Bioconjugate Chemistry

Communication

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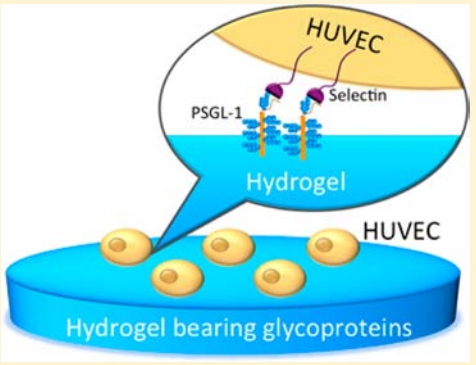
Preparation of Biointeractive Glycoprotein-Conjugated Hydrogels through Metabolic Oligosacchalide Engineering

Yasuhiko Iwasaki,* Aki Matsunaga, and Shuetsu Fujii

Department of Chemistry and Materials Engineering Faculty of Chemistry, Materials and Bioengineering, Kansai University, 3-3-35 Yamate-cho, Suita-shi, Osaka 564-8680, Japan

Supporting Information

ABSTRACT: In the current study, synthetic hydrogels containing metabolically engineered glycoproteins of mammalian cells were prepared for the first time and selectin-mediated cell adhesion on the hydrogel was demonstrated. A culture of HL-60 cells was supplemented with an appropriate volume of aqueous solution of *N*-methacryloyl mannosamine (ManMA) to give a final concentration of 5 mM. The cells were then incubated for 3 days to deliver methacryloyl groups to the glycoproteins of the cells. A transparent hydrogel was formed via redox radical polymerization of methacryloyl functionalized glycoproteins with 2-methacryloyloxyethyl phosphorylcholine and a cross-linker. Conjugation of the glycoproteins into the hydrogel was determined using Coomassie brilliant blue (CBB) and periodic acid−Schiff (PAS) staining. The surface density of P-selectin glycoprotein ligand-1 (PSGL-1) on the hydrogels was also detected using gold-colloid-labeled immunoassay. Finally, selectin-mediated cell adhesion on hydrogels containing glycoproteins was demonstrated. Selectin-mediated cell adhesion is considered an essential step in the progression of various diseases; therefore, hydrogels having glycoproteins could be useful in therapeutic and diagnostic applications.



A cell membrane is composed of a phospholipid bilayer incorporating membrane proteins and covered with dense carbohydrate chains.[1] All components are dynamically arranged in the plasma membrane and regulate highly specific biointerfacial interactions. In particular, carbohydrates located on the outermost surface of the cell membrane contribute to various forms of communication between living cells and their environment.[2] To mimic the biological roles of carbohydrates, various synthetic polymers with pendant carbohydrates have been prepared.[3] The multivalency of carbohydrates in the glycopolymers allows them to exhibit strong interactions with specific molecules such as proteins.[4] Kobayashi and co-workers first prepared synthetic glycopolymers and clarified that galactose (Gal) residues are a preferred polymer conjugate for interacting with hepatocytes and Gal-recognition lectins.[5] Whitesides and co-workers also synthesized glycopolymers containing sialic acid, which is recognized by influenza viruses.[6,7] Dendritic and hyperbranched glycopolymers have been specifically designed to facilitate the intermolecular affinity of glycopolymers to receptor proteins.[8] Recently, the biological and therapeutic functions of carbohydrates have been clarified and many glycopolymer bioconjugates have also been designed.[9] Although these approaches are very successful for use in the regulation of cell−material interactions, almost all synthetic glycopolymers have simple carbohydrate residues because it is difficult to replicate the complex natural structure of carbohydrates via organic synthesis. In addition, some glycopolymers may not interact uniquely with specific cells and instead show affinity to a broad range of substrates. In the current study, synthetic hydrogels incorporating metabolically engineered glycoproteins of mammalian cells were prepared for the first time and selectin-mediated cell adhesion on the surface of synthetic hydrogels was demonstrated, as shown in Figure 1.

