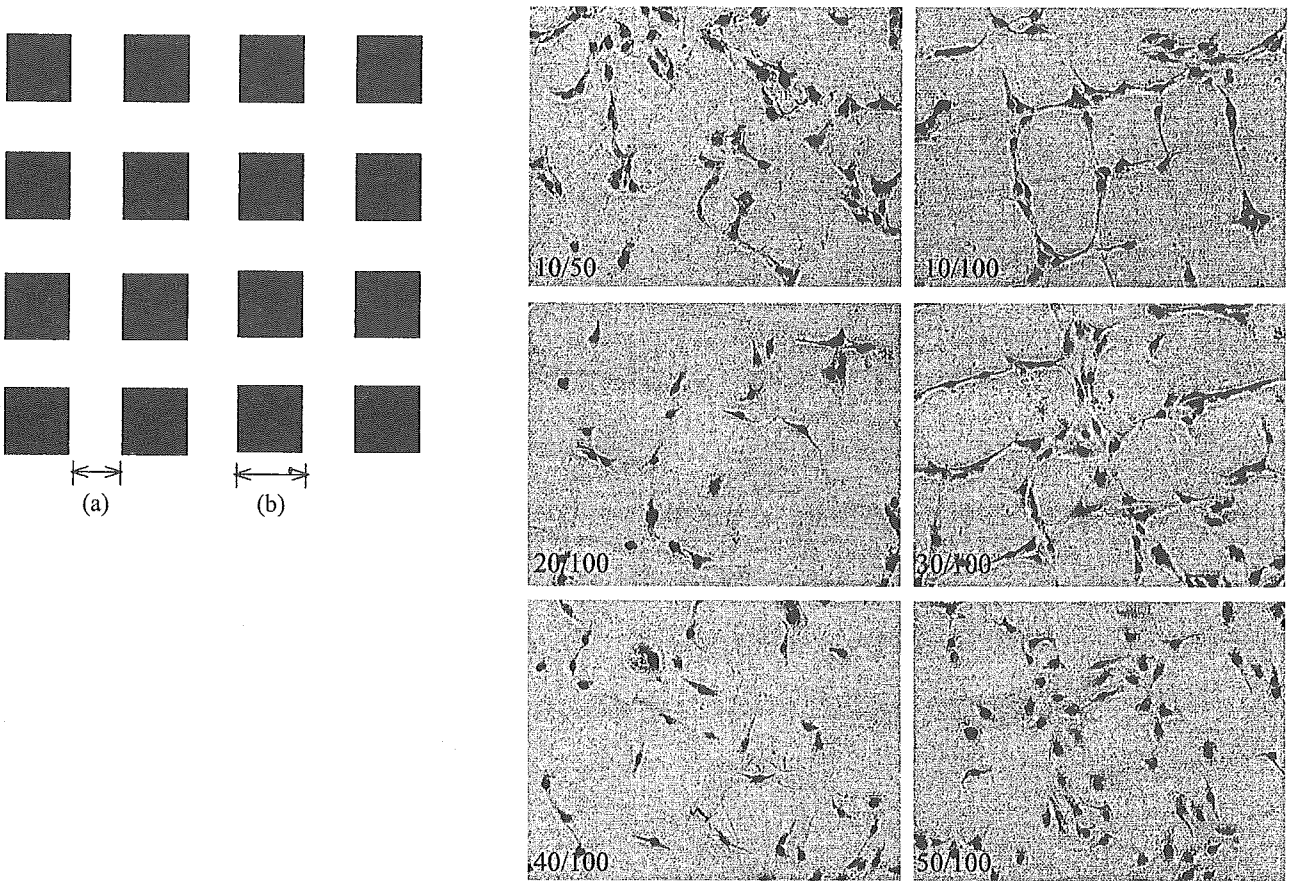

Figure 6. Photomasked and micropattern-cultured HUVECs on glass plates micropattern-immobilized with VEGF. The numbers in the photos indicate the width of immobilized regions (a) and the gap width of the lattice (b). The surface densities of gelatin and VEGF were both 21.2 ng/mm^2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

meant that 2% of surface immobilized with VEGF was covered with cells. Thus, the cells increased 50-fold over the number seeded. Such a dramatic increase has not been observed on other VEGF-immobilized surfaces.[13] In addition, approximately half of the cells on these surfaces survived for 7 to 10 days after reaching confluence. Thus, the immobilized VEGF appears to have maintained its activity for 14 to 17 days, including the time needed for cells to reach confluence.

When HUVECs were cultured in the presence of anti-VEGF antibody, the numbers of cells were significantly lesser than in the absence of antibody after 9 days (Fig. 5). Inhibition by the antibody was reduced with increased concentrations of immobilized VEGF. No such effect was observed on the gelatin-immobilized surface not treated with antibody. Thus, the immobilized VEGF specifically interacted with the HUVEC.

Figure 6 shows the micropatterning of HUVEC growth. Cells grew only on the VEGF-immobilized regions. When the lattice size was small, a clear form was not observed because of cell aggregation. However, when the lattice size was relatively large, networks of HUVECs formed on the surface.

VEGF is a key physiological regulator of angiogenesis during embryogenesis, skeletal growth, and reproductive functions.[23] Taguchi and colleagues[13] immobilized VEGF 165 on poly(acrylic acid)–grafted polyethylene film via a reaction between the amino group of VEGF and the carboxyl group, using water-soluble carbodiimide. They found that coimmobilization of fibronectin and VEGF enhanced the growth of HUVECs. Stone and colleagues[24] synthesized a VEGF–BSA covalent complex and this retained its chemotactic and proliferative properties. They suggested that bare prosthetic surfaces lined with VEGF might support endothelial cell proliferation and migration, and thereby offer new strategies to improve graft reconstruction and patency.

Here we showed that VEGF actively supported HUVEC growth in the immobilized state by specific interaction and by blocking with anti-VEGF antibody. As this photoimmobilization technique is applicable for any organic material, it will be possible to immobilize VEGF on various substrates. Immobilized VEGF significantly enhanced the surface coverage with endothelial cells. This enhancement is very important, considering the scarcity of endothelial progenitor cells

in the body. In addition, using this photoimmobilization technique it should be possible not only to promote endothelialization but also to regulate the formation of a network of vascular capillaries *in vitro*.

References

1. Koike N, Fukumura D, Gralla O, Au P, Schechner JS, Jain RK. Tissue engineering: creation of long-lasting blood vessels. Nature 2004;428:138–139.
2. Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci U S A 1989;86:7311–7315.
3. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989;161:851–858.
4. Ito Y, Liu SQ, Imanishi Y. Enhancement of cell growth on growth factor-immobilized polymer film. Biomaterials 1991;12: 449–453.
5. Ito Y, Uno T, Liu SQ, Imanishi Y. Cell growth on immobilized cell-growth-factor. 8. Protein-free cell culture on insulin-immobilized microcarrier. Biotechnol Bioeng 1992;40:1271–1276.
6. Horwitz JI, Toner M, Tomkins RG, Yarmush ML. Immobilized IL-2 preserves the viability of an IL-2 dependent cell line. Mol Immunol 1993;30:1041–1048.
7. Ito Y, Zheng J, Imanishi Y, Yonezawa K, Kasuga M. Protein-free cell culture on artificial substrata immobilized with insulin. Proc Natl Acad Sci U S A 1996; 93:3598–5601.
8. Kuhl PR, Grifith-Cima LG. Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. Nat Med 1996;2:1022–1027.
9. Ito Y, Chen G, Imanishi Y. Artificial juxtacrine stimulation for tissue engineering. J Biomat Sci Polym Ed 1998;9:879–890.
10. Kim EJ, Kang IK, Jang MK, Park YB. Preparation of insulin-immobilized polyurethanes and their interaction with human fibroblast. Biomaterials 1998;19:239–249.
11. Ito Y. Surface micropatterning to regulate cell functions. Biomaterials 1999;20:2333–2342.
12. Bromberg L, Salvati L. Bioactive surfaces via immobilization of self-assembling polymers onto hydrophobic materials. Bioconj Chem 1999;10:678–686.
13. Taguchi T, Kishida A, Akashi M, Maruyama I. Immobilization of human vascular endothelial growth factor (VEGF165) onto biomaterials: an evaluation of the biological activity of immobilized VEGF165. J Bioact Comp Polym 2000;15:309–320.
14. Kim YJ, Kang IK, Huh MW, Yoon SC. Surface characterization and in vitro blood compatibility of poly(ethylene terephthalate) immobilized with insulin and/or heparin using plasma glow discharge. Biomaterials 2000;21:121–130.
15. Kang IK, Choi SH, Shin DS, Yoon SC. Surface modification of polyhydroxyalkanoate films and their interaction with human fibroblasts. Int J Biol Macromol 2001;28:205–212.
16. Ito Y, Chen G, Imanishi Y, Morooka T, Nishida E, Okabayashi Y, Kasuga M. Differential control of cellular gene expression by diffusible and non-diffusible EGF. J Biochem 2001;129:733–737.
17. Gumusderelioglu M, Turkoglu H. Biomodification of non-woven polyester fabrics by insulin and RGD for use in serum-free cultivation of tissue cells. Biomaterials 2002;23:3927–3935.
18. Kapur TA, Shoichet MS. Immobilized concentration gradients of nerve growth factor guide neurite outgrowth. J Biomed Mater Res 2004;68A:235–243.
19. Ishikawa T, Terai H, Kitajima T. Production of a biologically active epidermal growth factor fusion protein with high collagen affinity. J Biochem 2001;129:627–633.
20. Hayashi M, Tomita M, Yoshizato K. Production of EGF-collagen chimeric protein which shows the mitogenic activity. Biochim Biophys Acta 2001;1528:187–195.
21. Zisch AH, Zeisberger SM, Ehrbar M, Djonov V, Weber CC, Ziemiecki A, Pasquale EB, Hubbell JA. Engineered fibrin matrices for functional display of cell membrane-bound growth factor-like activities: study of angiogenic signaling by ephrin-B2. Biomaterials 2004;25:3245–3257.
22. Ito Y, Hasuda H, Yamauchi T, Komatsu N, Ikebuchi K. Immobilization of erythropoietin to culture erythropoietin-dependent human leukemia cell line. Biomaterials 2004;25:2293–2298.
23. Ferrara N, Gerber H-P, LeCounter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–676.
24. Stone D, Phaneuf M, Sivamurthy N, LoGerfo FW, Quist WC. A biologically active VEGF construct in vitro: implications for bioengineering-improved prosthetic vascular grafts. J Biomed Mater Res 2002;59:160–165.

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Photo-reactive polyvinylalcohol for photo-immobilized microarray

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Abstract

A new photo-reactive polymer, polyvinylalcohol modified with phenylazido groups, was synthesized as a microarray matrix. The polymer is soluble in water and spin-coated onto glass plate. Aqueous solutions of proteins were micro-spotted onto the coated glass and were fixed by ultraviolet light irradiation. Subsequently, cell adhesion on the photo-immobilized protein microarray was investigated. Non-specific adhesion of cells onto non-protein-spotted regions was reduced in comparison with the previously prepared microarray chip (Biomaterials 24 (2003) 3021). The adhesion behavior of cells depended on the kind of immobilized proteins and the type of cells. The microarray will be useful for cell diagnosis and for the selection of biomaterials to regulate cell behavior.

