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出版情報：Chemical Communications : chem comm. 57 (83), pp.10871-10874, 2021-09-15. Royal Society of Chemistry
バージョン：
権利関係：

COMMUNICATION

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The "carbohydrate module method" is a promising approach for oligosaccharide mimetics using polymeric materials. However, it is difficult to predict the optimal structure for a particular oligosaccharide mimetic, and an efficient strategy for the synthesis and evaluation of glycopolymers is desirable. In this study, a screening of glycopolymers for the "carbohydrate module method" by a combination of photoinduced electron/energy transfer-reversible addition-fragmentation chain-transfer (PET-RAFT) polymerization and surface plasmon resonance imaging (SPRI) is demonstrated. The facile and fast screening of synthetic glycomimetics was achieved, and the glycopolymer with the optimal structure as a GM1 mimetic strongly interacted with the cholera toxin B subunit.

Carbohydrates are present on cell surface and are involved in various biological phenomena, such as pathogen infections and cancer metastasis.[1] These biological phenomena result from carbohydrate–protein interactions.[2] Carbohydrates that are presented densely on the cell surface have the effect of amplifying the interaction with a corresponding protein (lectin), and this is called the cluster glycoside effect.[3,4] In particular, oligosaccharides are a crucial class of carbohydrates that play an important role in the body. For example, GM1 ganglioside (one of the major monosialic glycosphingolipid, see Figure S1) is related to neurodegenerative diseases, cell differentiation, and pathogen infection.[5,6] Although the important functions of oligosaccharides are useful for various biological applications, difficulties in the synthesis and extraction of oligosaccharides makes them expensive to produce. Thus, there is a demand for alternative materials that can be obtained in large quantities and at low cost.

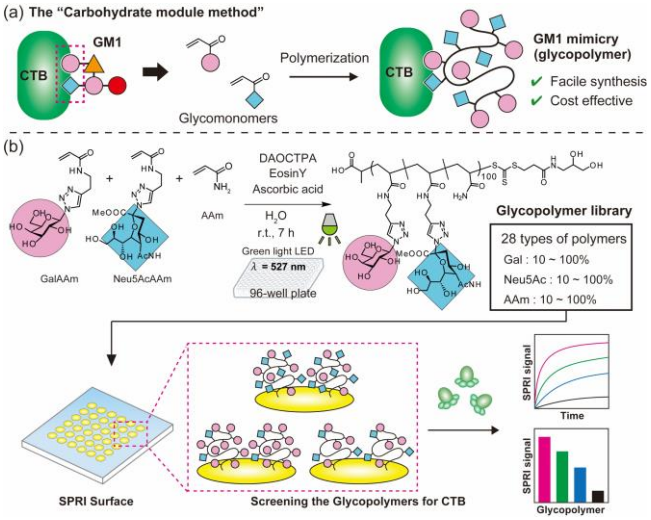

Figure 1. Schematic illustrations of the "carbohydrate module method" (a). Preparation of the glycopolymer library by PET-RAFT polymerization and screening of the library by SPRI (b).

The "carbohydrate module method" is a promising approach for mimicking oligosaccharides using polymeric materials.[7,8] In this method, each monosaccharide contained in an oligosaccharide is regarded as a "carbohydrate module". These monosaccharides are introduced into the side chains of synthetic polymers, and the resultant glycopolymers are expected to mimic the function of oligosccharides.[9,10] Synthetic glycopolymers can be easily prepared either by one-step polymerization of glycomonomers or by introduction of glycounits into the polymer backbones.[11] Previously, our group has demonstrated that glycopolymers modified with galactose and neuraminic acid, which are terminal residues of GM1 ganglioside, interacted with the cholera toxin B subunit (CTB), and that the function of GM1 could be reproduced using the "carbohydrate module method".[12] However, it is difficult to predict the appropriate structures for glycopolymers that will be effective mimics of the natural oligosaccharides. In terms of

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Electronic Supplementary Information (ESI) available: [Materials, characterization, ^{1}H and ^{13}C NMR spectra of the compounds, condition of the RAFT polymerization, UV-vis absorbance spectra, plots of Mayo-Lewis equation, results of XPS measurement, and SPRI signal curves]. See DOI: 10.1039/x0xx00000x

Table 1. Glycopolymer library prepared by PET-RAFT polymerization.[a]

