CHEMISTRY
AN ASIAN JOURNAL

DOI: 10.1002/asia.201100319

Fluorophore-labeled, Peptide-based Glycoclusters: Synthesis, Binding Properties for Lectins, and Detection of Carbohydrate-Binding Proteins in Cells

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Dedicated to Professor Eun Lee on the occasion of his retirement and 65th birthday

Abstract: A facile and efficient solid-phase synthesis of linear peptide-based glycoclusters with various valences and different spatial arrangements of the sugar ligands is described. The synthetic strategy includes 1) solid-phase synthesis of fluorophore-labeled, alkyne-containing peptides, 2) coupling of azide-linked, unprotected mono-, di-, and trisaccharides to the alkyne-conjugated peptides on a solid support by click chemistry, and 3) release of the fluorophore-labeled glycoclusters from the solid support. By using this methodology, 32 fluorescent glycoclusters with a valence ranging from 1 to 4 and different spatial arrangements of the sugar ligands were prepared. Lectin-binding properties of the glycoclusters were initially examined by using microarrays immobilized by various lectins. These glycoclusters were then employed to detect the cell-surface carbohydrate-binding proteins in bacteria. Finally, the uptake of glycoclusters by mammalian cells through receptor-mediated endocytosis was evaluated. The results, obtained from the in vitro and in vivo studies, indicate that the binding affinities toward immobilized and cell-surface proteins are highly dependent on the valence and spatial arrangements of the sugar ligands in glycoclusters.

Keywords: carbohydrates · click chemistry · cluster compounds · glycoproteins · oligosaccharides

Introduction

Glycans, which are found mainly in the form of glycoconjugates such as glycoproteins, proteoglycans, and glycolipids inside or on the surface of cells, participate in a wide range of physiological and pathological processes through interactions with proteins.[1,2] For example, glycan–protein interactions play a pivotal role in cell adhesion, signaling, and trafficking. Intriguingly, glycan-mediated biomolecular interactions are also implicated in the development of various diseases. Bacteria, viruses, and parasites infect hosts by initial adhesion to host cells through interactions of the pathogenic proteins with the host cell-surface glycans.[3] In addition, tumor metastasis and leukocyte-mediated inflammation also take place through these biomolecular interactions.[4] Therefore, the understanding of the molecular basis of glycan–protein recognition events is of great importance to elucidate the complex biological processes involving glycans.

It is generally accepted that multivalent interactions between glycans and proteins enhance an otherwise weak binding affinity of monomeric sugars with receptors.[5] Enhancements in binding affinity by multivalent interactions (often referred to as the cluster glycoside effect) are attributed to the chelate effect, clustering of carbohydrate-binding proteins, or statistical rebinding.[5b,c] To achieve a binding enhancement through the multivalent glycan–protein interactions, a variety of synthetic glycoclusters with diverse spatial arrangements and different numbers of glycan ligands have been prepared. These glycoclusters include linear and cyclic neoglycopeptides, glyconanoparticles, glyconanotubes, glycodendrimers, glycolipid micelles, glycoproteins, and glycopolymers.[5,6] In these glycoclusters, the nature of the spacer between the glycan moieties affects the binding enhancement achievable by proper complementarities between the clustered glycans and the oligomeric receptors. However, structural information on multivalent glycan–protein interactions with atomic resolution is not sufficient for the rational

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201100319.

design of glycoclusters. Thus, a series of glycoconjugates with various spacer lengths and a different valence of glycans are prepared for investigating multivalent interactions with receptors or detecting proteins in cells.

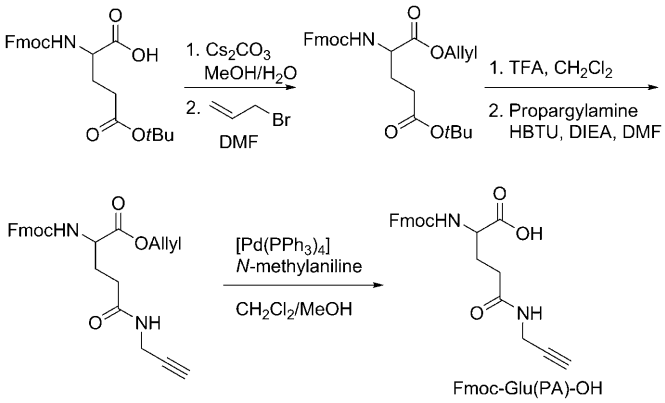
To readily obtain glycoclusters with diverse spatial separation and various valences of sugars, we have developed an efficient solid-phase synthetic strategy for fluorophore-labeled, peptide-based glycoclusters, which can be utilized to fluorescently detect proteins in vitro and in vivo. By using this strategy, 32 fluorescent glycoclusters with a valence (the number of glycans attached to glycoconjugates) ranging from 1 to 4 and different spatial arrangements of the glycans were obtained. Microarray and fluorescence microscopy analyses using these glycoclusters indicate that the valence and spatial arrangements of sugar epitopes exert influence on the binding affinities for immobilized and cell-surface proteins.