Keywords: Biochip; Microarray; Photo-immobilization; Cell adhesion; Cell chip

1. Introduction

Microarray technology has become a crucial tool for large-scale and high-throughput biological science and technology. It allows fast, easy and parallel detection of thousands of addressable elements in a single experiment under the same conditions [1,2]. DNA microarray approaches have demonstrated a rapid and economic way to interpret gene function [3,4]. In recent years, there have been considerable advancements in the preparation of small-molecule arrays [5–7], peptide arrays [8–11], protein arrays [12,13], polysaccharide arrays [14–16], antigen arrays [17–19], antibody arrays [20–22], and tissue arrays [23,24]. The microarray is important not only for genomics or proteomics but also for cellomics, where very few studies have been carried out. Recently, Ziauddin and Sabatini [25] prepared transfected cell microarrays from cDNA microarrays after addition of a lipid transfection reagent and adherent mammalian cells. In addition, microarrays of different kinds of antibody and proteins have been used for the assay of lymphocytes and cells, respectively [26,27]. Such microarrays of proteins are useful for cell surface profiling. By using the chip it is possible to observe cell behavior on a single surface under the same condition.

Although microarraying proteins is important, no universal immobilization method for the preparation of arrays has been developed. Non-covalent immobilization (physical adsorption) or chemical coupling reactions (using amino or carboxyl groups in the materials) were usually used. However, the former is not suitable for stable immobilization and the latter is limited by the structure of the immobilized materials. Therefore, a photo-immobilization method was devised for the preparation of microarray chips for immunological [28] and cell adhesion assays [27]. Although the immobilization method does not regulate the molecular orientation of the immobilized molecules, any materials can be immobilized on a substrate because of the radical reactions. Because all molecules are randomly immobilized, the activity was considered to be averaged on each immobilized molecule.

Previously, we have microarrayed proteins using photo-reactive poly(acrylic acid) as a matrix on a polystyrene tissue culture plate [27]. However, since cells significantly adhered to areas without immobilized proteins (glow-discharged polystyrene surface), it was

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difficult to differentiate between immobilized and non-immobilized areas. In other words, the S/N ratio was low. Therefore, in the present study, a new photo-reactive polymer inhibiting non-specific cell adhesion was developed.

2. Materials and methods

2.1. Preparation of photo-reactive polyvinylalcohol

The synthetic scheme of photo-active polyvinylalcohol is shown in Fig. 1. First 2-(2-(4-azidophenyl)vinyl)-4-(3-pyridylmethylene)-1,3-oxazolin-5-on (Az-8) was synthesized as follows. 2-(3-(4-Azidophenyl) prop-2-enoylamino)acetic acid (9 g), 3-pyridylaldehyde (4 g), acetic anhydride (12 g), sodium acetate (0.6 g), and cyclohexane (35 g) were mixed and allowed to stand for 6 h at 70°C. After heating, the mixture was cooled and isopropyl alcohol (30 g) was added to the mixture, which was then again allowed to stand for 15 h at room temperature. The resulting precipitate was recovered by filtration at room temperature and the solid material washed with cooled methanol (30 g). Finally, the product was dried under vacuum. Approximately 7 g of Az-8 was obtained. The product had an absorbance at 390 nm.

Subsequently, Az-8 (7 g) was dissolved in tetrahydrofuran (70 ml) and 3 g of aminobutylaldehyde dimethylacetal was added dropwise to the solution at 5–10°C. After 2 h, the absorbance at 390 nm in the reactant suspension disappeared and a new absorbance at 316 nm appeared. Water (200 ml) and aqueous ammonium solution (2 ml) were added to the solution and the mixture was stirred for 2 h. The resulting precipitate was filtered from the solution. Approximately 3.5 g of 2-(3-(4-azidophenyl)prop-2-enoylamino)-*N*-(4,4-dimethoxybutyl)-3-(3-pyridyl)prop-2-enamide (Az-8-γAB) was obtained. ^{1}HNMR, 8.97 (s, 1H), 8.55 (s, 1H), 8.42 (d, 1H, *J=4*), 7.73 (d, 1H, *J=8*), 7.51 (d, 1H, *J=15.6*), 7.39 (d, 2H, *J=8.8*), 7.24 (dd, 1H, *J=4,8*, 8.0), 7.08 (t, 1H, *J=5.2*), 6.96 (d, 2H, *J=8.4*). 6.60 (s, 1H), 6.51 (d, 1H, *J=15.6*), 4.36 (t, 1H, *J=4.8*), 3.31 (s, 6H), 3.31–3.28 (m, 2H), 1.66–1.59 (m, 4H). Elemental analysis showed; C, 61.05; H, 5.78; N, 18.56, Calculated; C, 61.32; H, 5.82; N, 18.66.

Finally, the photo-reactive polyvinylalcohol (Azido-unit pendant Water-soluble Photopolymer, AWP) was synthesized as follows. Fifty grams of polyvinylalcohol (degree of polymerization was 1700 and 88% of hydroxyl groups were converted to acetal, EG-30, Nippon Gosei Co., Ltd) was dissolved in 300 ml of water at 80°C for 1 h and Az-8-γAB (3.2 g) was added to the solution with 1.5 g of phosphate acid and 100 ml of water at 60°C. The mixture was allowed to stand for 24 h at 60°C. After the reaction, 30 g of ion-exchange resin (WA-20, Mitsubishi Chem. Co., Ltd.) was added to the solution and allowed to stand for 3 h to remove the phosphoric acid until the pH of the solution reached 6.5. The resin was removed by polyester filter (400 mesh). The solution contained 10% (w/v) AWP and had a viscosity of 2825 cP/25°C.

GPC measurement was performed using GS 320HQ (Shodex Asahipak; column size, 7.6 × 500 mm^2; eluent, mixture of 1.428% NaCl water/acetonitrile (7/3); flow rate, 0.5 ml/min; temperature, 50°C; detection absorbance, 290 nm).

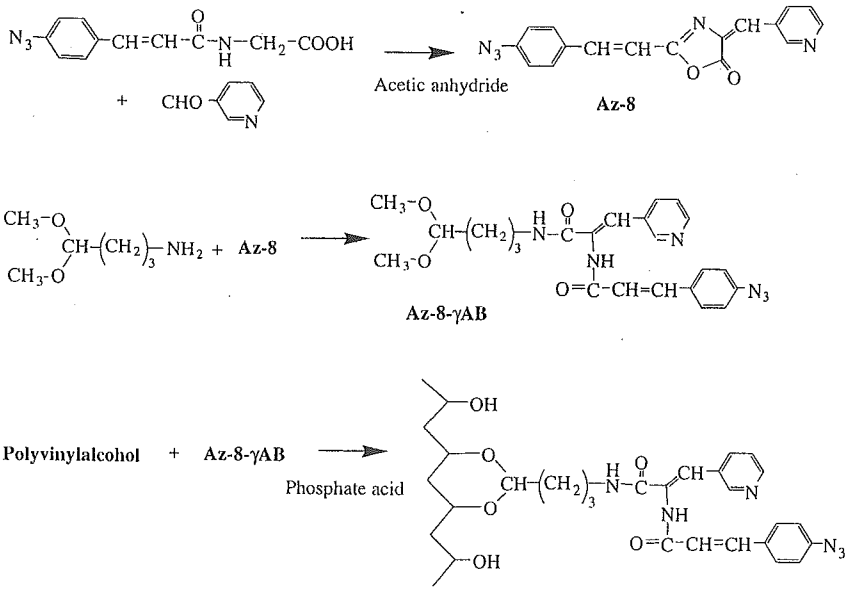

Fig. 1. Synthetic scheme of photo-reactive polyvinylalcohol.

2.2. AWP coating and characterization

An aqueous solution of AWP (1 wt%) was spin-coated onto a glass plate at 350 rpm for 2 s, followed by 1400 rpm for 30 s. The coated plate was air-dried at 40°C for 5 min. The thickness of coated AWP was measured by surface roughness measurement (Toyo Seimitsu Co., Ltd., Surfcom920B) after scratching the surface to a 0.1 μm width. Percentage of the remaining resin layer was determined by the thickness before and after UV-irradiation.

2.3. Protein microarray

A micro-spotter (Stampman®) produced for DNA microarraying by the Nippon Laser Electric Co. (Nagoya, Japan) was used for the microarray experiment as previously reported [27]. The arrayer dip pen was incubated in an aqueous solution located in one of the wells of the plate, lifted from the well, and placed on a cell culture dish for a prescribed time. Subsequently, the pen was moved to a washing dish and washed as described below. The dip pen was incubated in a 0.1% sodium dodecylsulfate solution for 5 s, water for 5 s, and ethanol for 5 s. Finally, it was dried in vacuo for 14 s. The dried pen was then incubated in the next aqueous solution. These processes were controlled by personal computer and were repeated for the complete microarray construction.

A protein microarraying was carried out as shown in Fig. 2. First, the glass plate was spin coated with the prescribed concentrations of AWP. After AWP coating, an aqueous solution of protein at various concentrations was cast onto the coated plate. Subsequently, the plate was irradiated with ultraviolet light (153 mW/cm^2) using an UV Spot Light Source L5662 (Hamamatsu Photonics Co., Hamamatsu, Japan). The plate was then washed five times with water. Except for the light-irradiation, all processes were carried out at 4°C.

2.4. Cell adhesion assay

COS-7 cells were cultured in Dulbecco modified Eagle's medium (DMEM, Sigma) with 10% fetal bovine serum (FBS) and RAW264, HepG2 and STO cells were cultured in a minimum essential medium (MEM, Sigma) with 10% FBS and 1% non-essential amino acids (Invitrogen Life Technologies). The cells were harvested with a 0.25% trypsin solution containing 0.5 mM EDTA. The recovered cells were washed with culture medium and suspended in each medium (4.0×10^4 cells per 12 well culture plate, Iwaki). The cell suspension was added to the protein microarrayed dishes, and allowed to stand for 2 h or 3 d at 37°C. After incubation, the dishes were washed three times with phosphate-buffered saline, and cells were directly observed by phase-contrast microscopy (× 40, Olympus).

