Synthesis of Glycopolymers Carrying 3'-Sialyllactose for Suppressing Inflammatory Reaction via Siglec-E

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Synthesis of Glycopolymers Carrying 3'-Sialyllactose for Suppressing Inflammatory Reaction *via* Siglec-E

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One of the new strategies to treat autoimmune diseases is to target Siglec, a membrane protein receptor with the ability to suppress immune responses. Herein, we synthesized glycopolymers carrying 3'-sialyllactose in various glycounit densities. RAW 264.7 macrophages transfected to express secreted alkaline phosphatase (SEAP) were used to evaluate the immunosuppression ability of the glycopolymers. The inhibition of the signal transmission was dependent on the glycounit densities of the glycopolymers, and was maximized at the moderate density (70%).

Keywords: Glycopolymers, Siglec, RAFT polymerization

Immunity is a biological response that eliminates antigens such as bacteria, viruses, and other pathogens, and plays an important role in biological activities.[1] In some cases, malfunctions of the immune systems cause autoimmune diseases where immune cells attack their own cells and tissues, resulting in inflammation.[2,3] To date, drugs targeting immune cells and inflammatory cytokines have been developed; however, there are various side effects (e.g., inhibition of normal cell proliferation, morbidity from other infections due to reduced inflammatory cytokines), and thus, the development of novel drugs that exhibit immunosuppression through another mechanism has been required.[4,5]

Sialic acid-binding immunoglobulin-like lectins (Siglec) are a family of immune checkpoint receptors expressed on cells of the immune and hematopoietic systems, and are emerging as targets for new immune response modulators.[6-9] Each of Siglec types recognizes the corresponding sialic acid residue as ligands, and plays roles as a regulator of immune cell signaling.[6,10-12] Siglec-9 has an immunoreceptor tyrosine-based inhibitory motif (ITIM) near the plasma membrane that transmits inhibitory functions to immune cells, and has been reported to suppress the production of the pro-inflammatory cytokines TNF-α and IL-6, and to increase the production of the inhibitory cytokine IL-10.[13-15] The Siglec molecules are usually masked by glycoconjugates on the cell surface, and therefore, dissociation of the binding of sialic acid, a natural ligand for Siglec (K_d = mM), by competitive ligands is required to control the transmit immunosuppressive signals.[6,16,17]

Glycoproteins that strongly interact with Siglec have been developed as the competitive ligands.[18-22] Since the accumulation of Siglecs on membranes is also thought to trigger signal transduction, polymeric molecules with multiple carbohydrates that can cross-link multiple Siglec molecules are attracting attention.[6,23,24] Glycopolymers are a class of polymeric glycoligands, and have advantages of easy synthesis.[25,26] Glycopolymers have multiple glycounits in one molecule, and increase the probability of binding to a lectin resulting in the strong interaction (the cluster glycoside effect).[27,28] Furthermore, the interaction of glycopolymers with target molecules can be controlled by designing the structures of the glycopolymers (polymer length and glycodensity) using controlled polymerization.[29-31] Bertozzi et al. reported the interaction of the glycopolymers with Siglec-7 and -9.[24,32] However, the effect of polymer structures on the interaction with Siglec-9 has not been studied in detail.

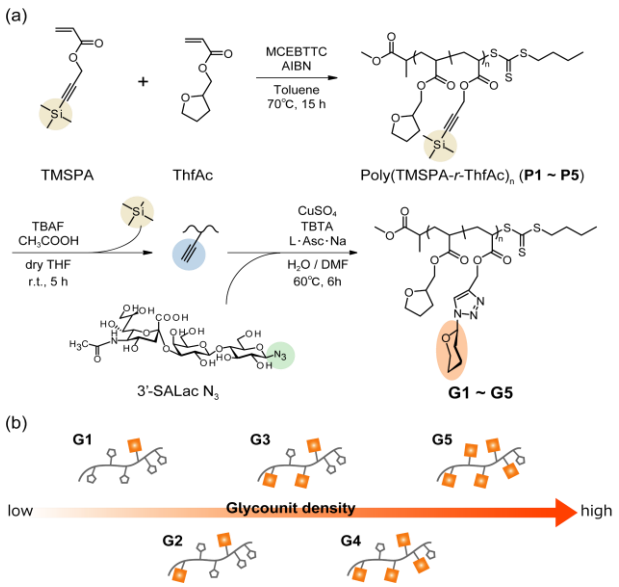

Figure 1. Schematic illustrations of the synthesis of glycopolymers carrying 3'-sialyllactose units.