Results and Discussion

Synthesis of Fluorophore-labeled Glycoclusters

Our synthetic procedure includes 1) assembly of fluorophore-labeled, alkyne-conjugated peptides on a solid support, 2) coupling of azide-linked, unprotected sugars to the alkyne-conjugated peptides on a solid support through click chemistry, and 3) release of the glycoclusters from the solid support. A linear peptide was used as a scaffold of glycoclusters because of its facile assembly on the solid support and the easy control of the spacer length between sugar ligands. Azide-containing sugars were attached to alkyne-linked peptides through click chemistry, as this reaction is highly compatible with a broad range of functional groups in various solvent systems, and the formed triazole functionality is stable under hydrolysis and redox conditions.[7]

Synthesis of peptides with a different number of alkyne groups and various spacer lengths was accomplished by a standard solid-phase peptide synthesis method using propargylamine-containing glutamic acid (Fmoc-Glu(PA)-OH) prepared according to Scheme 1. To control the spacer length, glycine, 4-aminobutanoic acid or 6-aminohexanoic acid was inserted into the alkyne-containing residues (see Scheme 1 in the Supporting Information). After assembly of peptides on the solid support (PS-PEG Rink amide linker resin) under HBTU-HOBt-DIEA conditions (HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIEA = diisopropylethylamine), a fluorophore of cyanine 3 (Cy3) was coupled to the peptides to fluorescently detect proteins in vitro and in vivo. Fluorophore-containing peptides attached to the solid support were then reacted with azidoethylated mono-, di-, and trisaccharides under click chemistry conditions ($CuSO_4$ and sodium ascorbate). Azidoethylated α-Man (α-mannose), α-Fuc (α-fucose), β-GlcNAc (β-*N*-acetylglucosamine), and β-Lac (β-lactose) were prepared as previously reported,[8] and azidoethylated NeuNAcα2,6LacNAc (*N*-acetylneuraminosyl-α-2,6-*N*-acetyllactosamine) was synthesized by enzymatic glycosylation of azidoethyl β-LacNAc by α-2,6-sialyltransferase in the presence of CMP-NeuNAc (cytidine monophosphate neuraminic acid). After click chemistry, the assembled fluorescent glycoclusters were released from the solid support by trifluoroacetic acid (TFA) and purified by reversed-phase HPLC. For lectin-binding studies using protein microarrays and cell experiments, a series of α-Man (**1**–**10**) and β-Lac (**11**–**20**) clusters with a valence ranging from 1 to 4 and different spatial arrangements of the sugar ligands were prepared (Figure 1). In the case of glycoclusters containing α-Fuc (**21**–**24**), β-GlcNAc (**25**–**28**), and NeuNAcα2,6LacNAc (**29**–**32**), mono- and tetravalent glycoclusters with different spacer lengths



Scheme 1. Synthesis of Fmoc-Glu(PA)-OH. Abbreviation: HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIEA = diisopropylethylamine.

Abstract in Korean:

형광물질이 결합된 펩타이드 기반의 당복합체: 합성, 렉틴에 대한 결합특이성, 세포에 있는 당결합 단백질 검출

당의 개수와 당 사이의 간격이 조절된 선형 펩타이드에 기반을 둔 당복합체를 빠르고 간편하게 고체상에서 합성할 수 있는 방법을 서술한다. 개발된 합성 전략은 (1) 형광물질이 결합된 알카인기를 포함한 펩타이드를 고체상에서 합성하는 방법, (2) 아지드가 결합된 단당류, 이당류, 삼당류를 알카인기를 포함한 펩타이드에 클릭 합성법을 이용하여 고체상에서 합성하는 방법, (3) 형광물질이 결합된 당복합체를 고체상에서 해리하는 방법을 포함한다. 이 방법을 이용하여 당 개수를 한 개에서부터 네 개까지 포함하고 당 사이의 간격이 다른, 형광물질이 결합된 당복합체를 32종 합성하였다. 먼저 다양한 렉틴이 고정된 마이크로어레이를 이용하여 당복합체의 렉틴 결합 특이성에 대해 조사하였다. 다음으로, 이들 당복합체를 이용하여 세포표면에 당결합 단백질을 가진 대장균을 검출하였다. 마지막으로 인간 세포가 수용체를 통해 당복합체를 흡수하는 것에 대해 조사하였다. 이상의 세포 내, 세포 외 실험을 통해 당복합체가 고체기질에 고정된 단백질이나 세포표면의 단백질과 결합할 때 당의 개수와 당 사이의 간격에 따라 결합하는 정도가 다르다는 것을 보여주었다.