Polymer	G (%)[b]	N (%)[b]	A (%)[b]	Conv. (%)[c]	M_n[d]	M_w/M_n[d]	Polymer	G (%)	N (%)	A (%)	Conv. (%)	M_n	M_w/M_n
G0N100	0	100	0	98	17900	1.52	G30N70	30	70	0	99	17600	1.58
G10N0	10	0	90	83	6900	1.51	G50N0	50	0	50	99	11800	1.41
G10N10	10	10	80	86	11900	1.46	G50N10	50	10	40	99	12000	1.37
G10N20	10	20	70	98	12900	1.55	G50N20	50	20	30	99	12200	1.59
G10N30	10	30	60	97	13700	1.49	G50N30	50	30	20	99	13900	1.54
G10N40	10	40	50	97	15200	1.52	G50N40	50	40	10	98	14400	1.53
G10N50	10	50	40	97	16200	1.53	G50N50	50	50	0	98	16900	1.44
G10N70	10	70	20	99	17700	1.46	G70N0	70	0	30	99	14800	1.43
G30N0	30	0	70	93	8600	1.50	G70N10	70	10	20	99	14900	1.46
G30N10	30	10	60	99	11800	1.49	G70N20	70	20	10	99	15200	1.52
G30N20	30	20	50	98	12500	1.58	G70N30	70	30	0	99	15800	1.59
G30N30	30	30	40	98	13800	1.48	G90N0	90	0	10	99	15200	1.45
G30N40	30	40	30	99	13900	1.54	G90N10	90	10	0	99	17700	1.42
G30N50	30	50	20	99	14400	1.58	G100N0	100	0	0	98	9900	1.54

[a] The ratio of [monomer]:[RAFT]:[eosin Y]:[ascorbic acid] = 100:1:0.01:1. [b] G, N, and A represent GalAAm, Neu5AcAAm, and AAm, respectively. [c] Monomer conversion was determined by ^{1}H NMR. [d] The relative molecular weight (M_n) and dispersity (M_w/M_n) values were determined by SEC analysis calibrated with a pullulan standard. The eluent was 100 mM $NaNO_3$ (aq).

glycounit ratio, for example, various glycopolymers with different ratios of "carbohydrate modules" need to be synthesized and then evaluated with target molecules. Thus, an efficient strategy for the synthesis and evaluation of glycopolymers is desirable to determine the optimal structure for a particular oligosaccharide mimetic.

In this work, we report the screening of glycopolymers for the "carbohydrate module method" by a combination of photoinduced electron/energy transfer-reversible addition-fragmentation chain-transfer (PET-RAFT) polymerization and surface plasmon resonance imaging (SPRI). PET-RAFT polymerization was developed by Boyer and co-workers and enables the fast and facile synthesis of many polymer libraries in the presence of oxygen.[13–16] Utilizing these features, application to multiple library synthesis and screening analysis was studied.[17–21] SPRI is a facile and fast method to evaluate the molecular interaction between a gold surface and an analyte. Glycopolymers prepared by RAFT polymerization have thiol groups at the termini and can be easily immobilized on a gold surface.[9,22] The purpose of the present study was to establish a method for synthesizing glycopolymers by aqueous PET-RAFT polymerization and to screen the interactions of the glycopolymers with target biomolecules by SPRI (Figure 1).

Glycopolymers were synthesized containing three monomers, acrylamide (AAm), galactose acrylamide (GalAAm), and *N*-acetylneuraminic acid acrylamide (Neu5AcAAm). The monomer composition was varied and a glycopolymer library was prepared by PET-RAFT polymerization (Figure 1). The monomer concentration [M] was fixed at 0.5 M. The monomer, RAFT agent (DAOCTPA), photooxidation-reduction catalyst (eosin Y), and reducing agent (ascorbic acid) were mixed at a ratio of 100: 1: 0.01: 1 in Milli-Q water (200 μL). Each mixture was added to a 96-well plate and irradiated with LED light (λ = 527 nm) for 7 h at room temperature. The wavelength of the irradiation light was selected based on the absorption peak of eosin Y, which is not overlapped with the absorption peak of DAOCTPA at 527 nm (Figure S3). The monomer conversion and molecular weights were determined by proton nuclear magnetic resonance (^{1}H NMR) and size exclusion chromatography (SEC) analysis, respectively (Table 1). In most cases, the monomer conversions were over 90%, and the polymerization proceeded successfully. The conversion was decreased to 86% and 83% when the composition of AAm was 80% and 90%, respectively. This result suggested that the propagation rate for AAm was relatively slower than that of GalAAm and Neu5AcAAm in the PET-RAFT polymerization with DAOCTPA. The amide group of AAm is not substituted, and AAm is expected to have a lower stability in the radical state than an *N*-substituted acrylamide.[23] The propagation rates of each monomer and the reactivity ratios were calculated by the Mayo-Lewis equation (Figure S4 and Table S1). The propagation rates with the *N*-substituted monomers were higher than those with AAm, and thus, the monomer sequence of the copolymers partly seemed to be gradient. Although the dispersity (M_w/M_n) values of the glycopolymers had a relatively broad range for a RAFT polymerization system, the ranges were still narrower than for a free radical polymerization system (Table 1). Th UV-vis spectra of the glycopolymers indicated the presence of a trithiocarbonate group at the polymer terminus (Figure S5). These results demonstrated that the PET-RAFT polymerization enabled the facile and fast preparation of a library with 28 types of glycopolymers without requiring degassing of the solutions. The synthesized glycopolymers were facilely purified by ultrafiltration.