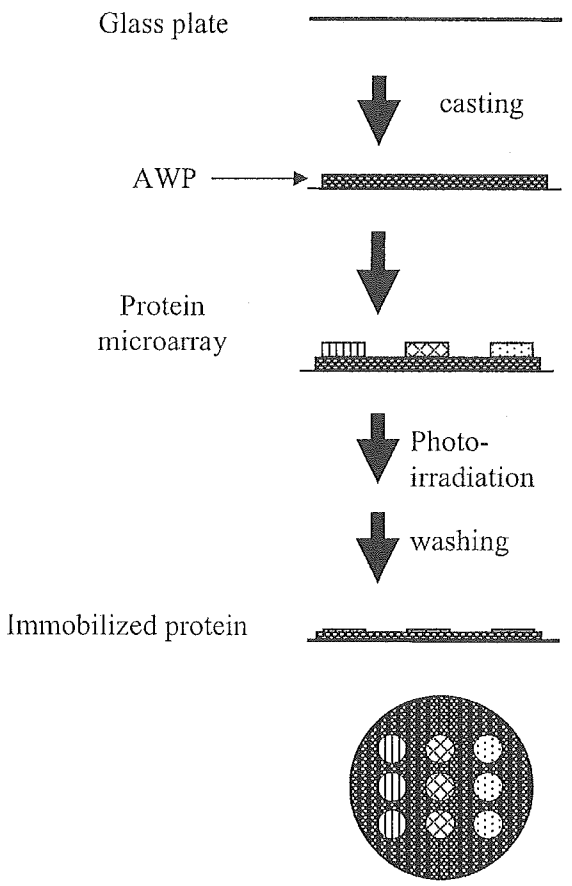

Fig. 2. Preparative scheme of protein microarray.

3. Results and discussion

3.1. Preparation of AWP

GPC charts of Az-8-γAB and AWP are shown in Fig. 3. The retention time of Az-8-γAB was 38.4 min. On the other hand, the retention time of AWP was 11.2 min. Aldehyde of Az-8-γAB, which was produced at acidic conditions, was observed at the retention time of 44.2 min. The GPC chart indicated that almost 100% of the Az-8-γAB reacted with the polyvinylalcohol.

Fig. 4 shows the ultraviolet spectrum of Az-8-γAB and AWP. The absorbance peak slightly shifted. However, there is no liquid dissolving both compounds, therefore a precise comparison based on the chemical structure is difficult. The content of incorporated azidophenyl groups in the polymer was the same as the feed content 0.7 mol%. The value was the same as that calculated from the calibration curve using Az-8-γAB.

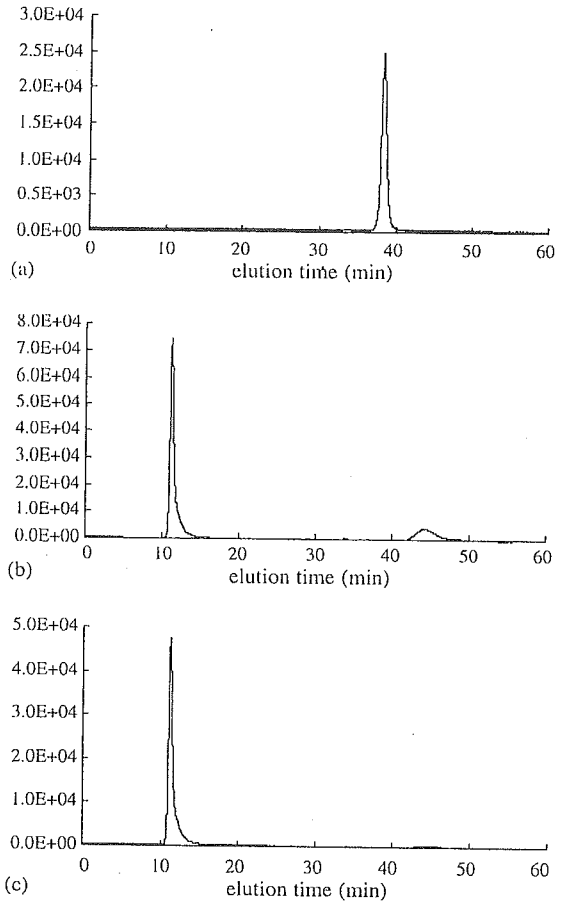

Fig. 3. GPC charts of a starting chemical, Az-8-γAB (a), AWP product after 3-h reaction (b), and after 24-h reaction (c).

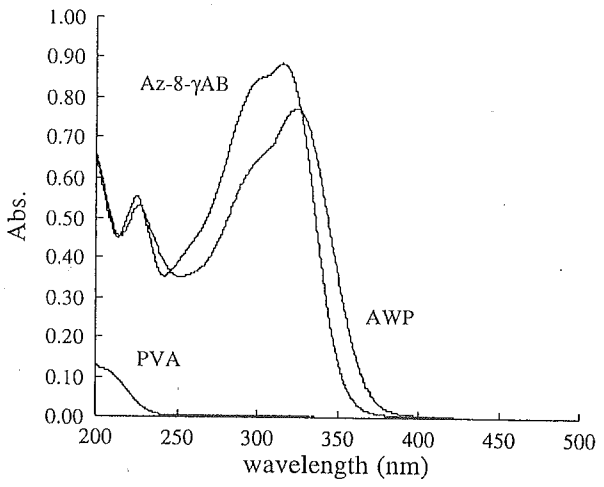

Fig. 4. UV spectra of Az-8-γAB, polyvinylalcohol, and AWP.

3.2. Polymer coating

AWP was spin-coated onto the glass plate. Fig. 5 shows the relationship between spinning velocity and the thickness of coated layer. The thickness decreased with an increase in the rotational velocity. In addition, the thickness depended on the concentration of AWP.

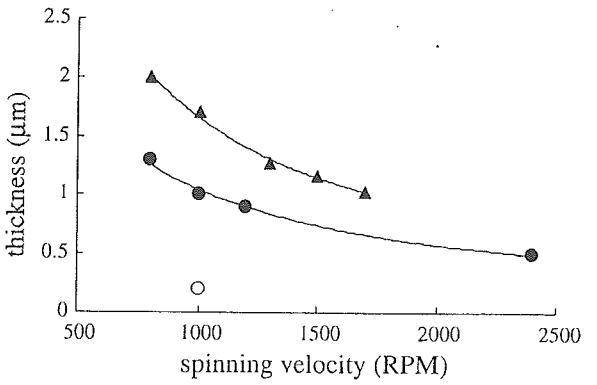

Fig. 5. Relationship between spinning velocity and thickness of formed layer. AWP concentration was 6.5 wt% (▲), 5.5 wt% (●), and 3.0 wt% (○).

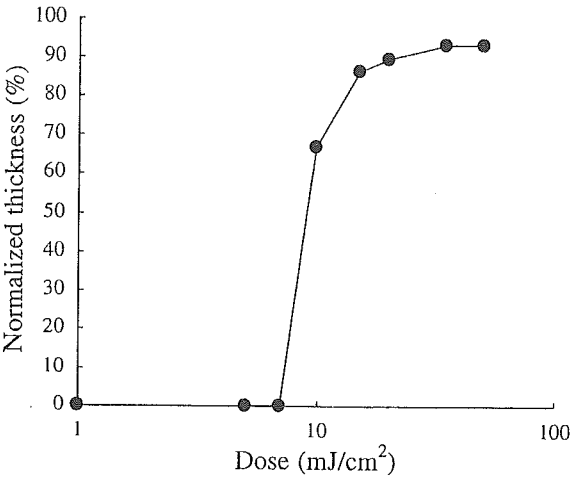

Fig. 6. Relationship between irradiation dose and amount of formed layer. The thickness was 1 μm.

Fig. 6 shows the relationship between the irradiation power and the thickness of the formed layer. The result demonstrated that AWP could be stably immobilized by the photo-irradiation.

3.3. Cell adhesion on the microarrayed surface

Fig. 7 shows the adhesion of HepG2 cells onto some protein-immobilized areas. On the glass surface, the cells adhered independently of immobilized or non-immobilized regions. However, on the AWP-coated glass surface cell adherence was dependent on the microarrayed proteins and the surface concentration. The background surface, which corresponded to the non-immobilized regions, did not allow adhesion of the HepG2 cell. Even after 3 d of culture, there was very little non-specific cellular adhesion. The difference between background and immobilized regions was enhanced with increased incubation time. The microarray on the AWP-coated glass plate showed that HepG2 cells did not adhere to the BSA-spotted surface,

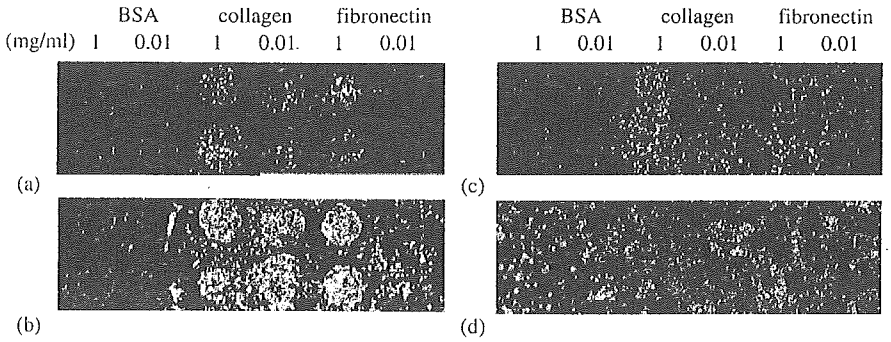

Fig. 7. Micrograph of HepG2 on bovine serum albumin (BSA), collagen, and fibronectin-spotted AWP-coated glass plate (a and b) or glass plate (c and d). The cells were incubated for 2 h (a and c) or 3 d (b and d) before washing.

Table 1
Cell adhesion on surfaces

	BSA	Collagen	Fibronectin
HepG2	—	+	+
COS-7	—	+	+ +
STO	—	+	+ +
Raw264	+	—	+

Dot array results are expressed as level of cell binding to each protein dot: (−) low, (+) moderate, and (+ +) high.

regardless of protein concentration. However, the HepG2 cells did adhere to the collagen-spotted surface, and the adherence was independent of the collagen surface concentration. HepG2 cells also adhered to the fibronectin-spotted surface, in a surface concentration dependent manner.

Table 1 shows the adhesion of COS-7, STO, and Raw264 cells on the microarrayed surface. COS-7 and STO cells adhered to the collagen- or fibronectin-immobilized regions in an immobilized amount dependent manner, but not to albumin-immobilized regions. On the other hand, Raw264 cells adhered to the albumin-immobilized regions more than the other cells and to the fibronectin-immobilized regions less than the other cells. Previously, we reported that Raw264 cells tended to adhere to various surfaces independent of the immobilized proteins because of their macrophage-like properties. Raw264 cells adhered to both albumin- and fibronectin-immobilized surfaces [27]. However, the present study demonstrated that the cells did not adhere to the collagen-immobilized surface. Protein dependence was also observed in the cells by increasing the number of arrayed proteins.

The present study demonstrated that a microarray can be conveniently and stably made by using a photo-reactive polymer and that cellular adhesion can be assayed using different concentrations of the micro-arrayed proteins. The non-adhesive polymer enhanced the difference between the background and protein-immobilized regions. Considering the potential of photo-immobilization, the photo-reactive polymer matrix will provide a universal method to prepare micro-arrays of various substances. In addition, the new non-cell adhesive non-ionic polymer, AWP, will be useful not only for microarray chips, but also in procedures requiring reduced interaction with biocomponents.