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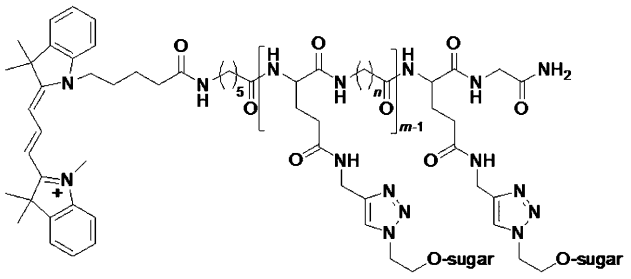

No	Symbol	No	Symbol	No	Symbol
1	Man-10	**12**	Lac-21	**23**	Fuc-43
2	Man-21	**13**	Lac-23	**24**	Fuc-45
3	Man-23	**14**	Lac-25	**25**	GlcNAc-10
4	Man-25	**15**	Lac-31	**26**	GlcNAc-41
5	Man-31	**16**	Lac-33	**27**	GlcNAc-43
6	Man-33	**17**	Lac-35	**28**	GlcNAc-45
7	Man-35	**18**	Lac-41	**29**	NeuNAcα2,6-LacNAc-10
8	Man-41	**19**	Lac-43	**30**	NeuNAcα2,6-LacNAc-41
9	Man-43	**20**	Lac-45	**31**	NeuNAcα2,6-LacNAc-43
10	Man-45	**21**	Fuc-10	**32**	NeuNAcα2,6-LacNAc-45
11	Lac-10	**22**	Fuc-41		

Figure 1. Structure of synthesized fluorophore-labeled neoglycopeptides: Sugar-*mn* (*m* = the number of a sugar ligand; *n* = the number of carbon between NH and CO groups of the tether).

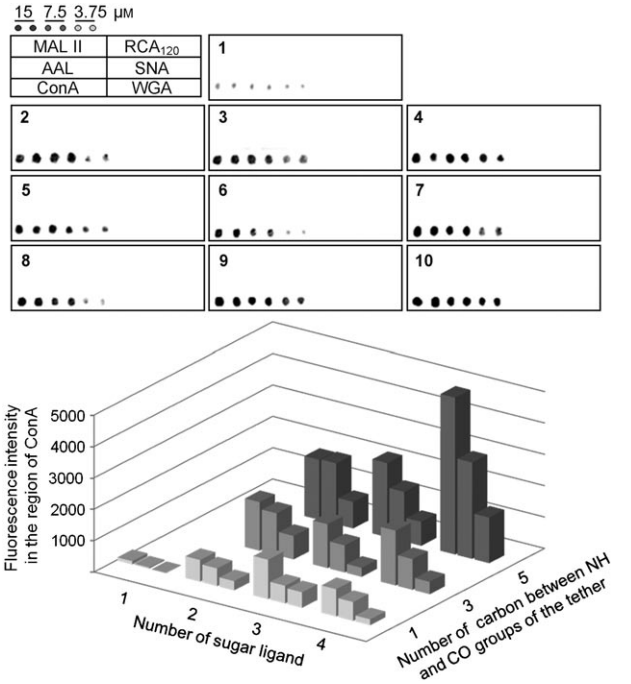

Figure 2. Fluorescence images of protein microarrays after probing with 20 μM of mannose-containing clusters (**1**–**10**). Graph: quantitative analysis of fluorescence intensity of protein microarrays.

were synthesized for lectin-binding experiments with protein microarrays.

Studies on Lectin-binding Properties of Glycoclusters Using Protein Microarrays

Carbohydrate and lectin microarrays have recently received considerable attention as powerful tools for the rapid analysis of glycan–protein interactions.[9] Binding properties of the fluorophore-labeled glycoclusters towards lectins were initially examined by using microarrays immobilized by various carbohydrate-binding proteins. Lectin microarrays used for this study were fabricated by printing six lectins (3.75, 7.5, and 15 μM) on *N*-hydroxysuccinimide (NHS) ester-derivatized glass slides, prepared according to the known procedure,[9g] with a pin-type microarrayer. The lectins include *Maackia amurensis lectin II* (MAL II, NeuNAcα2,3Gal binding protein), *Ricinus communis agglutinin I* (RCA_{120}, Gal binding protein), *Aleuria aurantia lectin* (AAL, Fuc binding protein), *Sambucus nigra lectin* (SNA, NeuNAcα2,6-Gal binding protein), *Concanavalin A* (ConA, α-Man/α-Glc binding protein), and *Wheat germ agglutinin* (WGA, GlcNAc binding protein).[10] After incubation of the printed slides for 5 hours at room temperature in a humid chamber, the slides were blocked with bovine serum albumin (BSA) to suppress nonspecific interactions. The resulting slides were treated with fluorescent glycoclusters **1**–**32** for 1 hour at room temperature to evaluate their lectin-binding properties.

