A Micropatterned Multifunctional Carbohydrate Display by an Orthogonal Self-Assembling Strategy

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Introduction

Engineering of microscopic surfaces using biomacromolecules such as carbohydrates[1−3] and proteins[4−6] and using cells on substrates is important as tools to develop biotechnologies such as biosensors,[7,8] bioelectronics,[9] and tissue engineering.[10] A variety of methods via covalent bond formation and supramolecular formation have been employed to immobilize biomacromolecules and to control the assembly. Supramolecular formation on surfaces is paid much attention because of the high specificity and the facile assembling control.[11,12] In addition, integration of supramolecular formation with lithography techniques is applicable for highly precise micropatterning of biomacromolecules.[13]

Recently, we have achieved immobilization and micropatterning of glycopolymers by supramolecular formation via hydrophobic interaction.[14] An amphiphilic glycoconjugate polymer (lactose-substituted styrene homopolymer abbreviated as PVLA)(Figure 1a) was displayed on self-assembled monolayer of octadecyltrimethoxysilane (ODS−SAM) prepatterned on silicon substrates. The micropatterned carbohydrate display was visualized by molecular recognition of a galactose-specific lectin RCA_{120} (*Ricinus communis* agglutinin 120) with FITC (fluorescein isothiocyanate) fluorophore. The glycopolymer PVLA functions not only as a glycocluster of artificial matrixes but also as controllable self-assembling materials.[15]

On the other hand, there are growing interests in micropatterning of multiple components to fabricate sophisticated substrates.[16−18] Since multiple micropatterning of biomacromolecules by lithography techniques is limited to simple surface organization with single biomolecules, supramolecular assembly of multiple components is a key step to the precise multiple micropatterning. Various interactions such as hydrophobicity, electrostaticity, host−guest complexation, and metal coordination can be applied to construct supramolecular assemblies selectively and independently.[19−22] Manipulation of more than two types of these interactions independently and simultaneously provides the "orthogonal self-assembling", resulting in a multiple micropatterning.

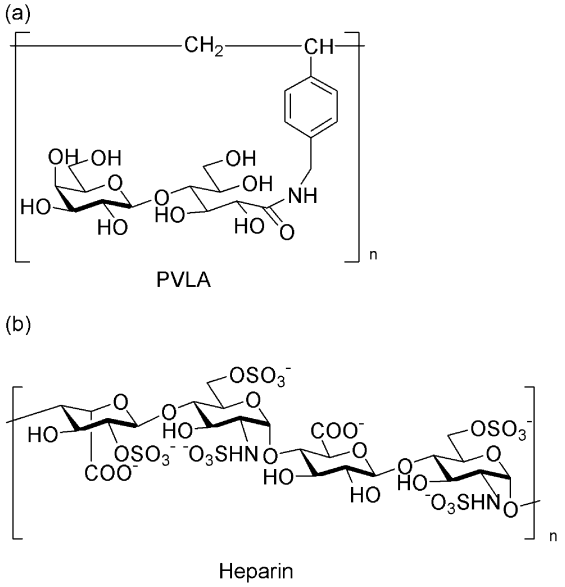

Figure 1. The chemical structures of (a) PVLA and (b) heparin.

In the present report, we expand the concept of orthogonal self-assembly to multifunctional micropatterning of biomacromolecules. Here, we applied two different types of glycopolymers of PVLA and heparin that can be self-assembled, respectively, by hydrophobic and electrostatic interactions. As shown in Figure 2, hydrophobic octadecyltrimethoxysilane (ODS) and cationic aminopropyltrimethoxysilane (APS) monolayers were immobilized and micropatterned on the substrate by the combination of photolithography and self-assembling processes. Then, the ODS/APS micropatterned substrate was simply immersed in these two glycopolymer solutions to display the orthogonal carbohydrates. Then, we tried the orthogonal assembly of proteins and cells on the micropatterned glycopolymers substrate. Proteins of RCA_{120} and basic fibroblast growth factor (bFGF) were micropatterned by the orthogonal carbohydrate recognitions. Hepatocytes and NIH 3T3 fibroblasts have been cocultured on the micropatterned glycopolymer PVLA/bFGF substrate. Heparin that is localized in extracellar matrixes (ECM) and cell envelopes participates in cell adhesion through bFGF,[23−25] while PVLA can be substrates for culture of hepatocytes through multivalent interactions between antennary galactoses and asialoglycoprotein receptors.[15]

Carbohydrates on cell surfaces serve as significant biological signals and play important roles in numerous intercellular recognition processes, for example, cell differentiation, cell adherence, immune response, infection from viruses, and cancer metastasis.[26] Therefore, carbohydrate-immobilized materials have recently attracted great interest. Micropatterned carbohydrates on solid surfaces are expected to be useful as tools to analyze carbohydrate−protein interactions and to fabricate the scaffolds of cell cultivation.