4. Conclusions

In the present study a new photo-reactive polymer, polyvinylalcohol modified with phenylazido groups, was synthesized and used as a microarray matrix. The polymer was spin-coated onto glass plates and aqueous solutions of proteins were micro-spotted and fixed by photo-irradiation. Non-specific adhesion of cells onto non-protein-spotted regions was reduced by using this polymer as a coating material, and the adhesion dependence of cells on the type of immobilized protein was clearly characterized. This type of microarray will be useful for cell-based diagnostics and for the selection of biomaterials that can regulate cell behavior.

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References

[1] Mitchell P. A perspective on protein microarray. Nat Biotechnol 2002;20:225–9.
[2] Zhu H, Snyder M. Protein chip technology. Curr Opin Chem Biol 2003;7:55–63.
[3] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270:467–70.

[4] DeRisi JL, Lyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 1997;278:680–6.
[5] MacBeath G, Koehler AN, Schreiber SL. Printing small molecules as microarrays and detecting protein–ligand interactions en masse. J Am Chem Soc 1999;121:7967–8.
[6] Falsey J, Renil JM, Park S, Li S, Lam KS. Peptide and small molecule microarray for high throughput cell adhesion and functional assay. Bioconj Chem 2001;12:346–53.
[7] Kuruville FG, Shamji A, Sternson SM, Hergenrother PJ, Schreiber SL. Dissecting glucose signaling with diversity-oriented synthesis and small-molecule microarrays. Nature 2002;416: 653–7.
[8] Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D. Light-directed, spatially addressable parallel chemical synthesis. Science 1991;251:767–73.
[9] Emili AQ, Cagoney G. Large-scale functional analysis using peptide or protein arrays. Nat Biotechnol 2000;18:393–7.
[10] Houseman BJ, Huh JH, Kron SJ, Mrksich M. Peptide chips for the quantitative evaluation of protein kinase activity. Nat Biotechnol 2002;20:270–4.
[11] Pellois JP, Zhou X, Srivannavit O, Zhou T, Gulari E, Gao X. Individually addressable parallel peptide synthesis on microchips. Nat Biotechnol 2002;20:922–6.
[12] MacBeath G, Schreiber SL. Printing proteins as microarrays for high through-put function determination. Science 2000;289: 1760–3.
[13] Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidingmaier S, Houfek T, Mitchell T, Miller P, Dean RA, Gerstain M, Snyder M. Global analysis of protein activities using proteome chips. Science 2001;293:101–2105.
[14] Wang D, Liu S, Trummer BJ, Deng C, Wang A. Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. Nat Biotechnol 2002;20:275–81.
[15] Houseman BT, Mrksich M. Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. Chem Biol 2002;9:443–54.
[16] Fukui T, Feizi T, Galustian C, Lawson AM, Chai W. Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Nat Biotechnol 2002;20:1011–7.
[17] Robinson WH, Digennaro C, Heber W, Haab BB, kamachi M, Dean EJ, Fournel S, Fong D, Genovese MC, Neuman de Vegvar HE, Skriner K, Hirschberg DL, Morris RL, Muller S, Pruijn G, van Venrooij WJ, Smolen JS, Brown PO, Steinman L, Utz PJ. Autoantigen microarrays for multiple characterization of autoantibody responses. Nat Med 2002;8:295–301.
[18] Messasoma L, Bacarese-Hamilton T, di Christina M, Rossi R, Bistoni E, Crisanti A. Antigen microarrays for serodiagnosis of infectious diseases. Clin Chem 2002;48:121–30.
[19] Fall BI, Eberlein-Konig B, Behrendt H, Niessner R, Ring J, Weller MG. Microarrays for the screening of allergen-specific IgE in human serum. Anal Chem 2003;75:336–562.
[20] Haab BB, Dunham MJ, Brown PO. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol 2001;2:R4.
[21] Huang RP. Detection of multiple proteins in an antibody-based protein microarray system. J Immunol Methods 2001;255:1–13.
[22] Lal SP, Christopherson RI, dos Remedios CG. Antibody arrays: an embryonic but rapidly growing technology. DDT 2002;7: S143–9.
[23] Kononen J, Bubendorf L, Kallioniemi A, Brlund M, Schraml P, Leighton S, Torhorst J, Mihaysch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.
[24] Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarray. Nat Med 2002;8:1323–7.
[25] Ziauddin J, Sabatini DM. Microarrays of cells expressing defined cDNAs. Nature 2002;411:107–10.
[26] Belov L, de la Vega O, dos Remedios CG, Mulligan SP, Christopherson RI. Immunophenotyping of leukemia using a cluster of differentiation antibody microarray. Cancer Res 2001;61:4483–9.
[27] Ito Y, Nogawa M. Preparation of a protein microarray using a photo-reactive polymer for a cell-adhesion assay. Biomaterials 2003;24:3021–6.
[28] Caelen I, Gao H, Sigrist H. Protein density gradients on surfaces. Langmuir 2002;18:2463–7.

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Photo-immobilization of a phospholipid polymer for surface modification

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Abstract

A photo-reactive polymer having a phospholipid polar group was prepared, and the polymer was photo-immobilized on polymeric surfaces, where its interactions with biocomponents were investigated. By using a photo-immobilization method, the polymer was used for surface modification of polyethylene and polypropylene, polymers whose surfaces were not treated in our previous development of the phosphorylcholine-derived polymer. The photo-reactive polymer was synthesized by a coupling reaction involving copolymer consisting of 2-methacryloyloxyethyl phosphorylcholine and methacrylic acid with 4-azidoaniline. When the polymer was unpattern immobilized on the surface, X-ray photo-electron spectroscopic analysis and static contact angle measurements were performed. It was shown that the surface was covered with phospholipid polar groups. Micropattern immobilization was carried out using a micropatterned photo-mask. Measurements using atomic force microscopy showed that the swelled micropatterned polymer was five times as thick as the dried one. Protein adsorption and platelet adhesion were reduced on the polymer-immobilized regions. Mammalian cells did not adhere, and formed aggregates on the immobilized regions. In conclusion, the photo-reactive phospholipid polymer was covalently immobilized on the conventional polymer surfaces and it tended to reduce interactions with proteins and cells.

Keywords: Photo-immobilization; Phospholipid polymer; Surface modification; Protein adsorption; Cell adhesion

1. Introduction

Lipid membranes are used as biomimetic systems, and are expected to become a key component of novel biomolecular materials [1–14]. In particular, lipid bilayer membranes on solid supports have been the subject of numerous publications [1–11]. The membranes prepared on various solids by optimized variations of the available deposition chemistries have been shown to accommodate a variety of proteins and enzymes in controlled orientations and in active conformations. The supported lipid bilayer is considered to mimic the native environment of membrane-associated biomolecules. These membranes are also promising surfaces for developing new biosensors and coating materials that resist non-specific interactions with proteins and cells.

The lipid head group of phosphorylcholine, a zwitterion, is a common group in the lipid molecules that form biological membranes, and is considered to play an important role as a surface material for biomedical devices by reducing interaction with proteins and cells. As a biomimetic polymer, 2-methacryloyloxyethyl phosphorylcholine (MPC)-containing polymer was synthesized by the group of Ishihara and Nakabayashi [15,16]. The polymer shows non-thrombogenicity, that is, suppression of non-specific protein adsorption, platelet adhesion, activation, and aggregation when the polymer contacted whole blood, even in the absence of anticoagulants.

Recently, some types of MPC-containing copolymers have been synthesized for coating [17] and covalent immobilization was achieved [18–20]. However, the

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covalent immobilization was limited to specialized surfaces. Therefore, in the present investigation, photo-immobilization was employed for covalent immobilization of the polymer on a broader variety of surfaces.

In addition, although many synthetic polymers have been devised to improve surface reducing interaction with proteins or cells, it is difficult to directly compare the surface properties of different polymers under precisely the same conditions. Therefore, a micropatterning method has been devised for lipids and polymers [21–23], and the method has proved useful for comparing the interaction of polymers with proteins and cells [24–26]. We modified the method of Matsuda and Sugawara [27], and have applied this method to some growth factors [28–30], sulfated hyaluronic acid [31], heparin [32], and thermo-responsive polymer [33]. In the present study, micropatterning of MPC polymer was performed, and the interactions with proteins and cells were investigated.

2. Materials and methods

2.1. Synthesis of photo-reactive MPC polymer

The polymer synthesis is illustrated in Fig. 1. MPC copolymer consisting of MPC (90 mol%) and methacrylic acid (10 mol%) was obtained from NOF Co. Ltd. (Tokyo, Japan), and is referred to as PMAc. The molecular weight of PMAc, as measured by gel permeation chromatography, was 2.2×10^5. Modification of PMAc was performed as follows: 4-azidoaniline (12.44 mg) and water-soluble carbodiimide (17.47 mg) were dissolved in 2 ml of PMAc solution (5 wt%) and 98 ml of water was added to the solution. The solution was left to stand for 24 h. After the reaction, the product was dialyzed with dialysis cassette (PIERCE, Rockford, IL) until no further release of azidoaniline through the cassette was confirmed by ultraviolet (UV) absorption. The resultant solution was freeze-dried. The azidophenyl-derivatized PMAc is referred to as Az-PMAc. Elemental analysis indicated that the amount of azidophenyl group in Az-PMAc was 6%.

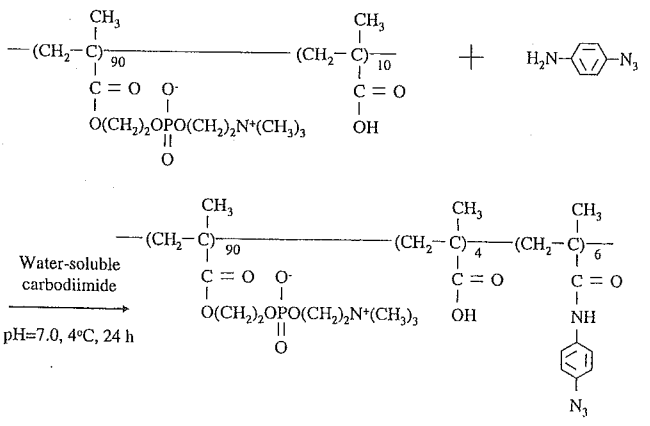

Fig. 1. Synthetic scheme for photo-reactive phosphorylcholine-containing polymer.

2.2. Micropatterning

The micropatterning method is illustrated in Fig. 2. An aqueous solution of Az-PMAc (1 wt%) was cast on polyethylene and polypropylene plates (diameter 22 mm), which were purchased from Sarstedt (Newton, NC) and from Nikkyo Technos Co. Ltd. (Tokyo, Japan), respectively, and air-dried at room temperature. Subsequently, the plate was covered with a photo-mask, which was manufactured by Toppan Printing Co. Ltd. (Tokyo, Japan) and was UV-irradiated with a UV lamp (UV Spot Light Source L5662, Hamamatsu Photonics, Hamamatsu, Japan) from a distance of 5 cm for 10 s ($16\,mW/cm^2$). When an unpatterned surface was prepared, the photo-mask was not employed. The plate was then repeatedly washed with distilled water.

