Preparation and Lectin Binding Property of Chitosan–Carbohydrate Conjugates[1]

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(Received October 4, 2000)

Chitosan–sialic acid conjugates were prepared using *p*-formylphenyl α-sialoside by reductive *N*-alkylation. The degree of substitution (DS) of conjugates could be controlled from 0.06 to 0.53 by the amount of sialoside. With the use of *p*-isothiocyanatophenyl α-sialoside, chitosan–sialic acid conjugates were also prepared with excellent efficiency. Chitosan–melibiose conjugates having α-galactosyl epitope were also prepared by reductive *N*-alkylation. These conjugates were transformed into water-soluble forms by *N*-succinylation and their protein binding property was tested using wheat germ agglutinin (WGA) or *Griffonia simplicifolia* (GSI-B_4) lectin. Strong immunodiffusion bands were observed in all of conjugates, thus demonstrating the specific binding of epitope in conjugate to each lectins.

Carbohydrates are ubiquitous components of cell wall membranes and occur as glycoproteins, glycolipids, proteoglycans, and capsular polysaccharides.[2] As such, they can participate in early intermolecular and intracellular events. Moreover, carbohydrate residues can serve as cell surface receptors for antibodies, viruses, and bacteria.[3] In order to mimic some of the above interactions, tremendous efforts have been directed at the syntheses of artificial carbohydrate-polymer conjugates.[4] Synthetic glycoconjugates constitute a wide family of carbohydrate derivatives including neoglycoproteins, neoglycolipids, clusters, and glycopolymers.[5] Glycoconjugates have been designed as models in carbohydrate-protein interaction studies,[3,6] vaccines,[7] inhibitors of cell adhesion by viruses, bacteria, mycoplasma, and toxin,[8] ligands in affinity chromatography,[9,10] diagnostic reagents, probes, targeted drug-delivery systems[11] or simply to confer upon enzymes and proteins improved thermal and proteolytic stabilities.

Chitosan **1** is a polysaccharide consisting of β(1-4)-2-amino-2-deoxy-D-glucopyranose (GlcN) repeating unit, it includes small amount of *N*-acetyl-D-glucosamine (GlcNAc) residues. At present, a number of interesting biological properties were reported for chitosan.[12] Chitosan itself is non-toxic and biodegradable.[13] Therefore, chitosan is an appealing bioactive polymer for further development. Additionally, sialic acids containing polymers[14] has been shown to be potent inhibitors of hemagglutination of human erythrocytes by influenza viruses.[14–16] *N*-Acetylneuraminic acid (Neu5Ac) is the most ubiquitous member of the sialic acid family of derivatives present on mammalian cell surface glycolipids and glycoproteins and is the key epitope recognized as being essential for a number of pathogenic infections.[17] Human antibodies against α-galactosyl (Gal) epitope, which occur naturally, are abundant (1–2%) and are responsible for acute rejection of xenotransplanted organs from lower animals.[18] Therefore, artificial glycopolymers having α-Gal epitope are also of much interest from the viewpoint of medical transplantation of pig livers, since they can block immune rejection.

We are now investigating the development of novel glycoconjugates based on chitosan. Recently, we reported in communications that chitosan–sialic acid or chitosan–melibiose conjugates show potent lectin binding properties.[1a,d] In this paper, we describe the preparation of some different types of chitosan–carbohydrate epitope conjugates and their lectin binding properties.

Results and Discussion

Chitosan–Sialic Acid Conjugates. Scheme 1 shows the reductive *N*-alkylation of chitosan (**1a**: NH_2 = 0.96, pK_a of NH_2 in chitosan = 6.5) with deprotected *p*-formylphenyl α-sialoside[19] **2a**. The results are summarized in Table 1. The sialic acid residue was successfully introduced to the amino groups of chitosan by reductive *N*-alkylation and gave chitosan-sialic acid conjugates **3** in good yields (76–100%) even under the acidic conditions. The DS of conjugate **3** could be controlled by the amount of aldehyde **2a**, in spite of the low reactivity of **2a** (below 48%). Highly substituted conjugate (DS = 0.53) was soluble in neutral water, although other conjugates of low DS (0.06–0.44) were not. Protected *p*-formylphenyl α-sialoside **2b** with *O*-acetyl and methyl ester groups was also successfully attached to chitosan and gave water-soluble conjugate **3** (DS = 0.85) in 70% yield (Scheme 2). Since water-insoluble conjugates **3** of low DS were not useful for biological evaluation, the remaining amino groups of conjugate were transformed by *N*-succinylation and gave succinylated conjugates **4** in 90–100% yields. A part of secondary amino groups in conjugate **3** was also succinylated. Under these mild aqueous conditions and basic work-up, however,