The current study utilizes the metabolic labeling process for sialic acids, which is one of the most robust methods for the surface engineering of living mammalian cells.[10−12] Sialic acid is the terminal residue of oligosaccharides, which cover the mammalian cell surface. The biosynthesis of sialic acids, beginning with uridine diphosphate (UDP)-*N*-acetyl glucosamine (UDP-GlcNAc), is produced by five consecutive reactions.[13] In the first reaction, UDP-GlcNAc 2-epimerase catalyzes the formation of *N*-acetyl mannosamine (ManNAc) from UDP-GlcNAc. ManNAc derivatives are then processed in the sialic acid biosynthesis of the non-natural functional groups delivered at the terminal position of the oligosaccharide.[14,15] Recently, various types of ManNAc derivatives containing nonreactive alkyl *N*-acyl groups, fluorinated substituents, and bioorthogonal functional groups have been synthesized and these molecules are applied for cell surface immobilization and imaging glycosylation.[16] Luchansky et al. verified the metabolic delivery of non-natural functional groups into sialic acid using liquid chromatography−mass spectrometry (LC-MS).[17,18] The metabolic conversion density of *N*-azidoacetyl mannosamine (ManNAz) to *N*-azidoacetylsialic acid (SiaNAz) bound in the

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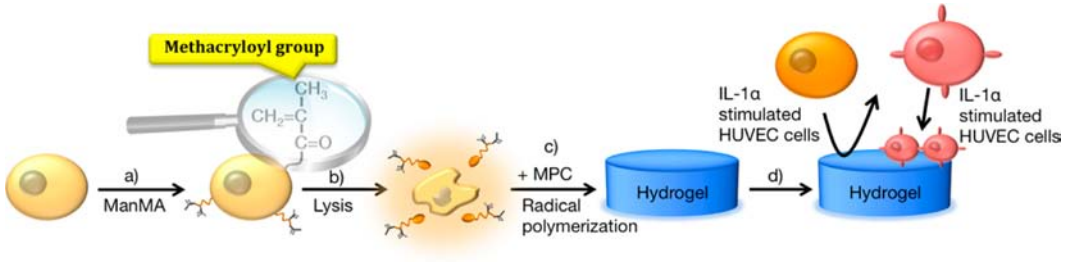

Figure 1. Preparation of a biointeractive hydrogel composed of mammalian glycoproteins: (a) metabolic oligosaccharide engineering with ManMA, (b) lysis of ManMA-treated HL-60 cells, (c) preparation of hydrogels via free radical polymerization of glycoproteins with MPC and MBA, (d) adhesion of HUVECs on hydrogels bearing glycoproteins.

cell membrane was quantified in a variety of cell types. Peracetylated sugar permeates the cell membrane more readily than does free sugar. Although peracetylated ManNAz was used, SiaNAz was found to constitute between 4% and 41% of the total sialosides, depending on the system.

Recently, we have synthesized methacryloyl-modified mannosamine (*N*-methacryloyl mannosamine, ManMA) and successfully delivered methacryloyl groups to sialic residues of mammalian cells.[19] This functional group is established as being useful for not only polymerization, but also other chemical reactions such as thiol−ene reactions.[20,21] The culture of HL-60 cells was supplemented with an appropriate volume of aqueous solutions of ManMA to give a final concentration of 5 mM. The cells were then incubated for 3 days to deliver methacryloyl groups to the carbohydrates of the cells. No adverse effect of 5 mM ManMA on cell viability and proliferation was observed.[22] The ManMA used in the current study is a free monosaccharide, and the membrane permeation of ManMA is expected to be worse than that of the peracetylated one.