Experimental Section

Self-Assembly of Glycopolymers and Molecular Recognition of Proteins. The treatments were carried out by immersing the corresponding substrates in the following aqueous solutions at 25 °C: PVLA

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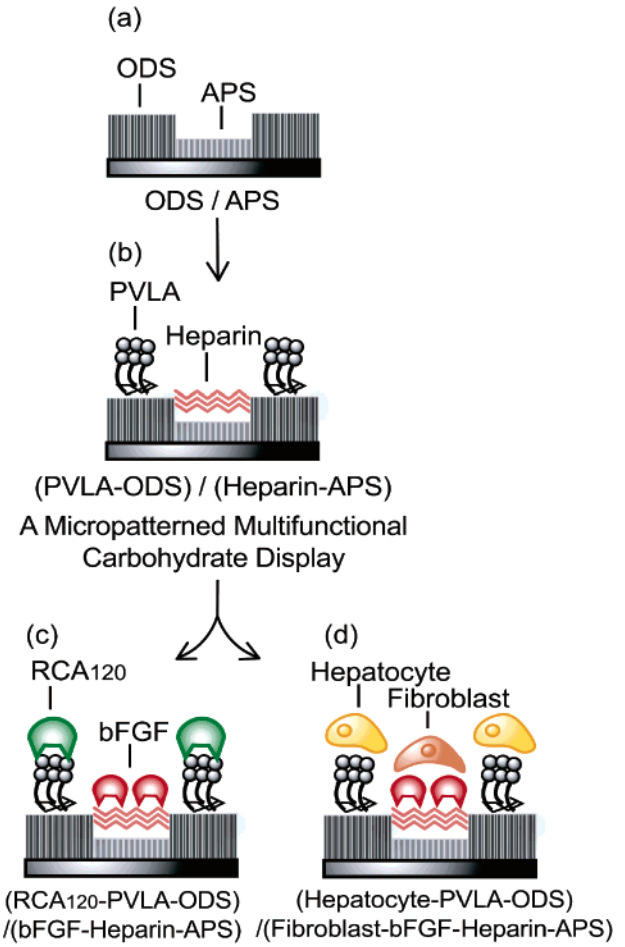

Figure 2. Schematic illustration of multiple biomacromolecular displays: (a) micropatterned ODS/APS substrate, (b) self-assembly of carbohydrates (PVLA and heparin), (c) molecular recognition of proteins (RCA_{120} and bFGF), and (d) coculture of cells (hepatocytes and fibroblasts).

(0.5 mg/mL, 3 h), heparin (0.05 mg/mL, 5 h), FTIC−RCA_{120} (0.1 mg/mL, 1 h), and TRITC−bFGF (0.01 mg/mL, 1 h). After the treatments, the substrates were washed with Milli-Q water and then were monitored by XPS (X-ray photoelectron spectroscopy) for glycopolymers and by fluorescence microscopy for proteins.

Culture of Hepatocytes and Fibroblasts.[27,28] Hepatocytes isolated from male Sprague−Dawley (SD) rats were obtained from KAC (Ritto, Japan). The cell viability was higher than 80%, as assessed by trypan blue exclusion. Hepatocytes were maintained at a concentration 5.0×10^5 cells/mL in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 UI/mL penicillin, 100 μg/mL streptomycin, 3% v/v FCS, and 4 μg/mL bovine insulin. The cells were seeded on the (PVLA−ODS)/(bFGF−heparin−APS) substrate and were incubated at 37 °C in 5% CO_2 atmosphere for 10 h. Nonadherent cells were removed by rinsing with phosphate-buffered solution (PBS).

NIH 3T3 fibroblasts were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), 1% NEAA, 1% NaPyr, 100 mU/mL of penicillin, and streptomycin. The fibroblasts were plated at a concentration of 1.0×10^6 cell/mL on the (PVLA−ODS)/(bFGF−heparin−APS) substrate. The substrates were incubated for 12 h in 10% FBS DMEM and then were rinsed with PBS to remove nonadherent cells.