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Table 1. Preparation of Chitosan–Sialic Acid Conjugates **3** by Reductive *N*-Alkylation

Entry	**2**	Yield[a]	DS	Reactivity[b]	Solubility
	equiv	%		%	in H_2O
1	0.2	100	0.06	30	No
2	0.4	77	0.10	25	No
3	0.6	76	0.29	48	No
4	0.8	91	0.34	43	No
5	0.9	74	0.44	48	No
6	1.2	84	0.53	44	Yes
7[c]	2.0	70	0.85	53	Yes

a) Yield was calculated by weight recovery from chitosan **1** according to the following equation:

$$\text{Yield} = \frac{(\text{weight of product})}{(\text{weight of chitosan})} \times \frac{(\text{FW of product})}{(\text{FW of chitosan})} \times 100$$

FW of product = FW of chitosan + (DS × FW of –CH_2PhNeu5Ac)

b) Reavtivity (%) = (DS)/(equiv of **2**).

c) Prepared by Scheme 2.

cyclic imide formation did not occur as judged from the two different signals at δ 2.54 (NHC(O)–CH_2) and 2.62 (CH_2–CO_2Na) in ^{1}H NMR.[20] Scheme 3 and Table 2 show the preparation of chitosan–sialic acid conjugates **6** with *p*-isothiocyanatophenyl α-sialoside **5**. In this case, conjugates **6** were obtained in excellent yields (91–100%). Noteworthy is the fact that the reactivity of isothiocyanate **5** was excellent (90–100%) compared with that of aldehydes **2a** or **2b** (25–53% in Table 1). Since these conjugates **6** of low DS (0.23 and 0.27) were insoluble in water, these were transformed into water-soluble conjugates **7** by *N*-succinylation. The chemical structures of

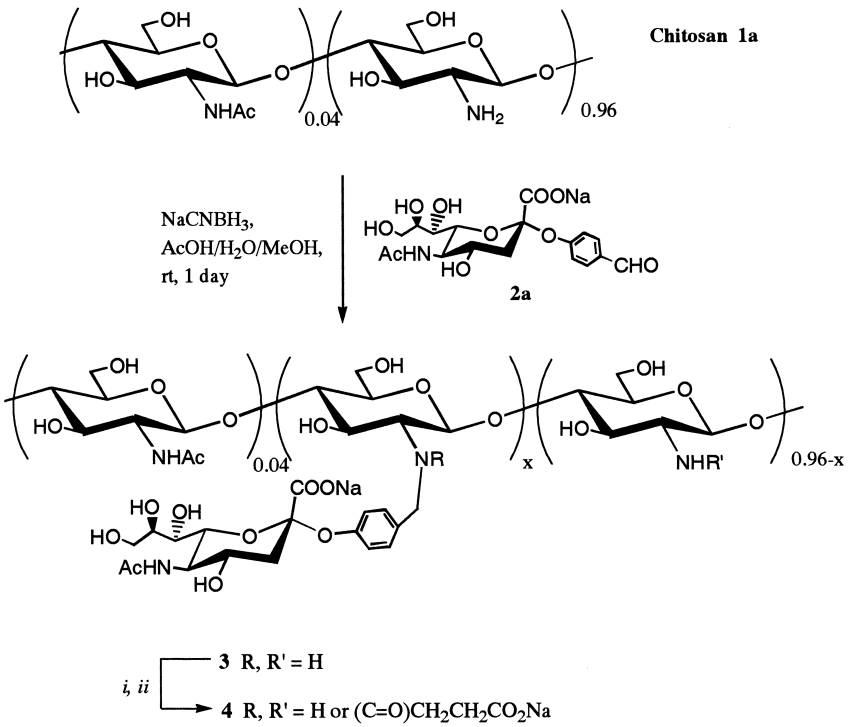
Table 2. Preparation of Chitosan–Sialic Acid Conjugates **6** with Isothiocyanate **5**

Entry	**5**	Yield[a]	DS	Reactivity[b]	Solubility
	equiv	%		%	in H_2O
1	0.23	91	0.23	100	No
2	0.30	100	0.27	90	No
3	0.40	97	0.38	97	Yes