2.3. Measurement of contact angle

The unpatterned sample was placed on the holder of a CA-W Automatic Contact Angle Meter (Kyowa Interface Science Co. Ltd., Saitama, Japan) and a drop of water (0.4 μl) was put on the sample surface. The contact angle of the drop on the surface was measured at room temperature. At least 10 contact angles on different areas were measured and averaged.

2.4. Measurement by X-ray photo-electron spectroscopy (XPS)

The unpatterned sample was inserted in the holder of an XPS, AXIS-HSi (Shimadzu/Kratos, Kyoto, Japan). After evacuation, the measurement was carried out under 3×10^{-9} Torr. The X-ray source was CuK_α, the applied voltage was 12 kV, and the electric current was

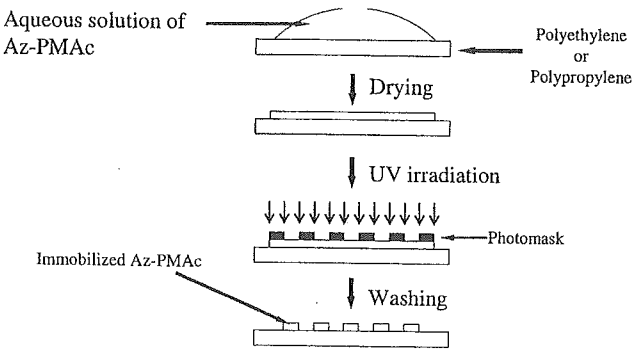

Fig. 2. Schematic illustration of micropatterning procedure.

10 mA. The take-off angle of the photo-electrons was 90°.

2.5. Measurement by atomic force microscopy (AFM)

The measurement was performed using an SPI-3800 (Seiko Instruments Inc., Chiba, Japan). The micropatterned sample was dried in vacuo for 1 day at room temperature and was set in a cell holder into which water could be injected. After observation of the dry sample, distilled water was injected into the sample cell and the same position was observed. The measurement was performed using the tapping mode with a nominal force constant of 0.09 N/m.

2.6. Interaction with proteins

Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin and FITC-labeled immunoglobulin were purchased from Sigma (St. Louis, MO). FITC-labeled fibrinogen was prepared as follows: a phosphate-buffered solution (PBS; 25 ml) containing human fibrinogen (500 μg) was added dropwise to a PBS (25 ml, pH 8.0) containing FITC (12.5 μg) and the pH of the mixture was adjusted to 9.0. The mixture was stirred at room temperature for 2 h. The resulting solution was dialyzed against double-distilled water using a Millipore dialysis tube (cut-off less than 10 000) at 4°C until the release of FITC became undetectable by fluorescence spectroscopy. Finally, the purified protein was lyophilized. All procedures were carried out in darkness.

The protein adsorption experiment was performed as follows: the sample plates were incubated in PBS containing the FITC-labeled albumin (10 mg/ml), FITC-labeled immunoglobulin (2 mg/ml), or the FITC-labeled fibrinogen (10 mg/ml) at 37°C for 10 min. After being washed with PBS, the sample was observed by fluorescence microscopy.

2.7. Interaction with platelets

Human whole blood was collected from healthy volunteers in a disposable syringe containing 3 ml of aqueous solution of 3.8 wt% sodium citrate. The citrated whole blood was immediately centrifuged for 15 min at 1200 rpm to obtain citrated platelet-rich plasma (PRP). The micropatterned sample plates were placed in contact with PRP and left for 60 min at 37°C. The PRP was removed with an aspirator, and the membrane was rinsed three times with PBS. Subsequently, 2.5 vol% glutaraldehyde in PBS was poured into each well containing the sample plates, and the samples were stored at room temperature for 2 h in order to fix the blood components on the sample plate. After it had been rinsed sufficiently with distilled water, the samples were freeze-dried. The surface of the sample plate was observed with a scanning electron microscope (SEM) after gold-sputtering treatment.

2.8. Cell culture

RAW264 (originating from leukemic mouse monocytes) cells were purchased from Riken Cell Bank (Tsukuba, Japan) and were cultured in minimum essential medium (Sigma, St. Louis, MO) with 10% fetal bovine serum and 1% non-essential amino acids (Invitrogen Life Technologies, Carlsbad, CA). The recovered cells were washed with the culture medium and suspended in each medium containing no serum (3×10^5 cells per 60 mm-diameter culture dish). The cell suspension was added to the sample plate, which was sterilized with 70% ethanol. The cells were incubated at 37°C under 5% v/v of CO_2 and were observed by a phase-contrast microscope equipped with a video camera.

3. Results and discussion

3.1. Synthesis of photo-reactive MPC polymer

The UV and fluorescence spectra of Az-PMAc are shown in Fig. 3. In the UV spectrum of the photo-reactive polymer, an absorption at 269 nm, which is assignable to the azidophenyl group, was observed.

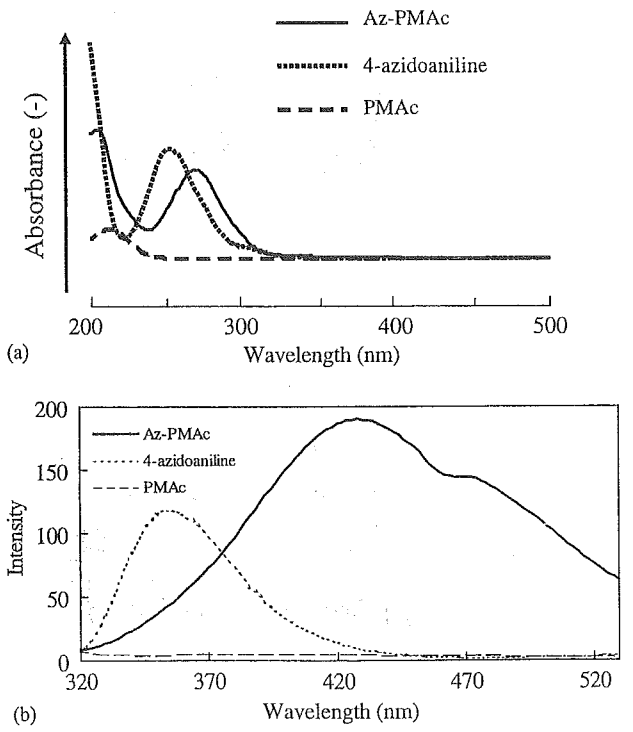

Fig. 3. UV (a) and fluorescence (b) spectra of azidoaniline, PMAc, and Az-PMAc.

Fig. 3a indicates that the absorption was slightly red shifted from the corresponding absorption of 4-azidoaniline, and Fig. 3b shows that the fluorescence was red shifted. These shifts may be due to electron delocalization of the azidophenyl group caused by amide bond formation. In previous studies, the peaks of photo-reactive hyaluronic acid and photo-reactive heparin were also red shifted from 4-azidoaniline [35,37].

3.2. Photo-immobilization

The Az-PMAc was coated on the plates and the coated surface was UV-irradiated with a photo-mask (Fig. 4). The surface pattern was the same as that of the photo-mask. The micropatterned surface was observed by phase-contrast microscopy (Fig. 4b) and by fluorescence microscopy (Fig. 4c). It is known that azido groups are decomposed by UV irradiation, and nitrene groups, which are highly reactive radical groups, are produced. The cast Az-PMAc formed molecular networks as a result of the produced radical groups. In addition, a micropatterned surface was formed both on polyethylene (Fig. 4c) and polypropylene (Fig. 4d) plates. The present result demonstrates that photo-immobilization is useful for covalent immobilization of MPC on various materials.

Previously, Prucker et al. [34] reported photo-chemical attachment of polymer films to solid surfaces via benzophenone derivatives. In their case, the amount of immobilized polymer on the surface reached saturation after about 10–20 min, when the light intensity was 100 mW/cm^2. In the present study, 10 s were enough for preparation of micropatterned immobilization, although the intensity was 16 mW/cm^2. Although the strength of binding of immobilized polymer to the surface has not been investigated, it was demonstrated that 10 s was enough for washing out of non-bound polymers.

The unpatterned PMAc surface on the polyethylene plate was made by UV-irradiation without a photo-mask. XPS measurement of the unpatterned surface demonstrated that the surface was covered with phospholipid polar groups (Fig. 5). In addition to the XPS spectrum of the previously reported MPC polymer coating surface [35,36], a new peak that was ascribed to the amide bond formed by reaction between PMAc and azidoaniline was found at 398 eV.

The water contact angle was measured on the unpatterned surface (Fig. 6). The contact angle on the

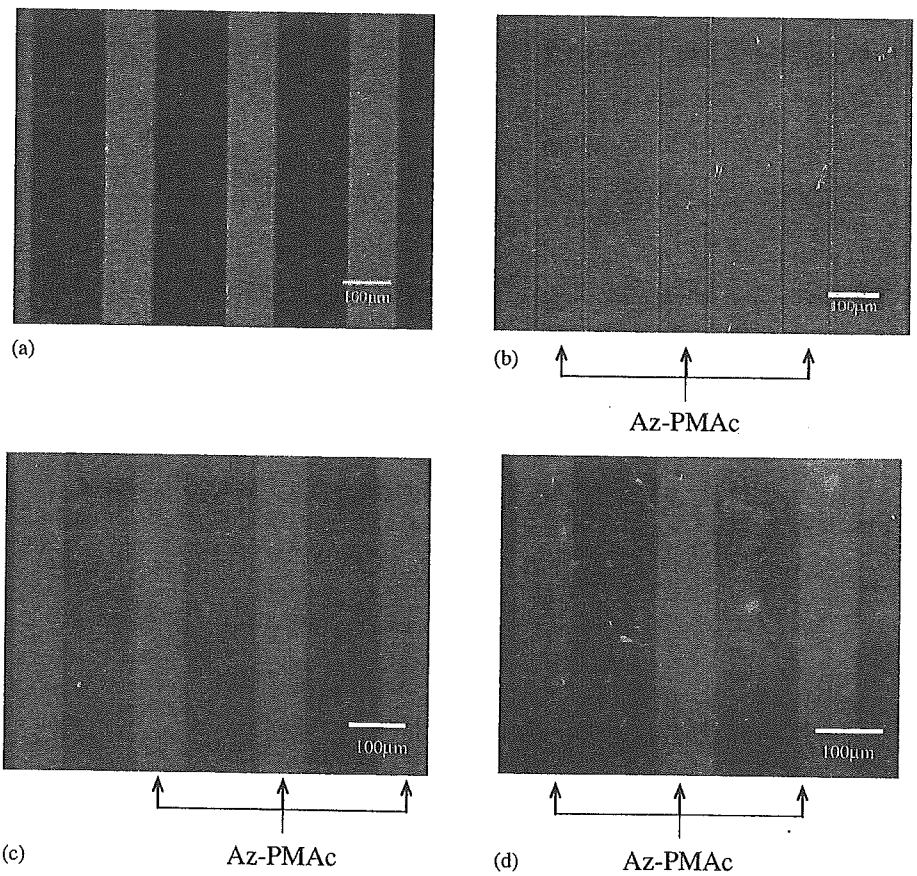

Fig. 4. Phase-contrast micrographs of photo-mask (a); and micropatterned surface of the polyethylene plate (b); fluorescence micrographs of micropatterned surface of the polyethylene plate (c); and of the polypropylene plate (d).