Coculture was carried out as follows: hepatocytes were incubated at a concentration 5.0×10^5 cells/mL in DMEM for 10 h and then fibroblasts were added and incubated at a concentration of 1.0×10^6 cells/mL for 12 h. The morphologies of these cultures were observed with an optical microscope (Olympus, Tokyo, Japan).

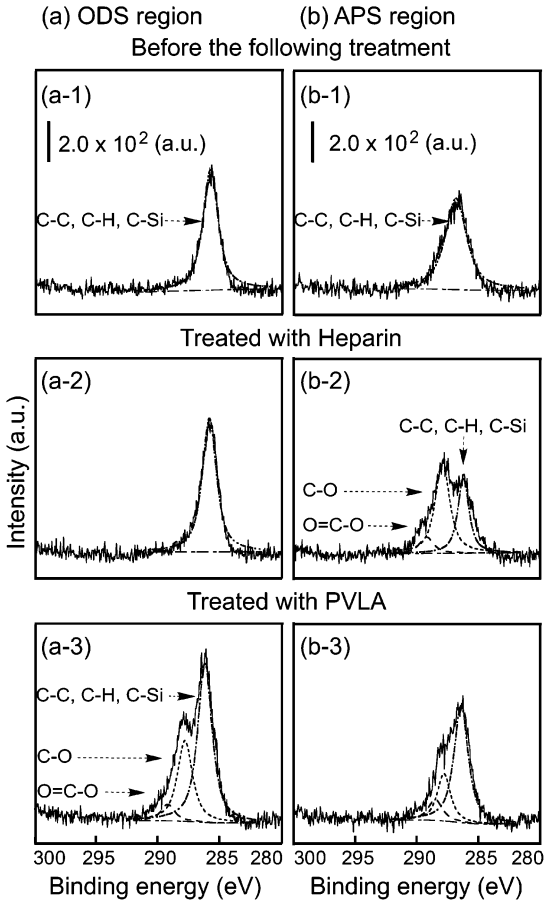

Figure 3. C1s XPS spectra of (a) ODS region on ODS/APS substrate and (b) APS region on ODS/APS substrate. (a-1) and (b-1) before the following treatment, (a-2) and (b-2) treated with heparin, (a-3) and (b-3) treated with PVLA.

Results

Self-Assembly of PVLA and Heparin on Micropatterned ODS/APS Substrates. The micropatterned ODS/APS substrates were incubated in aqueous solutions of two glycopolymers, heparin and PVLA. First, an ODS/APS substrate was immersed in an aqueous solution of heparin and the substrate was analyzed by XPS (Figure 3). The XPS spectra of each ODS and APS region of the micropatterned ODS/APS substrates on silicon are shown, respectively, in Figure 3a-1 and b-1. There appeared a strong signal around 288 eV along the APS region in Figure 3b-2, which was assignable to C−O and O═C−O bonds of heparin. The atomic ratio of [C−H]/[C−O]/[O═C−O] on the APS region was 39/46/15. On the other hand, a little signal due to C−O and O═C−O bonds was observed around 288 eV on ODS region in Figure 3a-2. These data indicated that heparin was adsorbed selectively on the APS region and negligibly on the ODS region of the ODS/APS substrate.

Then, a micropatterned ODS/APS substrate was treated with an aqueous solution of PVLA. A well-defined C−O peak at 288 eV and a shoulder O═C−O signal at 289 eV appeared on the ODS region in Figure 3a-3. The atomic ratio of [C−H]/[C−O]/[O═C−O] was 59/28/10, indicating the adsorption of PVLA. A shoulder peak of C−O was observed on the APS substrate in Figure 3b-3, and the peak intensity was about half of that on the ODS region. These data indicated that PVLA was not only adsorbed preferentially onto the ODS region but also less preferentially onto the APS region.