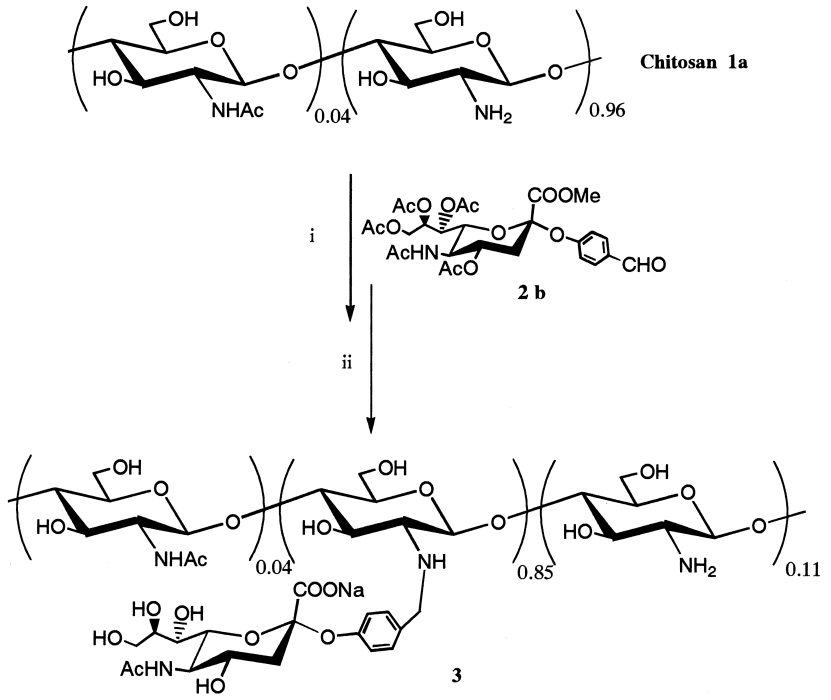
a), b) See Table 1.

these chitosan-sialic acid conjugates are summarized in Table 3. All of these conjugates were soluble in neutral water and structurally well defined by ^{1}H and ^{13}C NMR, and colorimetric analysis (570 nm) with ninhydrin. The protein binding properties of these conjugates were evaluated with WGA which is a plant lectin specific toward GlcNAc and Neu5Ac residues. In conjugates **3**, **4**, **6**, and **7**, strong immunodiffusion bands were observed when compared to a negative control (entry 8: *N*-succinylated chitosan), thus demonstrating the specificity of binding of Neu5Ac epitope in conjugates to WGA lectin. The very faint band shown in *N*-succinylated chitosan could be due to the small amount of GlcNAc residue (DS = 0.04) already present in the initial polymer chain.

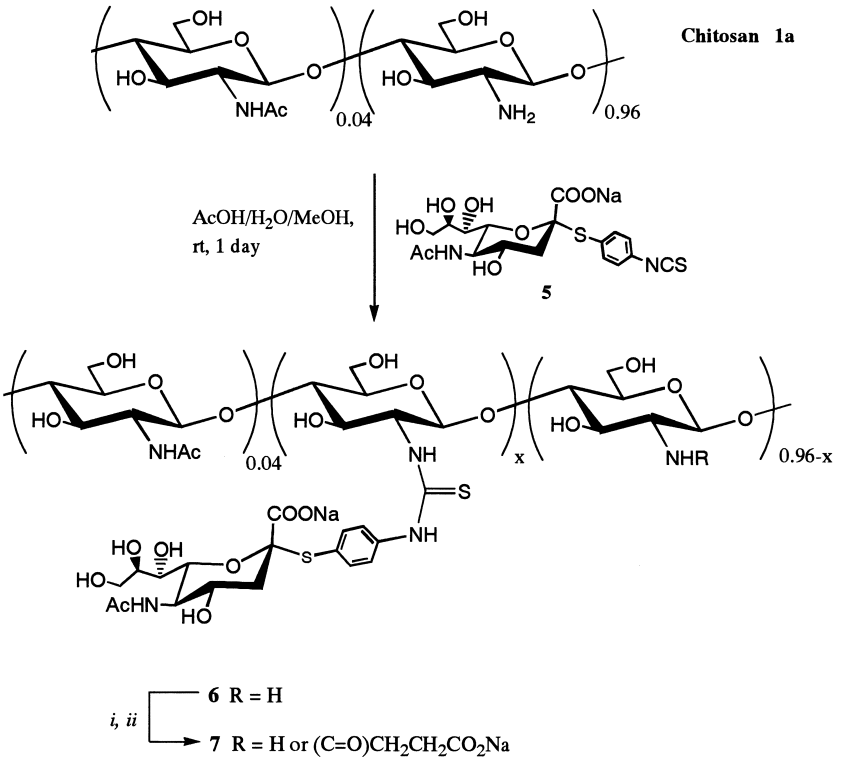
Chitosan–Melibiose Conjugates. Melibiose **8** is a disaccharide having 1–6 linked α-galactosyl (Gal) epitope, which is responsible for acute rejection of xenotransplanted organs.[18] As it was reported that reductive *N*-alkylation of chitosan could be achived with various reducing sugars,[20,21] we attempted the direct attchment of commercial melibiose **8** to two kinds of chitosan (Scheme 4, **1a**: NH_2 = 0.96 and **1b**: NH_2 = 0.80) and the results are summarized in Table 4. Highly substituted chitosan–melibiose conjugates **9** (DS = 0.58–1.17) were ob-