The results of microarray experiments using mannose-containing glycoclusters **1**–**10** (20 μM) showed that these probes exclusively recognized ConA, which is consistent with the established specificity of this lectin (Figure 2).[9d,10] As expected, monovalent probe **1** rarely bound to ConA owing to very weak interactions between a monovalent mannose and the protein. It was also revealed that as the tether lengthened, fluorescence intensities of mannose clusters associated with ConA increased gradually. The best mannose ligand among the tested probes for ConA was found to be tetravalent probe **10** with 5 carbons between the NH and CO groups of the tether. However, tetravalent probes **8** and **9**, with 1 and 3 carbons between the NH and CO groups, interacted with ConA more weakly than di- (**4**) and trivalent probes (**7**) with a longer spacer length, thereby suggesting that the linker length of mannose clusters has a significant effect on the ConA binding affinity.[6b] The microarrays treated with lactose clusters (**11**–**20**, 40 μM) indicated that these probes solely recognized RCA_{120} and the probes with short spacer lengths (**12**, **15**, **18**) interacted with the protein less tightly than those with longer spacers (Figure 3). In these microarray experiments, tri- and tetravalent lactose probes, with 3 and 5 carbons between the NH and CO groups of the tether, bound to RCA_{120} with similar binding affinities.

We then analyzed the fluorescence intensities of Fuc (**21**–**24**), GlcNAc (**25**–**28**), and NeuNAcα2,6LacNAc (**29**–**32**) clusters bound to proteins after incubation of the microarrays with 20 μM glycoclusters. Fluorescent signals from Fuc and GlcNAc clusters were observed for AAL and WGA attached to the surface, respectively (Figure 4). The protein microarrays incubated with NeuNAcα2,6-LacNAc clusters

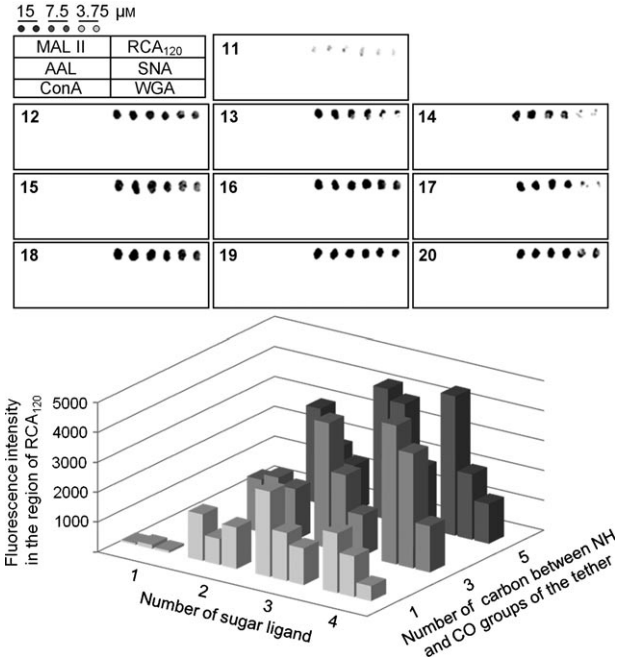

Figure 3. Fluorescence images of protein microarrays after probing with 40 μM of lactose-containing clusters (**11**–**20**). Graph: quantitative analysis of fluorescence intensity of protein microarrays.

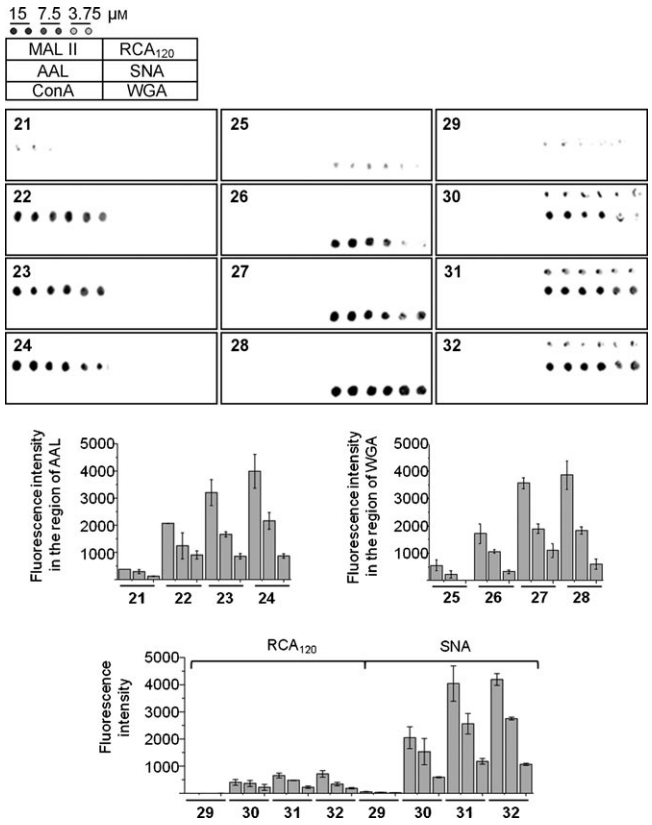

Figure 4. Fluorescence images of protein microarrays after probing with 20 μM of glycoclusters **21**–**32**. Graph: quantitative analysis of fluorescence intensity of protein microarrays.

