein adsorption were below 0.2 μg/cm², in particular, BPF adsorption was not detected (roughly zero). The amount of adsorbed proteins increased with composition of PLA unit in the polymer, indicating enhancement of affinity between proteins and polymer membranes. In PMBLA50 surface, the amount of adsorbed BSA, BγG, and BPF increased to be ca. 1.6, 1.0, and 1.3 μg/cm², respectively. Generally, the amount of maximum protein adsorption was calculated as mono-layered adsorption on the surface, and obtained as follows: BSA: 0.9 μg/cm², BγG: 1.8 μg/cm², and BPF: 1.7 μg/cm². From these calculated values, it is suggested that multi-layer BSA adsorption was located on the PMBLA50 and the other proteins (BγG and BPF) were adsorbed within mono-layer on the surface. On the other hand, 1–2.5 μg/cm² of protein adsorption was observed on PLGA membrane, indicating multi-layer protein adsorption. From this, it was considered that amount of protein adsorption on the PMBLA was relatively lower than that of PLGA surface, even if higher MaPLA content (PMBLA50), reflecting protein adsorption resistant MPC unit. The major factor on protein adsorption was to be PLA unit, which functional unit effectively increased protein adsorption. Cell adhesion would be favorable with increasing PLA composition in the polymer.

3.3. Cell adhesion on membranes

L-929 cell adhesion was evaluated as shown in Fig. 4. Primary seeding density was 6 × 10³ cells/cm² in each membrane. In the case of PMBLA5 membrane, the number of cell adhesion was determined to be below 500 cells/cm², then the data was shown using asterisk (*) in Fig. 4. PMBLA10 also showed low cell adhesion at 6 h. The PMBLA5 surface showed very low affinity to the cells. From the protein adsorption data, the PMBLA5 surface would be very inert to the BPF adsorption. The dominant factor regarding the cell adhesion was discussed as follows. Generally, cell adhesion on the membrane was induced via protein adsorption. The protein adsorption, particularly, fibrinogen and fibronectin such as cell adhesive proteins, were quite important, because receptors on the cell

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**Table 2**

| Abbreviation | Contact angle (°) | Before contacting water | After contacting water |
|--------------|------------------|-------------------------|------------------------|
| PMBLA5       | 85.4 ± 1.2       | 75.8 ± 2.3              |
| PMBLA10      | 88.9 ± 1.1       | 71.8 ± 1.1              |
| PMBLA30      | 82.5 ± 1.1       | 74.8 ± 1.8              |
| PMBLA50      | 81.3 ± 1.6       | 74.0 ± 0.9              |
| PLGA         | 97.8 ± 2.0       | 101.4 ± 2.2             |

*Mean values of the 10 measurements and standard deviations are indicated.
membrane recognized the adhesive proteins via RGDS (arginine–glycine–aspartic acid–serine) moiety, and the cell attached on the surface via protein adsorption layer. Therefore, the relationship between amount of protein adsorption and number of cell adhesion was observed with similar tendency. Taking these results into account, the number of cell adhesion was dependent on the amount of protein adsorption. It is considered that over 0.5 \( \mu \text{g/cm}^2 \) of protein adsorption was onset for cell adhesion at primary stage.

On the other hand, large number of primary cell adhesion was observed after 6 h; 1000 cells/cm\(^2\) of the fibroblast adhered on the PMBLA30 and PMBLA50, and PLGA showed good adherent property (3600 cells/cm\(^2\)). Among them, the difference on the primary adhesion was considered effect of phospholipid unit. However, cell proliferation after 5 days was observed except for PMBLA5. This result indicated that the phospholipid polymer surface with PLA segment showed biologically inert on primary stage. The adherent cells on the inert surface (PMBLA) would show nice cell proliferation as same as on PLGA. It is suggested that the protein adsorption from the serum in the medium, produced proteins from the adherent cells were very important for the cell growth. Furthermore, the cell proliferation was proportional to the PLA content in the polymer. The cell proliferation was also dependent on the amount of protein adsorption. These results suggest that the biologically inert surface on the phospholipid polymer is changeable by incorporation of MaPLA.