Scheme 1. Reagents and conditions: i, succinic anhydride, AcOH/H_2O/MeOH, rt 1 day; ii, 0.5 M NaOH, rt, 2 h.



Scheme 2. Reagents and conditions: i, $NaCNBH_3$, $AcOH/H_2O/MeOH$, rt, 1 day; ii, 0.5 M NaOH, rt, 2 h.



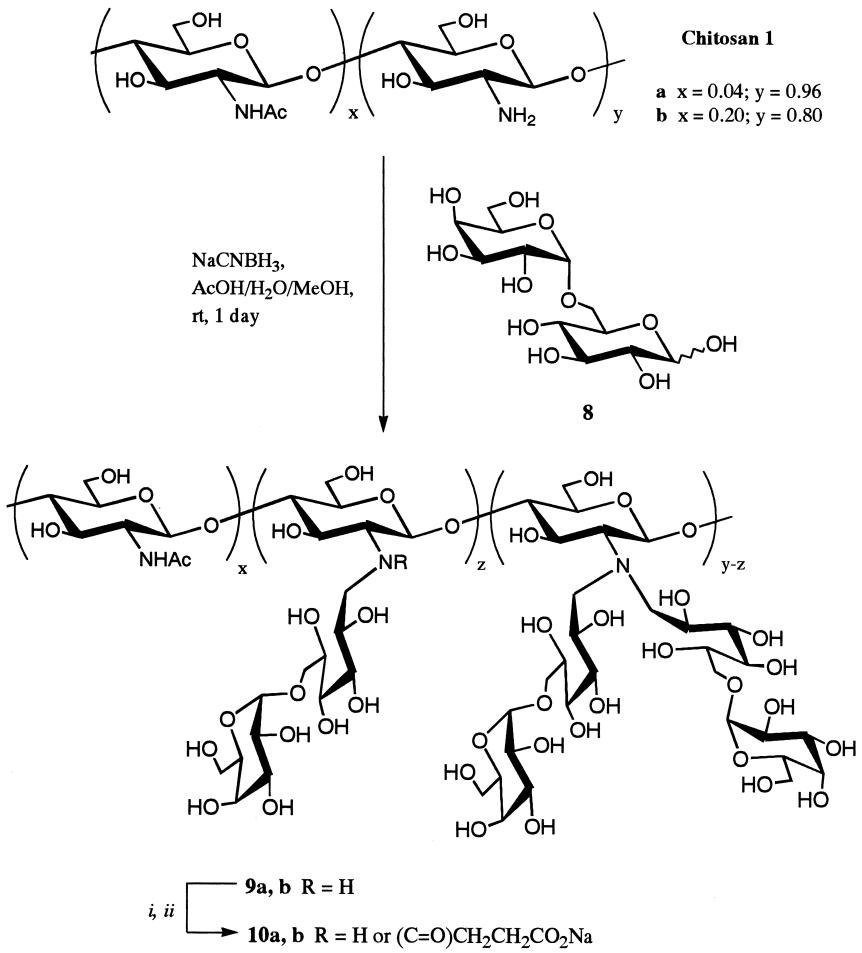
Scheme 3. Reagents and conditions: i, succinic anhydride, $AcOH/H_2O/MeOH$, rt 1 day; ii, 0.5 M NaOH, rt, 2 h.