We previously reported that PVLA hardly adsorbed to cystamine (ω-aminoethyl disulfide) terminated gold substrate.[29]

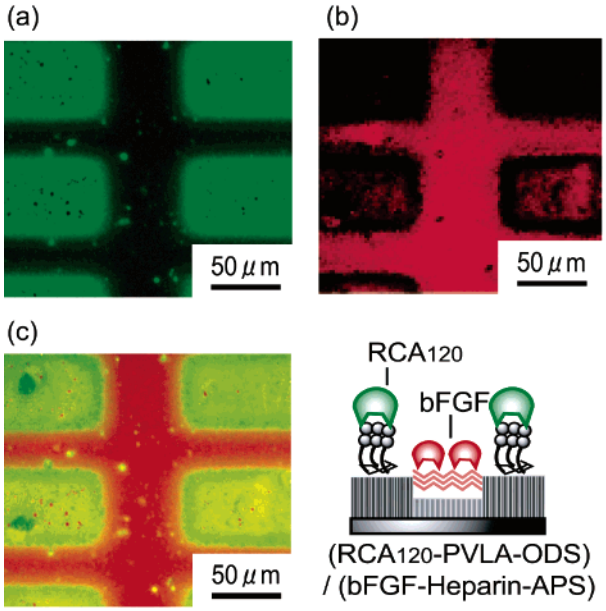

Figure 4. Fluorescence images of (RCA_{120}−PVLA−ODS)/(bFGF−heparin−APS) substrate: viewed with (a) a green filter and (b) a red filter and (c) their overlay.

Cystamine on gold substrate was reported to form well-defined cationic and hydrophilic monolayers with contact angles around 44°.[30] On the other hand, the thickness of the APS−SAM on a silicon substrate estimated by ellipsometry was 8 Å which was a little thicker than the value (5.5 Å) estimated by molecular modeling, and its contact angle was 65°, suggesting that APS formed a little disordered multilayer with a slight hydrophobic character.[31,32] We assume that the hydrophobicity of APS resulted in less selective adsorption of PVLA on the APS region on silicon substrate.

Molecular Recognition by RCA_{120} and bFGF. Successive micropatterning of RCA_{120} and bFGF could be performed most clearly when ODS/APS substrates were immersed in heparin, bFGF, PVLA, and RCA_{120} solutions in this order. The patterned fluorescence micrograph is shown in Figure 4. The green fluorescence of FITC−RCA_{120} was observed within the PVLA−ODS region, with an S/N ratio of 2.8 (Figure 4a). The red fluorescence of TRITC−bFGF was observed primarily within the heparin−APS region, with an S/N ratio of 1.8 (Figure 4b). The overlaid image (Figure 4c) of the FITC−RCA_{120} and TRITC−bFGF indicates that these proteins have specific affinities toward each glycopolymer. The micropatterning of the two glycopolymers as well as the two carbohydrate-binding proteins could be achieved by simply immersing the ODS/APS substrates in the glycopolymer and protein solutions.

Coculture of Hepatocytes and Fibroblasts. Cell adhesion on the multifunctional micropattern on glass substrates was examined with rat hepatocytes and NIH 3T3 fibroblasts. Prior to coculture, the substrate was treated with each cell. When hepatocytes were incubated on the (PVLA−ODS)/(bFGF−Heparin−APS) substrate for 10 h, a micropattern of round-shaped hepatocytes was observed along the PVLA−ODS region (Figure 5b). Even though the substrate was rinsed with PBS, hepatocytes stayed on the micropattern, indicating that hepatocytes adhered specifically and strongly on the PVLA−ODS region. When NIH 3T3 fibroblasts were incubated on the (PVLA−ODS)/(bFGF−Heparin−APS) substrate for 12 h, another characteristic micropattern appeared: spindle-shaped fibroblasts adhered and extended on the bFGF−heparin−APS region (Figure 5c). Hepatocytes and fibroblasts adhered specifically, respectively, on PVLA and bFGF with little nonspecific adsorption.

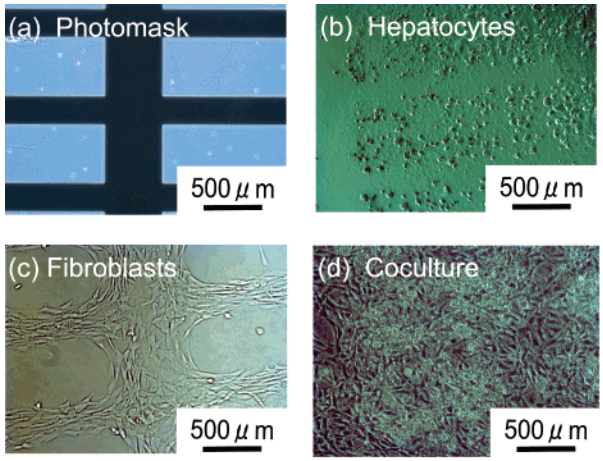

Figure 5. Cell adhesion to the multifunctional micropatterned (PVLA−ODS)/(bFGF−heparin−APS) substrate: (a) photomask, (b) culture of hepatocytes, (c) culture of NIH 3T3 fibroblasts, and (d) coculture of hepatocytes and NIH 3T3 fibroblasts.