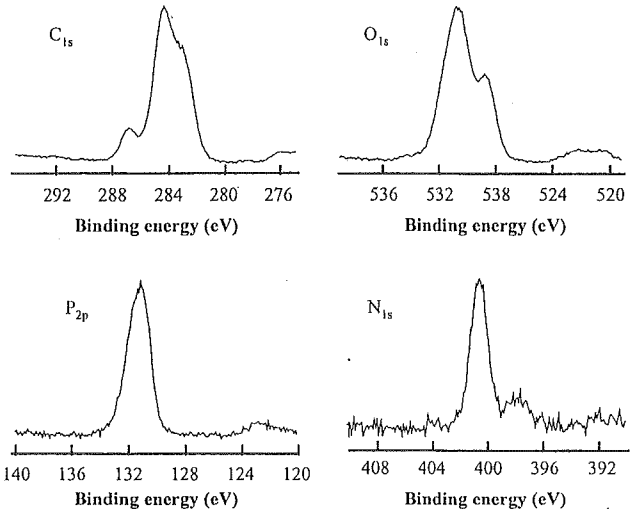

Fig. 5. XPS spectra of the Az-PMAc immobilized polyethylene surface.

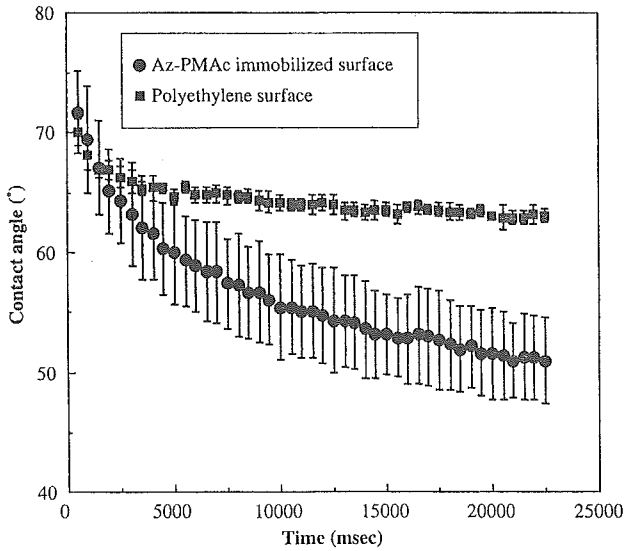

Fig. 6. Time course of static contact angle of water on the Az-PMAc-immobilized and non-immobilized polyethylene surface.

PMAc-immobilized surface rapidly decreased with time, although that on the polyethylene surface did not. It was demonstrated that a hydrophilic surface was formed by immobilization of the PMAc.

The surface was observed by AFM, as shown in Fig. 7. In the dried state, the thickness was about 800 nm. However, the PMAc layer rapidly swelled in water to a thickness of about 4000 nm. The hydrogel state of PMAc was formed after soaking for 10 min in water. Fig. 8 shows the force curves of the micropatterned surface. On the surface of bare polyethylene (2, 4, 6, 8, 10), the force abruptly increased with decreasing distance between the cantilever and the surface. On the other hand, on the Az-PMAc-immobilized region (1, 3,

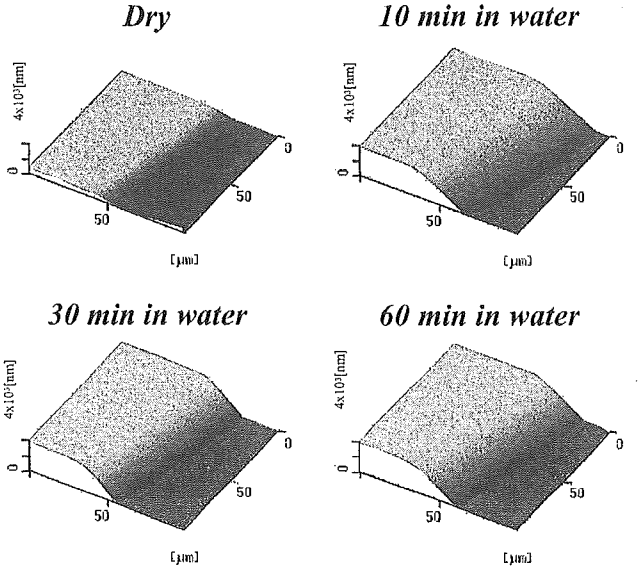

Fig. 7. AFM images of the Az-PMAc-micropatterned surface. The dried sample was measured and then incubated in water for different periods.

5, 7, 9) the force did not increase so abruptly with the decrease of distance. These results demonstrated that the Az-PMAc surface was so soft that force was not significantly produced on the surface.

3.3. Interaction with biological components

The sample plate was immersed in the protein solutions, and the protein-adsorbed sample was observed by fluorescence microscopy (Fig. 9). Albumin, immunoglobulin, and fibrinogen predominantly adsorbed onto the non-immobilized surface. The fluorescence intensity of adsorbed proteins is significantly higher than that of Az-PMAc alone. Previously, we reported that an MPC-adsorbed surface inhibited adsorption of proteins [37,38]. The present study confirmed the previous reports.

Human blood platelet adhesion onto the micropatterned surface was observed by SEM. The number of platelets on the PMAc-immobilized regions $(0.34 \pm 0.02) \times 10^3$ cell/μm^2 was significantly less than that on the non-immobilized regions $(1.13 \pm 0.12) \times 10^3$ cell/μm^2. The non-adhesiveness of MPC polymer has been reported previously [38]. The present study critically demonstrated this property.

The time course of behavior of RAW264 on the micropatterned surface is shown in Fig. 10. When the cells were added to the surface, they randomly distributed independent of the immobilized material. However, after 5 min, they began to aggregate on the Az-PMAc-immobilized surface; the cellular aggregates increased in size with time and eventually floated. On the

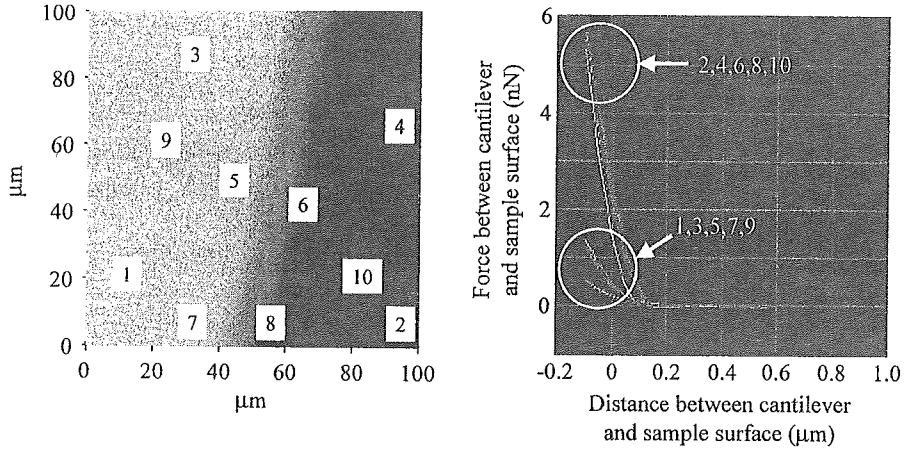

Fig. 8. Contact points of the cantilever with surfaces and the force curve between the cantilever and surfaces. The numbers represent the contact points of the cantilever. Points 1, 3, 5, 7, and 9 were on the Az-PMAc-immobilized surface and points 2, 4, 6, 8, and 10 on the bare polyethylene surface.

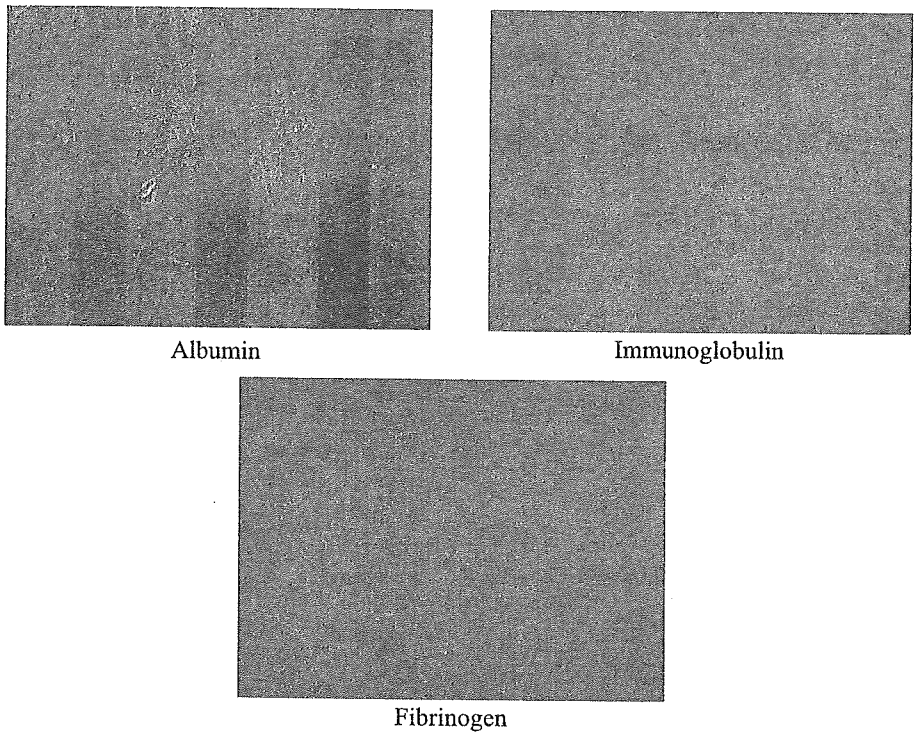

Fig. 9. Fluorescence micrographs of proteins (albumin, immunoglobulin, and fibrinogen) adsorbed onto the Az-PMAc-micropatterned polyethylene surface. The wavelengths of excitation and emission were 470 ± 20 and 525 ± 25 nm, respectively.

non-immobilized region the cells adhered and spread on the surface. The floated aggregates on the Az-PMAc-immobilized region were completely removed by mild shaking. It is known that RAW264 shows macrophage-like properties and tends to adhere to various materials [39]. It was demonstrated that PMAc inhibited the adhesion of even very adhesive cells.

The present study demonstrated photo-immobilization of a phospholipid polymer and visualized the interactions with biocomponents such as proteins, platelets, and cells. The photo-immobilization technique is useful for surface modification and the phospholipid polymer significantly reduced the interactions with proteins and cells.