tained in good yields (80–95%). With use of an excess amount (6.0 equiv/NH_2) of **8**, a part of *N*,*N*-dialkylated products was obtained, although only mono *N*-alkylation occurred with 3.0 equiv of **8**. The structure of *N*,*N*-dialkylated moiety was partly confirmed from two different signals at δ 4.85 (H-1 of N-monoalkylated GlcN residue) and 5.05 (H-1 of *N*,*N*-dialkylated GlcN residue) in ^{1}H NMR.[1b] In any case, the reactivity of **8** was relatively low (17–25%) due to the low content of aldehyde form in melibiose **8** in aqueous medium. To refine the reactivity and modified spacer moiety, the deprotected *p*-formylphenyl β-melibioside[1d] was used (Scheme 5). The reactivity was increased to 44–54% and gave conjugates **12** having

Table 3. Chemical Structure and Binding Assay of Chitosan–Neu5Ac Conjugates to WGA Lectin

Entry	Compd	Functional group (DS)[a]				Binding to lectin[b]
		–Neu5A	–Suc	$-NH_2$	–NHAc	
1	**3**	0.53	0	0.46	0.04	++
2	**4**	0.10	0.79	0.07	0.04	++
3	**4**	0.29	0.53	0.14	0.04	++
4	**4**	0.53	0.26	0.17	0.04	++
5	**6**	0.38	0	0.58	0.04	++
6	**7**	0.23	0.59	0.14	0.04	++
7	**7**	0.38	0.51	0.07	0.04	++
8	—[c]	0	0.50	0.46	0.04	±

a) DS (Neu5Ac and Suc) were estimated by ^{1}H NMR (method, see experimental part). DS(NH_2) was estimated by clorimetric method with ninhydrin at 570 nm. Since the signal of NHAc of chitosan was overlapped with that of Neu5Ac in ^{1}H NMR, DS(NHAc) of products were settled to 0.04 which was the same value of original chitosan. b) ++, strong band; ±, very faint band. c) *N*-succinylated chitosan.

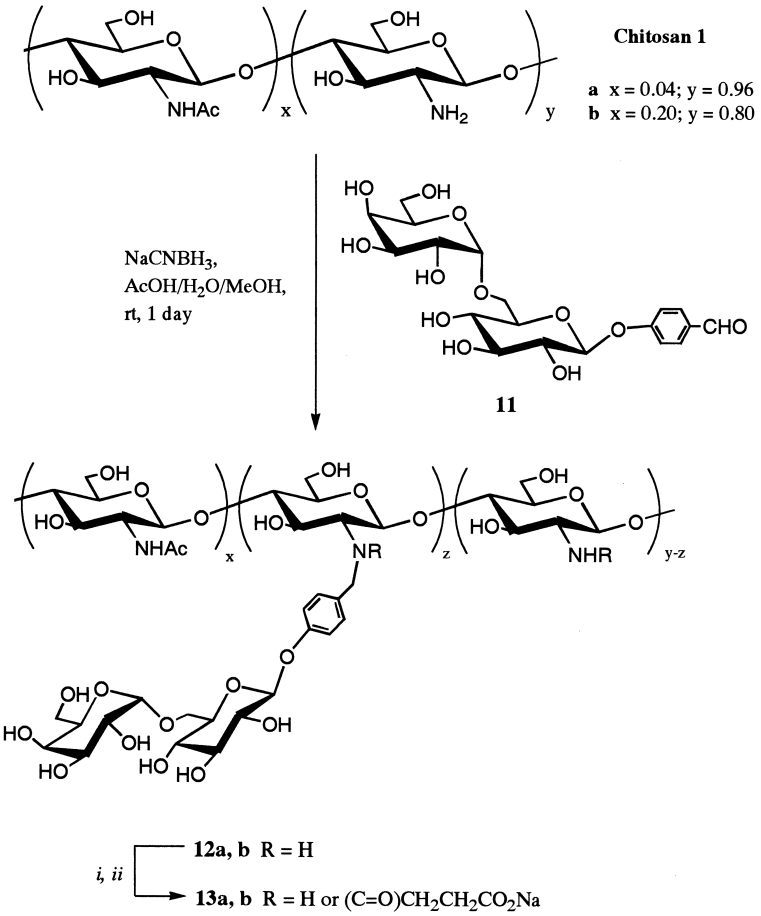


Scheme 4. Reagents and conditions: i, succinic anhydride, AcOH/H_2O/MeOH, rt, 1 day; ii, 0.5 M NaOH, rt, 2 h.