To investigate the influence of ManMA treatment on selectin-mediated cell−cell binding, the adhesion of ManMA-treated HL-60 cells on human umbilical vein endothelial cells (HUVECs) was studied. Previously, Murase and co-workers studied the effect of phenolic compounds on selectin-mediated cell−cell binding using HL-60 cells and HUVECs.[23] In this study, HUVECs were stimulated with an inflammatory cytokine, IL-1α, to generate an inflammatory condition in vitro and E-selectins were expressed in stimulated HUVECs with activation of nuclear factor κB (NF-κB). HL-60 cells preferentially adhered to the stimulated HUVECs, whereas no adhesion of HL-60 cells to the nonstimulated HUVECs was observed. The ManMA-treated HL-60 cells were also placed in contact with the IL-1α-stimulated HUVECs. Figure 2A shows phase-contrast and fluorescence micrographs of HUVECs in contact with HL-60 for 30 min. The HL-60 cells were prestained with calcein-AM to distinguish the two cell types. Phase-contrast micrographs showed that every adherent HUVEC adopted a spindle shape. On the nonstimulated HUVECs that were not treated with IL-1α, no adherent HL-60 cells were observed. In contrast, the HL-60 cells adhered well to the IL-1α-stimulated HUVECs. This adhesion is attributable to molecular binding between E-selectin and selectin-binding glycoproteins, such as P-selectin ligand glycoprotein-1 (PSGL-1). The selectin ligand glycoprotein is regularly expressed on the surface of leukocytes and plays an important role in leukocyte recruitment in the bloodstream during inflammation. E-selectins interact with PSGL-1 and other glycoproteins having α(2,3) sialylated and α(1,3) fucosylated carbohydrates structured similar to sialyl Lewis x (sLe^x) tetrasaccharide.[24−26]

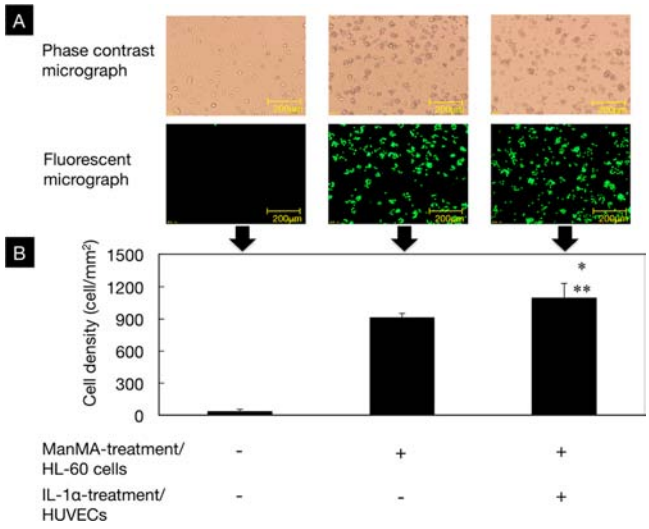

Figure 2. Adhesion of HL-60 cells on HUVECs: (A) phase-contrast and fluorescence micrographs of cells and (B) density of adherent HL-60 cells on HUVECs. *P < 0.01 vs ManMA(−)/IL-1α(−), **P > 0.01 vs control/IL-1α(+).

Selectin-mediated cell binding is influenced by metabolic oligosaccharide engineering with non-natural monosaccharide analogues.[16] Dafik and co-workers reported that the number of adherent HL-60 cells on E-selectin-coated surfaces decreased with an increase in the replacement degree of sialic acids on the cell surface with fluorinated congeners.[27] Marathe et al. also reported that fluorinated GalNAc was incorporated into the oligosaccharides of the selectin ligand PSGL-1 and reduced the degree of cell binding to selectins.[28] In this study, the number of ManMA-treated HL-60 cells adhered to IL-1α-stimulated HUVECs was not significantly different from that of untreated HL-60 cells, as shown in Figure 2B. Mahal et al. reported that the surface density of non-natural functional groups delivered to the cell surface increased with an increase in the dose (0−40 mM) of *N*-levulinoyl mannosamine (ManLev), which is one of the bioorthogonal ManNAc derivatives.[15] Therefore, non-natural sialic acid may not be completely replaced at a 5 mM dose of ManMA, possibly resulting in the selectin-mediated adhesion of ManMA-treated HL-60 cells on HUVECs.