showed fluorescent signals in the regions of SNA and RCA_{120}. In this case, these glycoclusters interacted with RCA_{120} with much weaker binding affinities in comparison with SNA, which is consistent with the previous finding.[11] Analysis of fluorescence intensities of the microarrays also indicated that monovalent glycan probes (**21**, **25**, **29**) very weakly bound to the lectins under incubation conditions and tetravalent probes with longer spacers recognized the corresponding lectins more tightly than those with short ones. Overall, the results obtained from protein microarrays show that the binding affinities of glycoclusters to proteins are highly dependent on the valence and spatial arrangements of sugars.

Detection of Cell-surface Proteins in Bacteria

It has been known that many bacteria express carbohydrate-binding proteins on the cell surface.[12] Pathogenic properties of the bacteria are caused by the initial binding to host cells through the recognition events of bacterial proteins and host glycans. To investigate the binding properties of glycoclusters toward cell-surface lectins in bacteria, 25 μM of multivalent mannose clusters **2**–**10** were incubated for 1 hour with *E. coli* ORN178 strain, which produces a mannose-binding protein, a gene product of *fimH*, on pili.[13] After washing to remove unbound mannose clusters, bacterial cells were imaged by using confocal fluorescence microscopy. It was found that a valence of a sugar ligand in glycoclusters had influence on the binding to the cells. Tri- and tetravalent glycoclusters (**5**–**10**) recognized the cells more tightly than divalent ones (**2**–**4**) (Figure 5a and Figure 1a in the Supporting Information). Trivalent mannose probes (**5**–**7**) showed a slightly lower binding affinity with the cells than those with tetravalent probes (**8**–**10**). We also found that glycoclusters **2**, **5**, and **8**, with short spacer lengths, weakly interacted with proteins in bacteria, in comparison with those with a longer tether. These findings indicate the importance of the valence of a sugar ligand and the nature of the tether in glycoclusters for the strong binding to bacterial cells. Finally, the specific binding of mannose clusters to bacterial proteins was investigated by competition experiments. Binding of tetravalent mannose probes (**8**, **9**, and **10**, 25 μM) to bacterial proteins was inhibited by pre-incubation of the bacteria with 20 mM mannose for 1 hour (IC_{50} of free mannose to inhibit **10** binding to mannose-binding proteins on *E. coli* ORN178 was measured to be 400 μM, see Figure 1b, in the Supporting Information), thereby demonstrating that the mannose probes selectively interact with mannose-binding proteins on the bacterial cell surface (Figure 5b).

Evaluation of Uptake of Lactose-containing Clusters by Mammalian Cells

Hepatocytes abundantly express an asialoglycoprotein receptor (ASGP-R) on the cell surface, which binds to terminal galactose or *N*-acetylgalactosamine on glycoproteins through multivalent interactions.[14] This binding event trig-