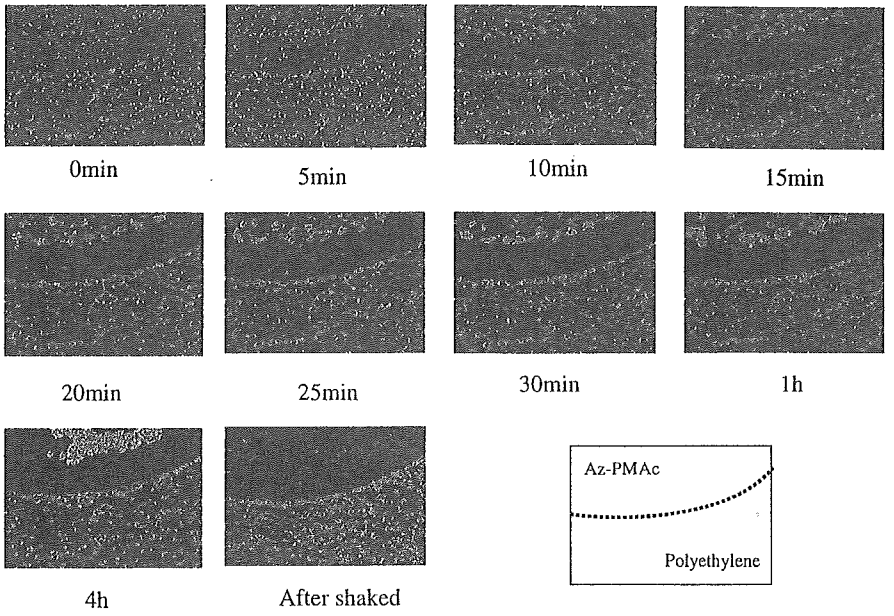

Fig. 10. Time course of behavior of RAW264 cells on Az-PMAc-immobilized and non-immobilized region (polyethylene surface), as observed by phase-contrast microscopy with a video camera.

Acknowledgements

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References

[1] Shen WW, Boxer SG, Knoll W, Frank C. Polymer-supported lipid bilayers on benzophenone-modified substrates. Biomacromolecules 2001;2:70–9.
[2] Naumann CA, Prucker O, Lehmann T, Rühe J, Knoll W, Frank CW. The polymer-supported phospholipid bilayer: tethering as a new approach to substrate-membrane stabilization. Biomacromolecules 2002;3:27–35.
[3] Mennicke U, Salditt T. Preparation of solid-supported lipid bilayers by spin-coating. Langmuir 2002;18:8172–7.
[4] Devadoss A, Burgess JD. Detection of cholesterol through electron transfer to cholesterol oxidase in electrode-supported lipid bilayer membranes. Langmuir 2002;18:9617–21.
[5] Andersson AS, Glasmästar K, Sutherland D, Lidberg U, Kasemo B. Cell adhesion on supported lipid bilayers. J Biomed Mater Res 2003;64A:622–9.
[6] Richter RP, Brisson A. Characterization of lipid bilayers and protein assemblies supported on rough surfaces by atomic force microscopy. Langmuir 2003;19:1632–40.
[7] Baumgart T, Offenhäuser A. Polysaccharide-supported planar bilayer lipid model membranes. Langmuir 2003;19:1730–7.
[8] Ross EE, Rozanski LJ, Spratt T, Liu S, O'Brien DF, Saavedra SS. Planar supported lipid bilayer polymers formed by vesicle fusion. 1. Influence of diene monomer structure and polymerization method on film properties. Langmuir 2003;19:1752–65.
[9] Sengupta K, Schilling J, Marx S, Fischer M, Bacher A, Sackmann E. Mimicking tissue surfaces by supported membrane coupled ultrathin layer of hyaluronic acid. Langmuir 2003;19:1775–81.
[10] Zhao J, Tamm LK. FTIR and fluorescence studies of interactions of synaptic fusion proteins in polymer-supported bilayers. Langmuir 2003;19:1838–46.
[11] Schneider J, Barger W, Lee GU. Nanometer scale surface properties of supported lipid bilayers measured with hydrophobic and hydrophilic atomic force microscope probes. Langmuir 2003;19:1899–907.
[12] Sun XL, Liu H, Orban JM, Sun L, Chaikof EL. Synthesis and terminal functionalization of a polymerizable phosphatidylethanolamine. Bioconjugate Chem 2001;12:673–7.
[13] Schuster B, Weighert S, Pum D, Sára M, Sleytr UB. New method for generating tetraether lipid membranes on porous supports. Langmuir 2003;19:2392–7.
[14] Elliot JT, Burden DL, Woodward JT, Sehgal A, Douglas JF. Phospholipid monolayers supported on spun cast polystyrene films. Langmuir 2003;19:2275–83.
[15] Ishihara K, Ueda T, Nakabayashi N. Preparation of phospholipids polymers and their properties as polymer hydrogel membrane. Polym J 1990;22:355–60.
[16] Ishihara K, Iwasaki Y. Reduced protein adsorption on novel phospholipids polymers. J Biomater Appl 1998;13:111–27.
[17] Lewis AL, Hughes PD, Kirkwood LC, Leppard SW, Redman RP, Tolhurs LA, Stratford PW. Synthesis and characterization of phosphorylcholine-based polymers useful for coating blood filtration devices. Biomaterials 2000;21:1847–59.
[18] Lewis AL, Cumming ZL, Goreish HH, Kirkwood LC, Tolhurs LA, Stratford PW. Crosslinkable coatings from phosphorylcholine-based polymers. Biomaterials 2001;22:99–111.
[19] Court JL, Redman RP, Wang JH, Leppard SW, O'Byrne VJ, Small SA, Lewis AL, Jones SA, Stratford PW. A novel phosphorylcholine-coated contact lens for extended wear use. Biomaterials 2001;22:3261–72.
[20] Lu JR, Murphy EF, Su TJ, Lewis AL, Stratford PW, Satija SK. Reduced protein adsorption on the surface of a chemically grafted phospholipid monolayer. Langmuir 2001;17:3382–9.

[21] Yoshina-Ishii C, Boxer SG. Arrays of mobile tethered vesicles on supported lipid bilayers. J Am Chem Soc 2003;125:3696–7.
[22] Kam L, Boxer SG. Spatially selective manipulation of supported lipid bilayers by laminar flow: steps toward biomembrane microfluidics. Langmuir 2003;19:1624–31.
[23] Carlson JW, Bayburt T, Sligar SG. Nanopatterning phospholipid bilayers. Langmuir 2000;16:3927–31.
[24] Grooves JT, Mahal LK, Bertozzi CR. Control of cell adhesion and growth with micropatterned supported lipid membranes. Langmuir 2001;17:5129–33.
[25] Orth RN, Wu M, Holowka DA, Craighead HG, Baird BA. Mast cell activation on patterned lipid bilayers of subcellular dimensions. Langmuir 2003;19:1599–605.
[26] Sapuri AR, Baksh MM, Grooves JT. Electrostatically targeted intermembrane lipid exchange with micropatterned supported membranes. Langmuir 2003;19:1606–10.
[27] Matsuda T, Sugawara T. Photochemical protein fixation on polymer surfaces via derivatized phenyl azido group. Langmuir 1995;11:2272–6.
[28] Ito Y. Surface micropatterning to regulate cell functions. Biomaterials 1999;20:2333–42.
[29] Chen G, Ito Y. Gradient micropattern immobilization of EGF to investigate the effect of artificial juxtacrine stimulation. Biomaterials 2001;22:2453–7.
[30] Ito Y, Chen G, Imanishi Y, Morooka T, Nishida E, Okabayashi Y, Kasuga M. Differential control of cellular expression by diffusible and non-diffusible EGF. J Biochem 2001;129:733–7.
[31] Chen G, Ito Y, Imanishi Y, Magnani A, Lamponi S, Barbucci R. Photoimmobilization of sulphated hyaluronic acid for antithrombogenicity. Bioconjugate Chem 1997;8:730–4.
[32] Ito Y, Hayashi M, Imanishi Y. Gradient micropattern immobilization of heparin and its interaction with cells. J Biomater Sci Polym Ed 2001;12:367–78.
[33] Liu H, Ito Y. Gradient-micropattern-immobilization of a thermoresponsive polymer to investigate its effect on cell behaviors. J Biomed Mater Res 2003;67A:1424–9.
[34] Prucker O, Naumann CA, Rühe J, Knoll W, Frank CW. Photochemical attachment of polymer films to solid surfaces via monolayers of benzophenone derivatives. J Am Chem Soc 1999;121:8766–70.
[35] Ishihara K, Tanaka S, Furukawa N, Kurita K, Nakabayashi N. Improved blood compatibility of segmented polyurethanes by polymeric additives having phospholipid polar groups. I. Molecular design of polymeric additives and their functions. J Biomed Mater Res 1996;32:391–9.
[36] Ishihara K, Ishikawa E, Iwasaki Y, Nakabayashi N. Inhibition of fibroblast cell adhesion on substrate by coating with 2-methacryloyloxyethyl phosphorylcholine polymers. J Biomater Sci Polym Ed 1999;10:1047–61.
[37] Iwasaki Y, Fujiike A, Kurita K, Ishihara K, Nakabayashi N. Effect of reduced protein adsorption on platelet adhesion at the phospholipid polymer surfaces. J Biomater Sci Polym Ed 1996;8:151–63.
[38] Iwasaki Y, Ishihara K, Nakabayashi N, Khang G, Jeon JH, Lee JW, Lee HB. Platelet adhesion on the gradient surfaces grafted with phospholipid polymer. J Biomater Sci Polym Ed 1998;9:801–16.
[39] Ito Y, Nogawa M. Preparation of a protein-array using a photoreactive polymer for a cell adhesion assay. Biomaterials 2003;24:3021–6.

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The tissue response to an alkylene bis(dilactoyl)-methacrylate bone adhesive

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Abstract

Gluing is an attractive technique to fix small bone fragments. However, to date no bone adhesive could be established successfully for all day clinical use.

The purpose of this experimental study was to investigate the biocompatibility of a new bone glue based on alkylene bis(dilactoyl)-methacrylate in 36 rabbits. Monocondylar osteotomy of the distal femur was performed and bone glue was applied into the osteotomy gap in 24 rabbits. The remaining 12 animals served as controls. In all rabbits the osteotomy was subsequently stabilized by K-wire osteosynthesis. Six animals of the glue group and 3 controls were euthanized after 7, 21, 42, and 84 days, respectively. Fracture healing and degradation pattern of the glue was studied using histological, histomorphometrical, scanning electron microscopical, and radiological methods.

Good resorption of the glue by mononuclear and multinucleated giant cells without prolonged inflammatory processes was observed in the glue group. Histomorphometrical analysis did not reveal any significant differences in fracture healing between the glue and control group at any time. Complete remodelling of the former osteotomy gap was found in all rabbits after 84 days.

This bioresorbable bone adhesive exhibited good biocompatibility and its degradation did not interfere with physiological fracture healing.

Keywords: Bone adhesive; Polymethylmethacrylate; Biocompatibility; Degradation; Bone remodelling; Histomorphometry

1. Introduction

Gluing is an attractive alternative technique to fix bone fragments in orthopaedic and trauma surgery. There are several advantages of this technique compared to nailing or plating.