To characterize the polymerization ability of methacryloyl-functionalized glycoproteins, redox-initiated free radical polymerization with 2-methacryloyloxyethyl phosphorylcholine (MPC) was performed. MPC is a highly hydrophilic monomer that is useful for creating biocompatible materials because the phosphorylcholine unit avoids nonspecific interaction with plasma proteins and cells.[29] The increase in size of the

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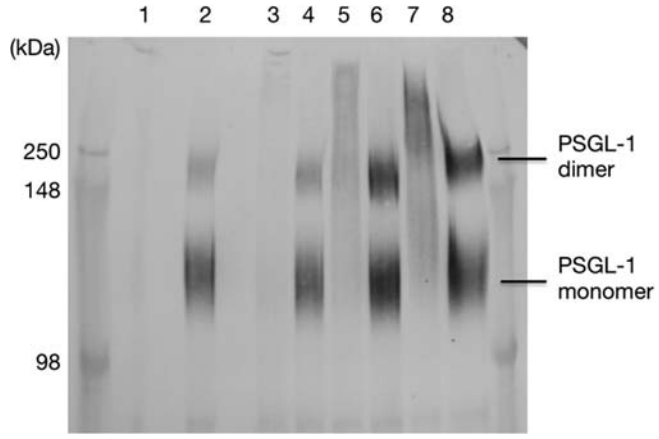

Polymerization condition

Lane	ManMA-treatment	MPC (mM)
1	+	1000
2	-	1000
3	+	500
4	-	500
5	+	100
6	-	100
7	+	50
8	-	50

[Lysate]=500 μg/ml

Figure 3. Western blot analysis data representing PSGL-1 antibody reactive bands. The lysate was mixed with various concentrations of MPC and polymerization was performed using a redox initiator. Odd-numbered lanes: ManMA-treated HL-60 cells. Even-numbered lanes: untreated HL-60 cells.

glycoproteins was determined by Western blot analysis. ManMA-treated or untreated HL-60 cells were disrupted with ultrasonication, and insoluble substances were precipitated by centrifugation. The precipitate was dissolved in RIPA lysis buffer. The resulting solution was mixed with a given amount of MPC and radical polymerization was performed via redox initiation. Figure 3 shows Western blot analysis data for PSGL-1 of ManMA-treated and untreated HL-60 cells after polymerization. PSGL-1 is a disulfide-bonded homodimeric mucin-like glycoprotein expressed on leukocytes that interacts with both P- and E-selectin.[30] PSGL-1 consists of two identical 120-kD glycoprotein chains and has numerous sialylated, fucosylated O-linked oligosaccharide branches, many of which terminate in the sLe^x determinant.[31] The even-numbered lanes of Figure 3 are the blot results for lysates from the native HL-60 cells (without ManMA-treatment). Bands of monomeric and dimeric PSGL-1 were observed, and the size did not change regardless of the concentration of MPC. In contrast, the increase in size of the PSGL-1 ManMA-treated cells were qualitatively recognized after polymerization (odd-numbered lanes). The original bands from the monomeric and dimeric PSGL-1 faded as the concentration of MPC increased. The electrophoretic mobility of PSGL-1 was completely reduced due to the large hydrodynamic size when the concentration of MPC exceeded 500 mM. In addition to the polymerization, similar results were observed via a thiol−ene click reaction. Conjugations of PSGL-1 of the HL-60 cells with thiol-terminated poly(ethylene glycol) (PEG)[19] and poly(*N*-isopropylacrylamide) (PNIPAM)[32] were also verified using Western blot analysis. These conjugations provided alternative functions such as selective adhesion and thermoresponsive association to the HL-60 cells. Both free radical polymerization and the thiol−ene click reaction are robust reaction processes in the preparation of polymeric materials. These reactions enable the chemical modification of glycoproteins.