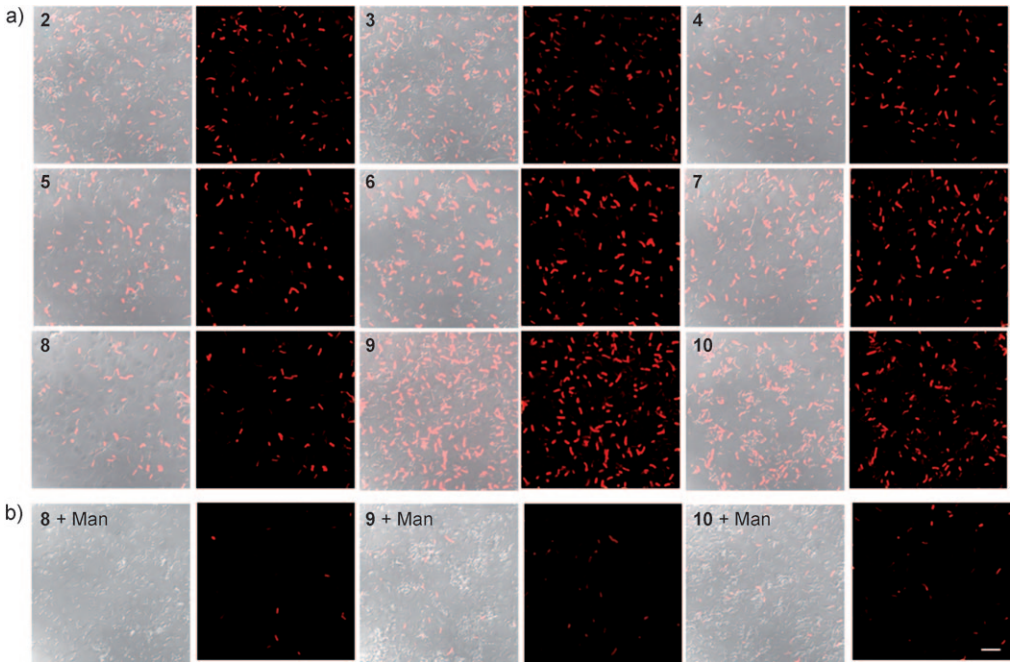

Figure 5. a) Confocal fluorescence microscopy images of *E. coli* ORN178 incubated with 25 μM mannose-containing clusters **2**–**10** for 1 h. b) Confocal fluorescence microscopy images of *E. coli* ORN178 pre-treated with 20 mM mannose for 1 h followed by further incubation with 25 μM mannose-containing clusters **8**–**10** for 1 h (bar = 10 μm).

gers the receptor-mediated endocytotic uptake of the glycoproteins into cells, which are then transported to the lysosome where they are degraded. Thus, ASGP-R is involved in the clearance of Gal/GalNAc-terminated glycoproteins from circulation into hepatocytes.

To analyze the uptake of lactose-containing probes by cells through receptor-mediated endocytosis, we used the hepatocellular carcinoma cell line HepG2 that produces abundant ASGP-R but not the mannose receptor.[14] The HepG2 cells were incubated with 20 μM of multivalent lactose probes **12**–**20** for 3 hours at 37 °C. After washing to remove the unbound glycoclusters, fluorescence intensity of cells was measured by using an ArrayScan Reader (Cellomics). Quantitative analysis of fluorescence intensity of the treated cells revealed that the number of lactose ligands in the glycoclusters was important for uptake by HepG2 cells (Figure 6). However, lactose ligands having the same valence with different lengths of the tether were internalized into cells to a similar degree, suggesting that the nature of the tether plays a very small role in the uptake of lactose-containing probes by cells.

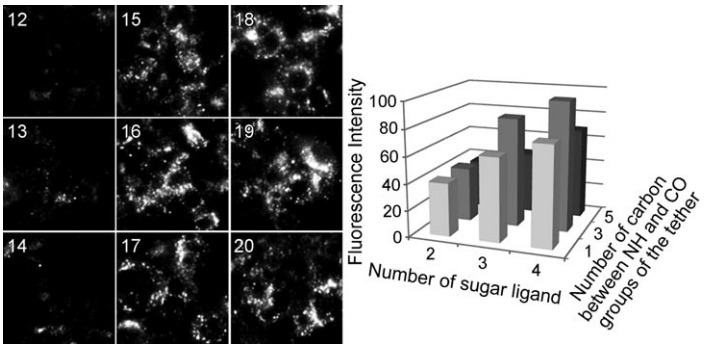

Figure 6. Fluorescence microscopy images of HepG2 cells incubated with 20 μM lactose-containing clusters **12**–**20** for 3 h. Graph: quantitative analysis of fluorescence intensity of the treated HepG2 cells (bar = 50 μm).

To further examine whether lactose-containing glycoclusters enter cells through ASGP-R-mediated endocytosis, HepG2 cells were pre-treated with galactose (2 or 20 mM) or lactose (2 or 20 mM) as a competitor for 1 hour at 37 °C, and then incubated with **20** (20 μM) for an additional 3 hours at 37 °C. The uptake of **20** by cells was inhibited by pre-incubation with galactose and lactose in a competitor concentration-dependent manner (Figure 7a and b), suggesting that galactose, lactose, and **20** share a common cellular receptor, ASGP-R. The results also showed that lactose was a more effective competitor than galactose, which is consistent with the previous finding that galactose has a lower binding affinity for ASGP-R than lactose.[15]

To ascertain the uptake of lactose probes by cells through receptor-mediated endocytosis, HepG2 cells were incubated with **20** (20 μM) for 4 hours at 4 °C, as the receptor-mediated endocytosis is suppressed at low temperature.[16] A remarkably reduced fluorescence intensity was observed, thus indicating that the uptake of **20** by cells was inhibited by low temperature incubation (Figure 7c). These results, together with competition results, suggest that lactose-containing glycoclusters are taken up by the HepG2 cells through ASGP-R-mediated endocytosis.