Herein, we synthesized glycopolymers carrying 3'-sialylactose (3'-SALac) in different glycounit densities for immunosuppression via interactions with Siglec-E, a true ortholog of Siglec-9. For effective introduction of the bulky trisaccharide into the polymer side chains, the glycopolymers were synthesized using "post-click" chemistry, which is a combination of controlled polymerization and "click" reaction.

Polymer precursors carrying protected alkyne groups were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization with 3-(trimethylsilyl)prop-2-yn-1-yl acrylate (TMSPA) and tetrahydrofurfuryl acrylate (ThfAc). ThfAc was chosen as the amphiphilic spacer monomer. The monomers, the RAFT agent methyl 2-(((butylthio)carbonothioyl)-thio)propanoate (MCEBTTC), and the initiator 2,2'-azobis(isobutyronitrile) (AIBN) were

Table1. Properties of RAFT polymerization of polymer precursors.

Polymers	Monomer feed ratio TMSPA (mol%)	ThfAc (mol%)	Conv.[a] (%)	DP[a] TMSPA (mer)	ThfAc (mer)	$M_{n,\,th}$[b] (g/mol)	M_n[c] (g/mol)	M_w/M_n[c] (-)
P1	10	90	98	8	91	15,900	13,600	1.53
P2	30	70	95	32	80	18,600	13,600	1.57
P3	50	50	91	42	47	15,200	14,000	1.69
P4	70	30	89	64	27	16,100	13,400	1.57
P5	90	10	89	76	13	16,100	11,300	1.53

Monomer concentration of each polymerization ([M]) was set at 5.0 M. The target degree of polymerization (DP) was set at 100 ([M]/[RAFT] = 100). The ratio of the initiator ([RAFT]/[AIBN]) was fixed at 50. (a) Monomer conversion (Conv.) and the degree of polymerization (DP) were determined from ^{1}H NMR. (b) Theoretical molecular weight was calculated by following the formula: $M_{n,\,th} = MW_{CTA} + MW_{TMSPA} \times DP_{TMSPA} + MW_{ThfAc} \times DP_{ThfAc}$ (c) Relative molecular weight and polydispersity index were determined by GPC analysis calibrated with a PMMA standard. The eluent was DMF with LiBr (10 mM).

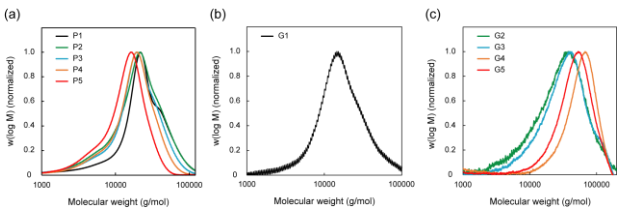

Figure 2. GPC traces of (a) P1 to P5 (eluent; DMF with 10 mM LiBr, standard; PMMA), (b) G1 (eluent; DMF with 10 mM LiBr, standard; PMMA), and (c) G2 to G5 (eluent; 100 mM $NaNO_3$ (aq), standard; pullulan).

dissolved in toluene with a stoichiometry of [monomer]: [RAFT]: [Initiator] = 100: 1: 0.2 ([monomer] = 5 M). The ratio of [TMSPA]: [ThfAc] was varied from 1: 9 to 9: 1, and poly(TMSPA-*r*-ThfAc) (P1 ~ P5) were obtained. Conversion rate and the degree of polymerization (DP) were determined by ^{1}H NMR. Molecular weight and polydispersity (M_w/M_n) were confirmed by gel permeation chromatography (GPC) analysis. The monomer conversions were over 89% and the polydispersities were below 1.69 for all conditions (Table 1). The DP values of each monomer in the obtained polymers were TMSPA: ThfAc = 8: 91 (P1), 32: 80 (P2), 42: 47 (P3), 64: 27 (P4), and 76: 13 (P5), indicating that the synthesized polymers had the designed monomer ratio. The GPC curves of P1 and P5 showed the shoulders on the high-molecular side and tailing on the low-molecular side, which were attributed to the polymerization properties of ThfAc and TMSPA, respectively (Figure 2a and S2). Although the dispersities were relatively broad as RAFT polymers, the synthesized polymer precursors carrying the protected alkyne groups in different densities were obtained. Sequentially, the TMS groups of the polymer precursors were deprotected using tetrabutylammonium fluoride in dry THF at room temperature. The complete deprotection of the TMS groups were confirmed by ^{1}H NMR (Figure S1-13–1-17).