The addition of *N*,*N*′-methylenebis(acrylamide) (MBA) into the polymerization reaction resulted in the formation of hydrogels containing glycoproteins. In the preparation of the hydrogels, the final concentrations of MPC and proteins were adjusted to 2.5 M and 1000 μg/mL, respectively. The MPC concentration for making hydrogels was optimized in reference to prior literature.[33] Figure 4A,B shows photographs of MPC hydrogels after staining with Coomassie brilliant blue (CBB) and periodic acid−Schiff (PAS) reagents, respectively. The

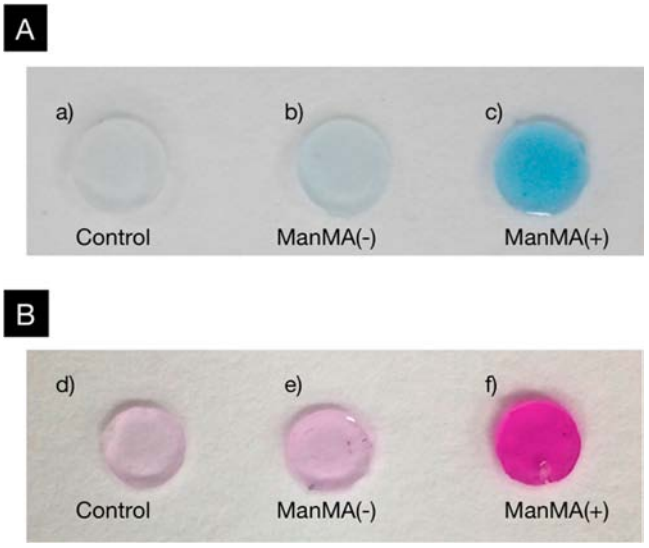

Figure 4. Photographs of hydrogels stained with (A) Coomassie brilliant blue (CBB) and (B) periodic acid−Schiff stains.

CBB and PAS staining methods are commonly used for staining proteins and carbohydrates, respectively. Before staining, the hydrogels were rinsed with RIPA lysis buffer and phosphate buffered saline (PBS) to remove any bound glycoproteins. The hydrogels prepared without cell lysate (Figure 4A-a,B-d) or with untreated HL-60 cell lysate (Figure 4A-b,B-e) did not exhibit staining after treatment with CBB and PAS reagents. In contrast, the dyes remained in the hydrogels prepared with ManMA-treated HL-60 cell lysate (Figure 4A-c,B-f). These results indicated that glycoproteins were covalently immobilized only in the hydrogels prepared with the lysate of the ManMA-treated HL-60 cells. Furthermore, no aggregation or precipitation of glycoproteins was observed in the hydrogels. The covalent conjugation to very hydrophilic polymer chains may help reduce the aggregation of glycoproteins.

The density of selectin-binding glycoproteins on the surface of the hydrogels was determined using a gold-colloid-labeled immunoassay.[34] The quantitative accuracy of this method was confirmed by comparing the number of gold colloid particles with the amount of target protein determined by radioimmuno-

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assay.[35] To minimize the nonspecific physical adsorption of antibodies into the hydrogel, a thin hydrogel film was prepared on a silicon wafer using a spin coater. After enhancement of the gold particle immobilized on a secondary antibody binding to a primary antibody of PSGL-1 with silver, elemental analysis of the hydrogel surface was performed with an X-ray photoelectron spectroscope (XPS; ESCA-3400, Shimadzu Co., Kyoto, Japan). The silver composition (Ag:Si atomic ratio determined by XPS) on the hydrogels prepared with lysate of ManMA-treated and untreated HL-60 cells is shown in Figure 5. The composition of the hydrogels prepared with lysate was

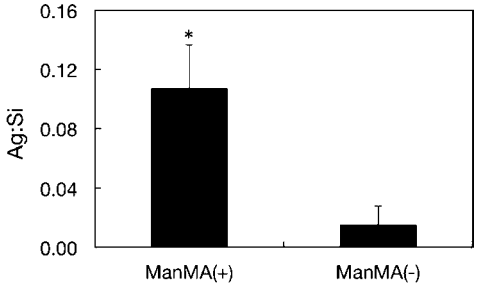

Figure 5. Surface composition of PLGL-1 assessed by XPS silver composition. *P < 0.01 vs ManMA(−).

normalized to that of the hydrogel prepared without lysates. The hydrogels prepared with the ManMA-treated HL-60 cells had a significantly higher silver composition than those prepared with untreated HL-60 cells.