Then, coculture of these two cells was examined. Figure 5d shows a round morphology of hepatocytes on PVLA−ODS region and another spindle morphology of fibroblasts on bFGF−heparin−APS region. Then, fibroblasts were observed to extend to the region of hepatocytes and to interpenetrate with each other, suggestive of the interaction between hepatocytes and fibroblasts.

Discussion

Orthogonal self-assembling technology has been developed by manipulating several types of physicochemical interactions independently and simultaneously to construct multiple micropatterning.[19−22] Hickman et al. set up the "orthogonal" concept by the modification of the two types of metal surfaces through multiple covalent bonds.[19] Demers et al. adapted a variety of interactions of molecules to prepare the orthogonal assembly of DNA and nanoparticles.[20] Recently, Xu et al. reported the orthogonal assembly of polymer and nanoparticles.[21] They applied the orthogonal self-assembly to the suitable lithographic templates. The orthogonal self-assembly provides the facile multiple micropatterns of organic molecules. We have extended the orthogonal concept to biomimetic, multifunctional, and supramolecular assemblies for biological applications.

The present paper has proposed the orthogonal self-assembling of two types of glycopolymers (PVLA and heparin), carbohydrate-binding proteins (RCA_{120} and bFGF), and cells (hepatocytes and fibroblasts) on micropatterned ODS/APS substrates. The ODS/APS substrates were prepared via the successive SAM formation and photolithography to display hydrophobic and cationic functionalities. The orthogonal functionalities enabled self-assembling of PVLA and heparin through hydrophobic and electrostatic interactions. Then, the two proteins could be micropatternd most effectively when ODS/APS substrates were immersed in heparin, bFGF, PVLA, and RCA_{120} solutions in this order. In addition, coculture of hepatocytes and fibroblasts could be performed on the micropatterned (PVLA−ODS)/(bFGF−heparin−APS) substrates, which was due to the biological recognition between asialoglycoprotein receptors on hepatocytes and multiantennary galactosides along PVLA and between bFGF receptors on fibroblasts and heparin-bound bFGF.

Conclusion

We could construct the three- and four-storied hierarchical structures composed of lipids, carbohydrates, proteins, and cells via orthogonal self-assembly strategy. This strategy can be a mimic of complex living tissues. Orthogonal relationship has been kept in these specific combinations of adsorption of glycopolymers, molecular recognition through carbohydrate-binding proteins, and adhesion of cells to attain the clear micropatterning. We expect that the present nanoprocessing using biomacromolecules, orthogonal in organization and hierarchy, will be essential in universal design for a variety of biotechnological tools such as cell culturing and biosensing.

Abbreviations

APS, 3-aminopropyltrimethoxysilane; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; ODS, octadecyltrimethoxysilane; RCA_{120}, Ricinus communis agglutinin 120; SAM, self-assembled monolayer; SD rat, Sprague-Dawley rat; TRITC, tetramethylrhodamine 5-isothiocyanate; XPS, X-ray photoelectron spectroscopy.