Neu5Ac-α(2-3)-Gal-β(1-4)-Glc (3'-SALac), which is recognized by Siglec-E (-9), was selected as a carbohydrate ligand to be introduced into the side chain.[13,33-35] The anomerically azidized 3'-SALac azide was introduced into the side chain of P1–P5 by Huisgen reaction (G1–G5, Figure 1). The yield of the addition reaction and the number of the glycounit in the polymer molecule (glycounit density) were confirmed by ^{1}H NMR (400 MHz). In all conditions, the yields of the glycounit addition were over 89%, and glycounit densities were 7, 26, 45, 65, and 76% for G1–G5, respectively (Table 2). It should be noted that an error of about 10% was observed in the integral values for the proton peaks. GPC analysis revealed the polydipersities were below 1.64, demonstrating that the reaction proceeded without any damage to the polymer main chains (Figure 2b and 2c). Thus, the combination of RAFT polymerization and Huisgen reaction enabled the synthesis of the glycopolymers carrying bulky 3'-SALac in various glycounit densities.

To evaluate the immunosuppression with synthesized glycopolymers, SEAP Raw 264.7 macrophage-like cells that express high levels of Siglec-E in response to LPS stimulation were used.[36,37] SEAP Raw 264.7 cells produce secreted alkaline phosphatase (SEAP) via the NF-κB pathway through TLR4 signaling in response to LPS stimulation.[38] Taking advantage of the fact that SEAP hydrolyzes the phosphate group of *p*-nitrophenylphosphate (pNPP) to produce a compound with an absorbance at 405 nm, the SEAP production was evaluated with enzyme-linked immunosorbent assay (ELISA). The low glycounit density of

Table 2. Properties of the glycopolymers synthesized by Husigen reaction.

Polymers	Alkyne conv.[a] (%)	Glycounit density[a] (%)	$M_{n,\,th}$[b] (g/mol)	M_n[c,d] (g/mol)	M_w/M_n[c,d] (-)
G1	>92	7	20,100	24,000	1.63
G2	>90	26	36,000	25,000	1.63
G3	>95	45	39,400	28,000	1.64
G4	>93	65	52,300	49,000	1.34
G5	>89	76	56,900	44,000	1.34

(a) Alkyne conversion and the glycounit density were determined from ^{1}H NMR. (b) Theoretical molecular weight was calculated by following the formula: $M_{n,\,th} = MW_{CTA} + MW_{(PA+3'\text{-}SALac)} \times DP_{(PA+3'\text{-}SALac)} + MW_{(PA)} \times DP_{PA} + MW_{ThfAc} \times DP_{ThfAc}$ (c) Relative molecular weight and polydispersity index of G1 were determined by GPC analysis calibrated with PMMA standard. The eluent was DMF with 10 mM LiBr. (d) Relative molecular weight and polydispersity index of G2 to G5 were determined by GPC analysis calibrated with a Pullulan standard. The eluent was 100 mM $NaNO_3$ (aq).