Firstly, gluing enables good fixation of small fragments, e.g. in comminuted fracture sites. Secondly, fixation of fragments by gluing is leading to more homogenous weight bearing distribution within the fracture site compared to pinning where load is mainly punctually transferred on the pins [1–3]. In consequence, the risk of implant failure by punctual stress-overload, like often seen in plating, due to high rigidity and stiffness of metallic implants can be reduced by gluing [4,5]. Moreover, in articular fractures glue seam can act as "subchondral spacer" to compensate joint surface displacement.

Several preconditions have to be met by a bone adhesive for its all day clinical use. It must have sufficient adhesive properties, good short- and long-term biocompatibility without interference with physiological fracture healing processes including fast biodegradability [3,6]. Furthermore, the criteria of a strong and flexible bond, adequate time of action, and possibility of sterilization should be fulfilled [7,8].

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なんでも固定化バイオチップ
—これまでのプロオテオーム研究は本当だったの?!—

タンパク質をはじめとする生体高分子をランダムな配向で
固定化できる光架橋技術。これにより，さまざまな
生体高分子の高感度な相互作用解析が可能になった。
世界初の臨床診断マイクロチップ・システムとして期待される。

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マイクロアレイ技術とは?

最近のライフサイエンスの研究には，材料の微細加工技術やナノテクノロジーが応用されつつあり，それによって新しい展開もみせはじめている。とくにDNAマイクロアレイ（DNAチップ）の技術は，バイオテクノロジーと微細加工技術が融合することで大きく進展した分野である。図1のように，その集積度は急速に増加し技術的な進展をみせる一方，さまざまな疾病の診断など臨床分野での利用が本格的に検討されはじめている。

遺伝子解析などのゲノミクスに用いられるDNAマイクロアレイ以外にも，プロテオミクス，グライコミクス，セロミクス，メタボロミクスと，バイオチップは解析の対象範囲を広げつつあり，マイクロアレイによって得られた情報を処理するバイオインフォマティクスの発展とともに，その重要度を増してきている。

DNAマイクロアレイ以外ですでに市販・利用されているバイオチップとしては，研究支援用の抗体マイクロアレイやタンパク質マイクロアレイなどがあるが，多様なタンパク質，糖，糖タンパク質，低分子化合物などを基板上に固定化したタイプのバイオチップについては，まだ開発途上にある[1]。

実は，タンパク質などの生体高分子をチップ上に固定化するには，DNAに比べてより困難がともなう。DNAは多様な遺伝情報を担っているものの，化学構造としてはほとんど類似した4つの塩基により構成されており，DNA鎖はほぼ均質な高分子と考えてよい。また，末端修飾も容易で，多種類のDNAを同一の方法で固定化することが原理的に可能である。

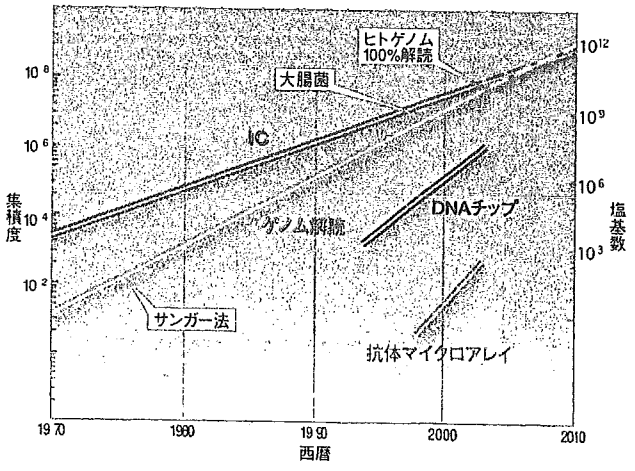

図1
コンピュータICとマイクロアレイチップの集積度の比較と遺伝子解析の向上

これに対してタンパク質は，構成するアミノ酸が化学的に多様であり，官能基の種類，数，存在場所も異なるため，それぞれが化学的に異なった性質をもつ。このため，同一の方法で一つの基板上に固定化するのは非常に困難である。

光固定化法

そこで筆者らは，この問題点を解決するために，タンパク質の光固定化法を考案した[2]。この方法は，三

つの特徴をもつ。

(1) ラジカル反応で架橋させるため，有機分子であれば「なんでも」固定化できる。

(2) 基板上への固定化に用いるマトリックスの成分を，生体膜成分やそれに近い性質の高分子にしたことで，非特異的な相互作用を弱めている。これにより高S/N比（signal to noise ration）の相互作用解析が可能になった。

(3) ランダムな配向性で固定化できる(図2)。これによって，従来の固定化法と比べ，生体分子のさまざまな部位をチップ上に提示することができるので，相互作用を網羅的に調べることができる。

この技術が広く応用されるようになると，従来の固定化技術を用いた生体分子相互作用解析は，はたして真の解析になっていたのか問題となるだろう。

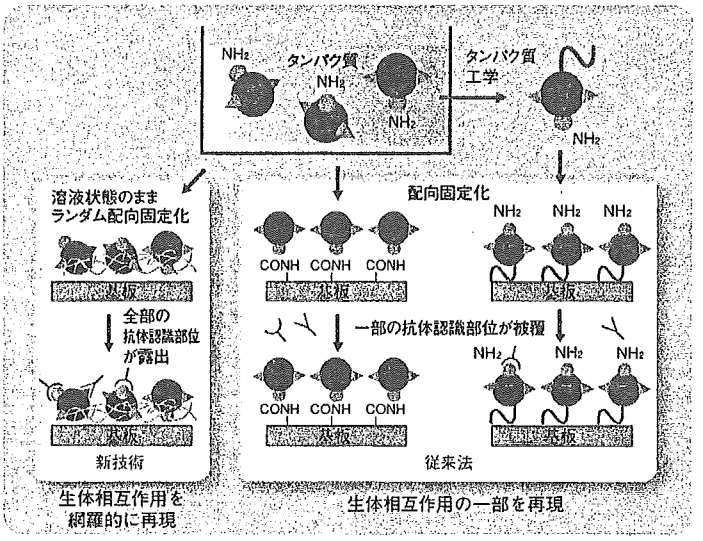

図2 光固定化法を用いたチップによる相互作用解析
光固定化法では，従来の化学固定化法や，組み換えタンパク質を用いた方法と異なり，生体分子のランダム配向固定化が可能。

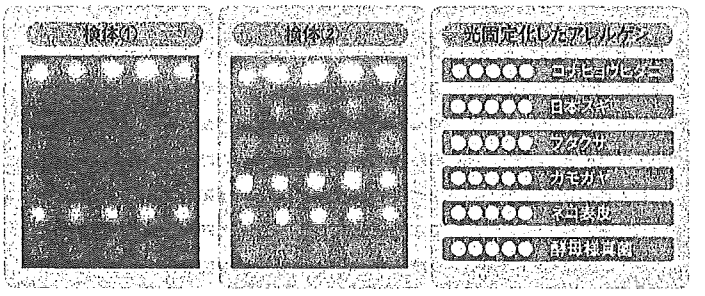

図3 アレルゲン・マイクロアレイによるアレルギー関連抗体IgEの化学発光法による検出

光固定化マイクロアレイの展開

光固定化法は，さまざまな有機分子の固定化に有効である。図3に，アレルギー診断用にアレルゲンを固定化した例を示す。血液中のポリクローナルな免疫グロブリンIgEは，同じタンパク質のさまざまな部位を認識するが，ランダム配向で固定化できる光固定化法は，そういったポリクローナルな抗体を検出するのに非常に有効な方法と考えられる。

現在，図4に示すような全自動測定装置を完成している。この装置では，チップおよび，測定したい溶液（血液）の入った試験管を装着するだけで，自動的に測定データを得ることができる。

光固定化法を表面プラズモン共鳴（SPR）用や水晶振動マイクロバランス（QCM）用チップに応用する試みを現在おこなっている。このような技術をもとに，1年以内に理研ベンチャーとしての起業を予定している。

さまざまな分子や細胞に応用可能な光固定化法は，今後，マイクロアレイチップのスタンダードとして大きく発展するものと期待している。

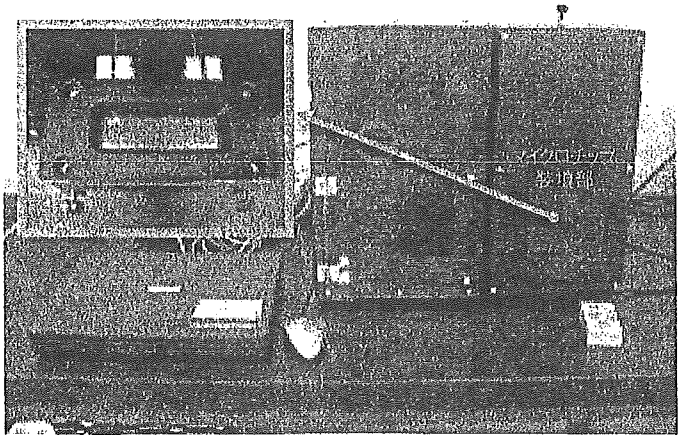

図4 光固定化を利用したバイオチップの全自動測定装置

参考文献
[1] 伊藤嘉浩「バイオテクノロジー総覧」通産資料出版会，p.662 (2005)
[2] 伊藤嘉浩，山内哲也，大村醒，高分子論文集，61，501(2004)

幹細胞の体外増幅

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In vitro expansion of stem cells

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The safe and rapid expansion of human stem cells is very important for realization of regenerative medicine. So far, various types of cell culture have been developed, and this technology has led to dramatic progress in life science through the use of sera or feeder cells derived from animals. However, to avoid contamination with pathogens, animal-derived materials should not be used in the culture medium when human stem cells are cultured for medical therapy. Therefore, development of completely artificial cell culture systems that do not require animal-derived materials is needed. First to increase the number of hematopoietic stem cells (HSCs) in cord blood, we cultured HSCs with native or immobilized human stromal cells that had been immortalized by gene transfection. In addition, biosignal molecules, including cytokines and membrane proteins, were immobilized for the HSC culture. Secondly it was found that human placenta feeder layers can support undifferentiated growth of primate embryonic stem cells.

1. はじめに

病気で機能障害や機能不全に陥った生体組織・臓器の治療のために近代的な人工臓器や臓器移植が発展してきた。しかし，人工臓器にはまだ不備が多く，臓器移植ではドナーの不足が深刻である。そのような中，80年代後半から，ハイブリッド人工臓器（培養皮膚）の考えを発展させた「ティッシュエンジニアリング」という考えが生まれた。これは，生分解性マトリックスを足場にして体内で組織再生を行わせようとするものであった。そして，90年代後半になると，クローン技術の発明と，ヒトES細胞の樹立という二つの大きな進展により「再生医療」という，幹細胞を積極的に

【キーワード】
造血幹細胞，ES細胞，ニッチェ，体外増幅，バイオリアクター

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