The trithiocarbonate group at the polymer terminus was reduced to a thiol in 1M KOH aqueous solution (Figure S4), and

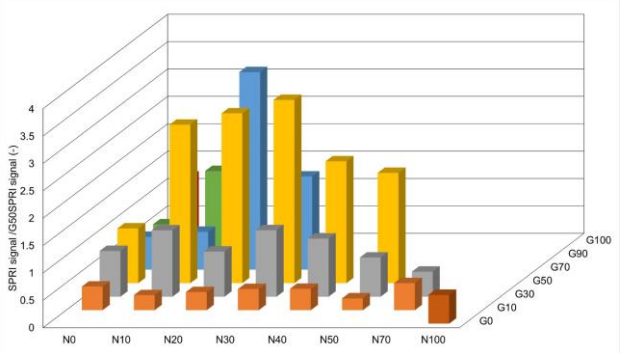

Figure 2. SPRI screening of the glycopolymer library: signal changes after addition of CTB solution (500 nM) to the glycopolymer-immobilized surfaces.

the glycopolymers were immobilized on a gold surface through the Au-thiol interaction for SPRI. The immobilization of the glycopolymers onto the gold surface was confirmed by X-ray photoelectron spectroscopy measurement. After polymer immobilization of **G100N0**, the peaks of C–C (284.8 eV), C–O and C–N (286.7 eV), and C=O (287.3 eV) bonds were increased compared with the unmodified surface (Figure S6). The peak at 286.7 eV derived from the C–O bonds of the carbohydrate units indicated the successful immobilization of the glycopolymers.

Screening of the glycopolymer library for interaction with CTB was performed by SPRI measurements. Each glycopolymer was immobilized on the gold surface spots of a SPRI chip. After equilibration with PBS(–) buffer (0.1 mL/min for 60 min), CTB solution (500 nM) was added and the signals were analysed. The surface of each glycopolymer showed SPRI signals, which indicated that the glycopolymers prepared by the "carbohydrate module method" interacted with CTB (Figure S7). The SPRI signals, normalized by the signals of **G50N0**, are shown in Figure 2. The homo-glycopolymer of galactose modules (**G100N0**) showed a higher SPRI signal than that containing all Neu5Ac modules (**G0N100**), indicating that galactose modules contributed to the interaction of the glycopolymers with CTB more than Neu5Ac units. This result is in agreement with reports that the galactose and Neu5Ac units of GM1 have different contributions to the interaction with CTB.[24,25] It is known that galactose and Neu5Ac units interact with the internal and shallow areas of the binding domains of CTB, respectively. The galactose units critically contribute to the interaction of GM1 with CTB, and Neu5Ac units enhance the interaction cooperatively. Thus, in the case of the "carbohydrate module method" for GM1 mimicry, the presence of both types of the carbohydrate modules was expected to be required for a strong interaction. As expected, the screening revealed that the glycopolymers containing 50% or 70% galactose units showed relatively high SPRI signals. In particular, the normalized signals of **G50N20**, **G50N30**, and **G70N20** were 3.1, 3.3, and 3.6, respectively. These values were more than three times higher than that of **G50N0**. These results demonstrated that the amount of adsorption of CTB on the SPR surface depended on the composition of the carbohydrate modules of the glycopolymers. Interestingly, the amount of