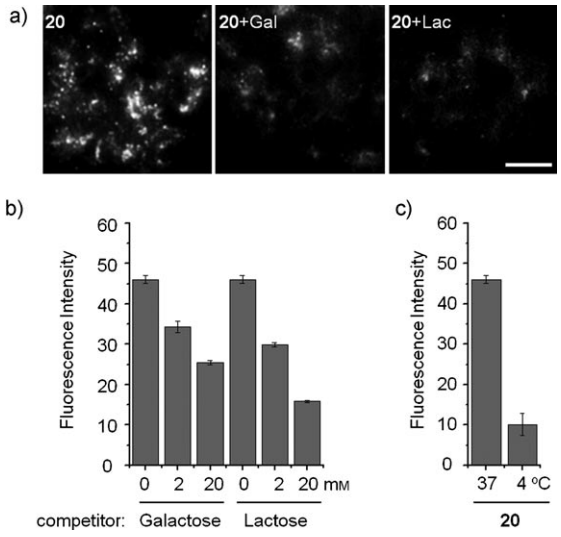

Figure 7. a) Fluorescence microscopy images of HepG2 cells incubated with 20 mM galactose or 20 mM lactose for 1 h at 37 °C, and then incubated with 20 μM **20** for additional 3 h at 37 °C (bar = 50 μm). b) Quantitative analysis of fluorescence intensity of HepG2 cells after incubation with a competitor and **20**. c) Quantitative analysis of fluorescence intensity of HepG2 cells incubated with **20** at 37 °C or 4 °C for 4 h.

Conclusions

We have developed an efficient method for the preparation of fluorophore-labeled glycoclusters with various valences and different spatial arrangements of the sugars. The synthetic fluorescent glycoclusters were employed for probing lectin-binding properties using protein microarrays, detection of proteins on the bacterial cell surface, and evaluation of uptake of glycoclusters by mammalian cells through receptor-mediated endocytosis. The results obtained from in vitro and in vivo experiments indicate that the binding affinities of free proteins and cell-surface proteins are highly dependent on the valence and spatial arrangements of sugar ligands in glycoclusters. We believe that this synthetic strategy can be expanded in the future for preparation of more diverse glycoclusters for basic biological research to understand glycan–protein interactions as well as for development of effective inhibitors to block these biomolecular interactions.

Experimental Section

Synthesis of Fluorophore-labeled Neoglycopeptides

Fluorophore-labeled neoglycopeptides were synthesized by Fmoc/*t*Bu strategy on PS-PEG Rink amide linker resin (0.25 mmol g^{-1}): Fmoc amino acid (3 equiv) was manually coupled on the resin (5.0 μmol) in the presence of HBTU (3 equiv), HOBt (3 equiv), and DIEA (6 equiv). Fmoc group was removed by treatment with 20% piperidine in *N*,*N*-dimethylformamide (DMF) and the resin was washed with DMF and CH_2Cl_2 several times. For coupling of Cy3 to the peptides, Cy3 (3.0 equiv) was preactivated with HOAt (3.0 equiv), HATU (2.9 equiv), and DIEA (6.0 equiv) in DMF (400 μL) for 10 min. The activated Cy3 was then added to the N-terminal amino-containing resin and shaken for 6 h. For coupling of sugars to the fluorophore-conjugated peptides by click chemistry, azide-linked mono-, di-, and trisaccharides (2.0 equiv per triple bond) dissolved in DMF (300 μL) were added to the fluorophore-labeled peptide resin and then sodium ascorbate (1.0 equiv per triple bond) and $CuSO_4$ (1.0 equiv per triple bond) in water (50 μL) was added to the reaction mixture. After 24 h, fluorophore-labeled glycopeptides were cleaved from the solid support by treatment with TFA-triethylsilane (TES; 98:2) for 2 h. The fluorescent neoglycopeptides were analyzed by analytical RP-HPLC with a gradient of 5–100% CH_3CN (0.1% TFA) in water (0.1% TFA) over 45 min and the purified products were characterized by MALDI-TOF MS (Table 1, see the Supporting Information).

Lectin-binding Properties of Glycoclusters using Protein Microarrays

Proteins (ConA, WGA, AAL, SNA, MAL II, RCA_{120}) used for this study were dissolved in sodium phosphate buffer (pH 8.2) containing 40% glycerol. Solutions of proteins (1 nL, 15 μM) from a 384-well plate were printed in a predetermined place on a NHS-derivatized glass slide prepared according to the procedure reported previously[9g] (a distance of 240 μm between the centers of adjacent spots) by using a pin-type microarrayer (Cartesian MicroSys™ 5100 PA). After completion of printing, the slide was placed into a humid chamber (60%) at room temperature for 5 h. Then, a compartmentalized plastic film, which is coated by adhesive on one side (thickness: 0.2 mm), was attached to the glass slide. The slide was inversely immersed into PBS (pH 7.4) containing 0.1% Tween 20 to prevent spot spreading and washed with gentle shaking by hand. The solution (15–20 μL) of PBS (pH 7.4) containing 0.1% Tween 20 and 1% BSA was dropped onto each block compartmented by a plastic film and then incubated for 0.5–1 h. The solution was removed by washing the slide with PBS containing 0.1% Tween 20 (30 mL, 3× 10 min). The fabricated protein microarrays were used immediately to get reproducible results.