Finally, the IL-1α stimulated and nonstimulated HUVECs were placed in contact with hydrogels to investigate selectin-mediated cell adhesion. The density of the adherent cells on the hydrogels is summarized in Figure 6. Of the MPC hydrogels prepared without lysate and those prepared with lysate of untreated HL-60 cells (without ManMA-treatment), very few IL-1α stimulated HUVECs were observed. In contrast, a large number of IL-1α stimulated HUVECs adhered to the hydrogels prepared with the lysate of the ManMA-treated HL-60 cells. In addition, the nonstimulated HUVECs did not adhere to the hydrogels prepared with the lysate of the ManMA-treated HL-60 cells. This result coincides well with the cell adhesion results (Figure 2) and suggests that glycoproteins of mammalian cells could be transferred to synthetic hydrogels while preserving the binding ability to the selectin expressed on the surface of the HUVECs.

In this study, we successfully prepared a hydrogel containing natural glycoproteins of leukemia cells and demonstrated the selectin-mediated adhesion of cytokine-stimulated endothelial cells on the hydrogel. In vivo, selectin-mediated cell adhesion is considered an essential step leading to inflammation, reperfusion injury, rheumatoid arthritis, metastasis, infection, and so forth.[36,37] Thus, novel synthetic materials that regulate these therapeutic targets would be beneficial. Metabolic oligosaccharide engineering with ManMA is a robust technique for creating biointeractive synthetic materials, and we have reported the first use of glycoproteins as monomers for conventional radical polymerization, which is the most practical polymerization method. Moreover, metabolic oligosaccharide engineering can be applied to a wide variety of cells. The relative dimensions, concentrations, and chemistries of all membrane components can vary depending on cell type and disease.[1] The polymeric materials supplied thorough the metabolic oligosaccharide engineering can uniquely interact with the specific biosubstrate and would be useful tools for biological, therapeutic, and diagnostic areas.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yasu.bmt@kansai-u.ac.jp.

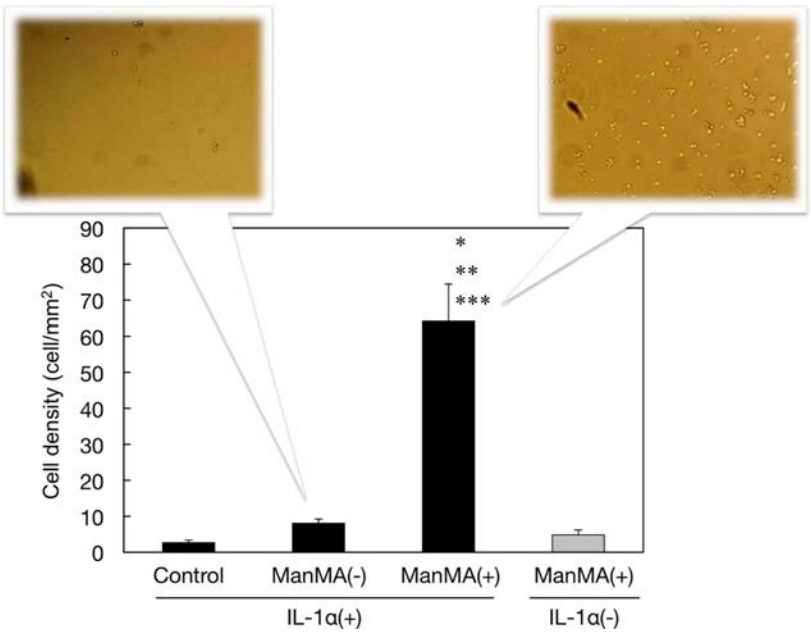

Figure 6. Density of adherent HUVEC cells on hydrogels. *P < 0.01 vs control/IL-1α(+); **P < 0.01 vs ManMA(−)/IL-1α(+); ***P < 0.01 vs ManMA(+)/IL-1α(−).

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Notes

The authors declare no competing financial interest.

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