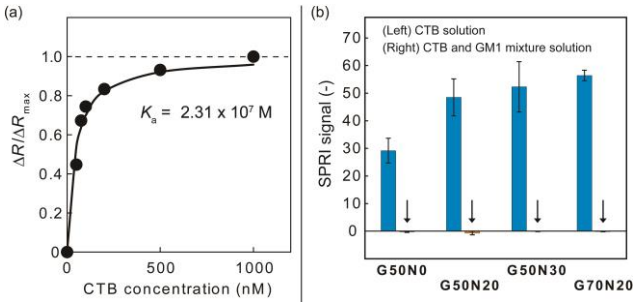

Figure 3. (a) SPRI signals of **G70N20** with different CTB concentrations, and the binding constant (K_a). The signal changes were normalized by the saturated value. (b) SPRI signals of the glycopolymers with CTB solution in the presence and absence of GM1. The blue and orange bars indicate the results for CTB solution (500 nM) and CTB solution (500 nM) with GM1 (10 μM), respectively.

adsorbed CTB dramatically changed depending on the composition of Neu5Ac modules. It is noteworthy that just the presence of both the carbohydrate modules in the polymer structures was insufficient and there was an optimal composition of carbohydrate modules required for good binding. To achieve the multiple binding of galactose and Neu5Ac modules to the domains of CTB, the two carbohydrate modules need be at an optimal distance (ca. 0.69 nm).[12,26] The carbohydrate modules were introduced into the glycopolymer structures in a random sequence, and the distance between the galactose and Neu5Ac modules were controlled by the composition of the glycopolymers. It was assumed that the content of the monomer sequences with the optimal distance of the two carbohydrate modules was relatively high in the structures of **G50N20**, **G50N30**, and **G70N20**. Although a simple prediction of the relationship between the composition of the carbohydrate modules and the adsorption amount of CTB was difficult, the glycopolymer screening revealed candidates with optimal structures as GM1 mimetics produced by the "carbohydrate module method". To determine the strength of the interaction of the glycopolymers with CTB, the binding constant of **G70N20** (which showed the highest SPRI signal) was measured. The CTB concentration was varied from 10 to 500 nM. The plots of the SPRI signals at each concentration were fitted with a Langmuir isotherm curve (Figure 3a). The apparent binding constant (K_a) of **G70N20**-immobilized surface was 2.31 × 10^7 M^{-1}. This value was lower than that of the GM1-immobilized surface (K_a = 10^{10} M^{-1}),[27] but was still relatively high for a GM1 mimetic polymer.

To demonstrate that the glycopolymers recognized the carbohydrate binding domains of CTB, an inhibition assay with the natural ligand GM1 was performed.[22] The binding constant of GM1 to CTB is high,[24,27] and the carbohydrate binding domains of CTB were expected to be covered by GM1 under the experimental conditions (CTB = 500 nM and GM1 = 10 μM). The glycopolymers (**G50N0**, **G50N20**, **G50N30**, and **G70N20**) did not show SPRI signals for the solution mixture of CTB and GM1 (Figure 3b), demonstrating that the glycopolymers recognized the carbohydrate domains of CTB. This result indicated that the glycopolymers interacted with CTB as GM1 mimetics, and that

the “carbohydrate module method” provided the synthetic glycomimicry of GM1 using polymeric materials.

In conclusion, a combination of PET-RAFT polymerization and SPRI measurements enabled the preparation of multiple candidates for glycomimicry of the GM1 ganglioside. To synthesize the glycomimetics, glycopolymers were prepared by the “carbohydrate module method” with two types of carbohydrate modules, galactose and Neu5Ac. PET-RAFT polymerization enabled the facile and fast preparation of a glycopolymer library. One-time screening of the SPRI measurements revealed that the interactions of the glycopolymers with CTB were dependent on the composition of the carbohydrate modules, and that the presence of both types of carbohydrate modules in an optimal ratio was essential for a strong interaction. Because the relationship between the composition of the carbohydrate modules in the glycopolymers and the interactions with CTB was unpredictable, the fast and simultaneous screening using SPRI measurements was a useful way to determine the optimal structure of glycopolymers that effectively mimicked the function of GM1. Furthermore, this facile and quick technique may be suitable for screening glycomimetics for pathogens with variable or unknown nature, such as the influenza virus and the SARS-COV-2.

Author contributions

T. Uemura, M. Nagao and Y. Miura designed the experiments. T. Uemura performed the experiments. M. Nagao and T. Uemura wrote the manuscript. T. Horiuchi and Y. Hoshino made contribution to the discussions during the work. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgement

This work was supported by AMED under Grant Number (JP21ak0101132h0002), a Grant-in-Aid for Scientific Research (B) (JP19H0276), and a Grant-in-Aid for Scientific Research on Innovative Areas (JP20H05230 and JP20H04825).

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