Solutions (15–20 μL) of fluorophore-labeled neoglycopeptides (20–40 μM) in PBS (pH 7.4) containing 0.1% Tween 20 (in the case of ConA, 0.5 mM $MnCl_2$ and 0.5 mM $CaCl_2$ were added to the solution) were dropped into each block compartment of the plastic film and then incubated for 1 h. The unbound glycoclusters were removed by washing the slide with PBS containing 0.1% Tween 20 (30 mL, 3×10 min). The slide was scanned using an ArrayWoRx scanner.

Detection of Bacterial Cells

E. coli ORN178 cells were grown overnight at 37 °C in LB medium in order to attain an optical density of approximately 1.0 (~10^8 cells mL^{-1}) at 600 nm (OD_{600}). The cell culture was centrifuged at 13000 rpm for 30 sec, washed with PBS buffer and spun down twice, and finally suspended in PBS buffer. *E. coli* ORN178 cells were incubated with 25 μM mannose-containing clusters (**1**–**10**) in PBS for 1 h at room temperature with gentle shaking and then centrifuged to collect the cell pellet. The supernatant was discarded and the pellet was resuspended in the same buffer. The process was repeated three times to remove unbound glycoclusters. The *E. coli* cells were imaged by using confocal fluorescence microscopy (LSM510 META, Carl Zeiss, Berlin, Germany). Fluorescence intensity was quantitatively measured by using NIS-Elements D 3.2 software (Nikon, Japan).

For determination of IC_{50}, *E. coli* ORN178 strain was incubated with various concentrations (0, 0.1, 0.3, 0.5, 0.7, 1, 5, 10 mM) of free mannose in PBS for 1 h at 37 °C and then treated with 25 μM **10** in PBS for 1 h. After washing twice with PBS, fluorescence intensity was quantitatively measured by using a Typhoon 9410 scanner (GE, Germany) and ImageQuant software.

Evaluation of Uptake of Lactose-containing Clusters by Mammalian Cells

The hepatocellular carcinoma cell line HepG2 that expresses ASGP-R was seeded in a 24-well plate at a density of ~10^5 cells per well and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). After 24 h, the cells were incubated with 20 μM lactose-containing glycoclusters (**12**–**20**) in culture media for 3 h at 37 °C. After washing twice with Dulbecco's Phosphate Buffered Saline (DPBS, without calcium and magnesium) to remove unbound glycoclusters, the cells were fixed with 3.7% formaldehyde containing 4,6-diamidino-2-phenyllindole (DAPI,

1 μg mL^{-1}) as the internal control for the ArrayScan VTI HCS Reader. After washing twice with DPBS, the cells were imaged by fluorescence microscopy (Eclipse TE2000-S, Nikon, Japan) or ArrayScan VTI HCS Reader with a 20X objective (Cellomics, USA). Fluorescence microscopy: the excitation wavelength range was from 510 to 560 nm for Cy3 and from 330 to 380 nm for DAPI. ArrayScan VTI HCS Reader: Target Activation BioApplication was used to acquire and analyze the images. Images of 1000 cells for each probe were analyzed to obtain the average cell number per field, fluorescence area and intensity per cell.

For competition experiments, HepG2 cells were seeded in a 24-well plate at a density of ~10^5 cells per well and cultured in DMEM supplemented with 10% FBS. After 24 h, the cells were incubated with galactose (2 or 20 mM) or lactose (2 or 20 mM) for 1 h at 37 °C. The cells were then treated with **20** (20 μM) in culture media and incubated for 3 h at 37 °C. After washing twice with DPBS, the cells were fixed with 3.7% formaldehyde containing 1 μg mL^{-1} DAPI. After washing twice with DPBS, the cells were imaged and evaluated by fluorescence microscopy or ArrayScan VTI HCS Reader with a 20X objective.

For confirmation of the receptor-mediated uptake mechanism, HepG2 cells were incubated with **20** (20 μM) for 4 h at 4 °C to block receptor-mediated endocytosis. After washing twice with DPBS, the cells were fixed with 3.7% formaldehyde containing 1 μg mL^{-1} DAPI. After washing twice with DPBS, the cells were imaged and evaluated by ArrayScan VTI HCS Reader with a 20X objective.

Acknowledgements

This work was supported by grants from the National Creative Research Initiative and WCU programs (R32-2008-000-10217-0). *Escherichia coli* ORN 178 and ORN 208 strains were kindly provided by Professor P. E. Orndorff (North Carolina State Univ.).

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Received: March 31, 2011
Published online: June 1, 2011