The cell morphology was observed with phase contrast microscopy as shown in Figs. 5 and 6. The cell morphology was clearly shown as a round shape on MPC incorporated membrane after 6 h. On the other hand, spread morphology has already observed on PLGA membrane after 6 h. The difference on the cell morphology was based on the difference of cell–material interaction via protein adsorption layer. It is considered that the adhesive properties between the membrane surface and the cells would be weaker due to the small amount of protein adsorption. After 5 days, spread morphology was observed on the membrane except for PMBLA5. Cell proliferation was also observed on the PMBLA10, PMBLA30, and PMBLA50, which surfaces showed the small amount of cell adhesion at 6 h. From these results, the phospholipid polymer with PLA...
segment showed changeable cell adhesion property by PLA composition, and the surface property on phospholipid polymer (PMBLA) would reach PLGA surface as well-known PLA polymer.

4. Conclusions

The novel phospholipid polymers with PLA segment were synthesized for the preparation of cytotocompatible interface. The polymer membrane surfaces involved the MPC unit and PLA segment, indicating the higher hysteresis on contact angle measurement and XPS analysis. The surface properties were spontaneously changed by chain rearrangement of MPC unit after contacting water. The number of cell adhesion increased with incorporation of PLA composition. And the membrane with higher PLA composition showed good cell proliferation, even when the small number of cell adhesion at primary stage. The phospholipid polymer membrane surface would be active with increasing MaPLA content for cell adhesion. These results are of great important for the design of cytocompatible interface.

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References

[1] R. Langer, J.P. Vacanti, Tissue engineering, Science 260 (1993) 920–926.
[2] S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, M. Hashida, In vitro cytotoxicity of macromolecules in different cell culture systems, J. Control. Rel. 34 (1995) 233–241.
[3] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Meth. 65 (1983) 55–63.
[4] T.L. Bonfield, J.M. Anderson, Functional versus quantitative comparison of IL-1β from monocytes/macrophages on biomedical polymers, J. Biomed. Mater. Res. 27 (1993) 1301–1305.
[5] A. Kishida, S. Kato, K. Ohmura, K. Sugimura, M. Akashi, Evaluation of biological response to polymeric biomaterials by RT-PCR analysis, Biomaterials 17 (1996) 1301–1305.
[6] Y. Kadoma, N. Nakabayashi, J. Masuhara, Synthesis and hemolysis test of the polymer containing phosphorylcholine groups, Kobunshi Ronbunshu 35 (1978) 423–427.
[7] K. Ishihara, T. Ueda, N. Nakabayashi, Preparation of phospholipid polymers and their properties as hydrogel membrane, Polym. J. 23 (1990) 355–360.
[8] K. Ishihara, Novel polymeric materials for obtaining blood-compatible surface, TRIP 5 (1997) 455–461.
[9] K. Ishihara, Bioinspired phospholipid polymer biomaterials for making high performance artificial organs, Sci. Technol. Adv. Mater. 1 (2000) 131–138.
[10] S. Sawada, Y. Shindo, S. Sakaki, A. Watanabe, Y. Iwasaki, S. Kato, M. Akashi, K. Ishihara, N. Nakabayashi, Perfect inhibition of inflammatory reaction on medical devices by coating with novel phospholipid polymer, Trans. Soc. Biomater. 25 (1999) 231.
[11] S. Sawada, S. Sakaki, Y. Iwasaki, N. Nakabayashi, K. Ishihara, Suppression of the inflammatory response from adherent cells on phospholipid polymers, J. Biomed. Mater. Res. 64A (2003) 411–416.
[12] J. Watanabe, K. Ishihara, Phosphorylcholine and poly(ε-caprolactone) containing copolymers as substrates for cell adhesion, Artif. Organs 27 (2003) 242–248.