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Supporting Information Available. Text giving the characterizations of micropatterned ODS/APS substrates and molecular recognition by RCA_{120} and bFGF. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Feizi, T.; Fazio, F.; Chai, W.; Wong, C. H. *Curr. Opin. Struct. Biol.* **2003**, *13*, 637.
(2) Chen, G.; Ito, Y.; Imanishi, Y.; Magnari, A.; Lamponi, S.; Barbucci, R. *Bioconjucate Chem.* **1997**, *8*, 730.
(3) Shirahata, N.; Yonezawa, T.; Miura, Y.; Kobayashi, K.; Koumoto, K. *Langmuir* **2003**, *19*, 9107.
(4) Templin, M. F.; Stoll, D.; Schrenk, M.; Traub, P. C.; Vohringer, C. F.; Joos, T. O. *Trends Biotechnol.* **2002**, *20*, 160.
(5) Tanaka, M.: Wong, A. P.; Rehfeldt, F.; Tutus, M.; Kaufmann, S. *J. Am. Chem. Soc.* **2004**, *126*, 3257.
(6) Yap, F. L.; Zhang, Y. *Langmuir* **2005**, *21*, 5233.
(7) Willner, I.; Katz, E. *Angew. Chem., Int. Ed.* **2003**, *42*, 4576.
(8) Bieri, C.; Ernst, O. P.; Heyse, S.; Hofmann, K. P.; Vogel, H. *Nat. Biotechnol.* **1999**, *17*, 1105.
(9) Park, K.-Y.; Kim, M.-S.; Choi, S.-Y. *Biosens. Bioelectron.* **2005**, *20*, 2111.
(10) Itle, L. J.; Pishko, M. V. *Biotechnol. Prog.* **2005**, *21*, 1004.
(11) Daniel, M. C.; Astruc, D. *Chem. Rev.* **2004**, *104*, 293.
(12) Henry, M. *ChemPhysChem* **2002**, *3*, 607.
(13) Ito, Y.; Nogawa, M.; Sugimura, H.; Takai, O. *Langmuir* **2004**, *20*, 4299.
(14) Miura, Y.; Sato, H.; Ikeda, T.; Sugimura, H.; Takai, O.; Kobayashi, K. *Biomacromolecules* **2004**, *5*, 1708.
(15) Kobayashi, K.; Kobayashi, A.; Akaike, T. *Methods Enzymol.* **1994**, *247*, 409.
(16) Co, C. C.; Wang, Y.-C.; Ho, C.-C. *J. Am. Chem. Soc.* **2005**, *127*, 1598.
(17) Khademhosseini, A.; Suh, K. Y.; Yang, J. M.; Eng, G.; Yeh, J.; Levenberg, S.; Langer, R. *Biomaterials* **2004**, *25*, 3583.
(18) Yousaf, M. N.; Houseman, B. T.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5992.
(19) Hickman, J. J.; Laibinis, P. E.; Auerbach, D. I.; ZOU, C.; Gardner, T. J.; Whitesides, G. M.; Wrighton, M. S. *Langmuir* **1992**, *8*, 357.
(20) Demers, L. M.; Park, S. J.; Taton, T. A.; Li, Z.; Mirkin, C. A. *Angew. Chem., Int. Ed.* **2001**, *40*, 3071.
(21) Xu, H.; Hong, H.; Lu, T.; Uzun, O.; Rotello, V. M. *J. Am. Chem. Soc.* **2006**, *128*, 3162.
(22) Gardner, T. J.; Frisbie, C. D.; Wrighton, M. S. *J. Am. Chem. Soc.* **1995**, *117*, 6927.
(23) Kunou, M.; Koizumi, M.; Shimizu, K.; Kawase, M.; Hatanaka, K. *Biomacromolecules* **2000**, *1*, 451.
(24) Rapraeger, A. C.; Krufka, A.; Olwin, B. B. *Science* **1991**, *252*, 1705.
(25) Okada, B. M.; Thiery, J. P.; Jouanneau, J. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 263.
(26) Tayler, M. E.; Drickamer, K. *Introduction to Glycobiology*; Oxford University Press: New York, 2003.
(27) Zavan, B.; Brun, P.; Vindigni, V.; Amadori, A.; Habeler, W.; Pontisso, P.; Montemurro, D.; Abatangelo, G.; Cortivo, R. *Biomaterials* **2005**, *26*, 7038.
(28) Nagai, H.; Terada, K.; Watanabe, G.; Ueno, Y.; Aiba, N.; Shibuya, T.; Kawagoe, M.; Sato, M.; Senoo, H.; Sugiyama, T. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1420.
(29) Matsuura, K.; Tsuchida, A.; Okahata, Y.; Akaike, T.; Kobayashi, K. *Bull. Chem. Soc. Jpn.* **1998**, *71*, 2973.
(30) Caldwell, W. B.; Chen, K.; Mirkin, C. A.; Babinec, S. J. *Langmuir* **1993**, *9*, 1945.
(31) Chiang, C. H.; Ishida, H.; Koenig, J. L. *J. Colloid Interface Sci.* **1980**, *74*, 396.
(32) Petri, D. F. S.; Wenz, G.; Schunk, P.; Schimmel, T. *Langmuir* **1999**, *15*, 4520.

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