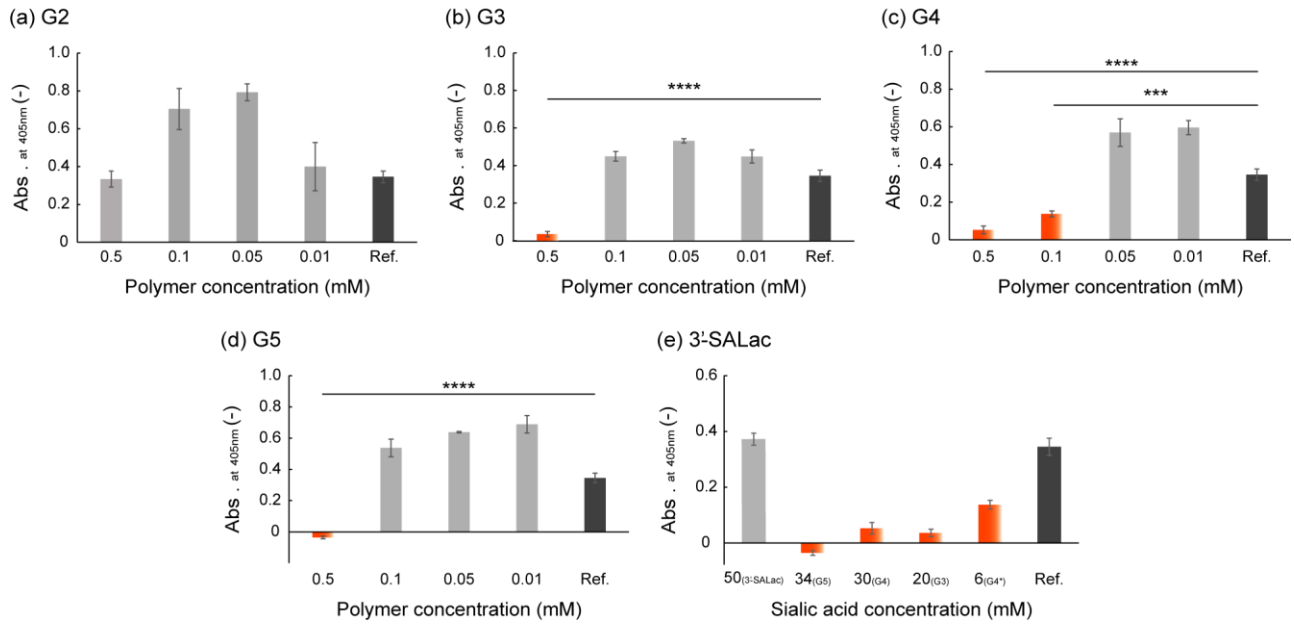

Figure 3. Suppression of LPS-induced SEAP production in RAW 264.7 macrophage-like cells by glycopolymers. Cells were incubated overnight and then treated with (a), (b), (c), and (d) glycopolymers (0.5, 0.1, 0.05, 0.01 mM) and (e) 3'-SALac (50 mM) for 30 min. After 30 min later, the cells were stimulated LPS (final conc. 100 ng/mL) for 24 h. The absorbance at 405 nm of the supernatants was then measured by ELIZA. The horizontal axis of (e) shows the concentration of 3'-SALac (mM), and the subscript of number means materials (G3, G4 and G5 are at 0.5 mM, G4* is at 0.1 mM of the polymer concentration). The sialic acid concentration of glycopolymers was calculated by following the formula: Sialic acid concentration = polymer concentration $\times$ DP($_{PA+3'SALac}$). In all graphs, "Ref" means without sugars and glycopolymers; that is 0 mM. All assays were performed in triplicate, and data are expressed as means ± 2*SD. ****, $p < 0.0005$ (two-sided); significantly different from the group treated with LPS alone by Student's t test

G1 (8%) resulted in its low solubility in buffer solution (PBS), and thus, G1 was not used in this assay. G2–G5 were dissolved in a mixture of solvents (DPBS (Ca(-), Mg(-) and medium) to final concentrations (0.5, 0.1, 0.05, 0.01 mM) to prepare glycopolymer solutions. As compared to the reference well (without glycopolymers), the wells treated with G3, G4 and G5 at high polymer concentration (0.5 mM) showed significant decrease in the absorbance at 405 nm (Figure 3b-d, $p < 0.0005$). The decrease in absorbance was 90, 85 and 99% for G3, G4 and G5, respectively. Furthermore, only G4 showed the decrease in absorbance at lower polymer concentration (0.1 mM, 60% decrease). There was no significant difference in the absorbance of G2 and 3'-SALac monomer (Figure 3a and e).

These results indicate that SEAP production was inhibited by glycopolymers with the glycounit density of 48% or higher, suggesting interactions of the glycopolymers with Siglec-E on the cell surface. Since 3'-SALac monomer and G2 (low glycounit density) did not show any suppression, a certain glycounit density was necessary for the expression of the suppressive ability. We presume that the high glycounit densities of G3–G5 (>48%) increased the binding probability of 3'-SALac to Siglec-E, resulting in the lower k_{off}. In addition, G4, with 70% glycounit density, showed inhibition of SEAP production at the lowest polymer concentration. This suggests that there is an optimal glycounit density or polymer conformation where the steric hindrance of the trisaccharide structure is avoided in binding to Siglec-E.[30]

In conclusion, we synthesized glycopolymers carrying 3'-SALac, which interacts with Siglec-E, using the combination of RAFT polymerization and Huisgen reaction. Then, the glycounit densities were varied from 7 to 76%. Evaluation of immunosuppression of the glycopolymers indicated that signal transduction via the NF-κB pathway was inhibited, and that the inhibition ability was dependent on the glycounit density. This signal suppression was achieved by the multivalency of the glycopolymers, and the moderate glycounit density maximized the ability. These results demonstrate the successful development of the polymeric glycoligand that targets Siglec-E, resulting in immunosuppression. In the future, we expect to develop materials with higher inhibitory ability by further design of polymer structures.

Acknowledgement

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Supporting Information is available on http://dx.doi.org/10.1246/cl.******.

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Graphical Abstract

Textual Information

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Authors' Names(required)	Takato Ishida, Masanori Nagao, Takahiro Oh, Takeshi Mori, Yu Hsohino, and Yoshiko Miura*

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