aromatic spacer in 89–95% yields. Since these conjugates **9** and **12** were insoluble in neutral water, these were transformed into *N*-succinylated conjugates **10** and **13** for the purpose of biological evaluation. The chemical structure and lectin binding assay of water-soluble *N*-succinylated chitosan–melibiose conjugates are summarized in Table 5. Some succinyl groups were also introduced onto the secondary amines of *N*-alkylated moieties of chitosan (DS of N(Sugar)–(Suc)). The protein binding properties of *N*-succinylated chitosan-melibiose conjugates **10** and **13** were evaluated with plant lectin *Griffonia simplicifolia* (GSI-B_4) which is known to be specific toward α-Gal residues. Strong immunodiffusion bands were observed for all conjugates **10** and **13**. A negative control (*N*-succinylated chitosan), on the other hand, showed no band against the

Table 4. Preparation of Chitosan–Melibiose Conjugates **9** and **12**

Entry	Chitosan		Reagent		Yield	DS	Reactivity	Compd.
	mg	NH_2		equiv	%		%	
1	100	0.96	**8**	3.0	95	0.58	19	**9a**
2	100	0.96	**8**	6.0	80	1.17	20	**9a**
3	200	0.8	**8**	3.0	84	0.76	25	**9b**
4	200	0.8	**8**	6.0	80	1.01	17	**9b**
5	40	0.96	**11**	0.5	95	0.22	44	**12a**
6	40	0.96	**11**	1.0	89	0.47	54	**12a**
7	100	0.8	**11**	0.5	90	0.26	52	**12b**
8	40	0.8	**11**	1.0	92	0.46	46	**12b**



Scheme 5. Reagents and conditions: i, succinic anhydride, AcOH/H_2O/MeOH, rt, 1 day; ii, 0.5 M NaOH, rt, 2 h.

lectin, thus demonstrating the specificity of the binding toward the α-Gal epitope in the conjugates **10** and **13**.

In conclusion, a variety of water-soluble and lectin binding chitosan–carbohydrate conjugates were successfully prepared in this study. Further biological properties of these promising conjugates will be investigated in near future.

Experimental

Materials. Two kinds of chitosan (**1b**, NH_2 = 0.8; DP = 140: Kyowa Tecnos Co. and its *N*-deacetylated product: **1a**, NH_2 = 0.96; DP = 140) were used in this study. Plant lectins of wheat germ agglutinin (WGA: *Triticum vulgaris*) and *Griffonia simplicifolia* (GSI-B_4) were purchased from Sigma Co.

General Methods. The ^1H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz AMX NMR spectrometer. Proton chemical shifts (δ) are given relative to internal 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (water soluble TMS: 0 ppm) for D_2O or 0.2 M DCl in D_2O solution (1 M = 1 mol dm^{-3}). Carbon chemical shifts are also given relative to water soluble TMS (0 ppm). Degree of polymerization (DP) of original chitosan was determined by GPC with pullulan as standard. The DS of amino group was determined by a colorimetric method with ninhydrin at 570 nm.

Preparation of Chitosan–Sialic Acid Conjugate (3, 4). Chitosan (**1a**: 100 mg) was dissolved in H_2O (10 mL), AcOH (100 mg), and MeOH (40 mL). A suitable amount of reported aldehyde[19] **2a** (Table 1) was added to the solution which was then

Table 5. Chemical Structures and Binding Assay of Chitosan–Melibiose Conjugates to *Griffonia simplicifolia* Lectin

Entry	Compd.	Mole fraction substitution (DS)[a]						Binding to lectin[c]
		NHAc	NH_2	NH Suc[b]	NH Sugar	N(Sugar)–(Suc)	N(Sugar)–(Sugar)	
1	**10a**	0.04	0	0.38	0.56	0.02	0	++
2	**10a**	0.04	0	0	0.25	0.50	0.21	++
3	**10b**	0.20	0.02	0.02	0.40	0.36	0	++
4	**10b**	0.20	0	0	0.29	0.30	0.21	++
5	**13a**	0.04	0.21	0.53	0.19	0.03	0	++
6	**13a**	0.04	0	0.49	0.43	0.04	0	++
7	**13b**	0.20	0	0.54	0.20	0.06	0	++
8	**13b**	0.20	0.02	0.32	0.14	0.32	0	++
9	d)	0.04	0.46	0.50	0	0	0	−

a) DS(NHAc) of products were almost same as original chitosan (0.04 and 2.0) by ^{1}H NMR. Other DS values were estimated by the same procedure in Table 3. b) Suc, $COCH_2CH_2CO_2Na$. c) ++, strong band; −, no band. d) *N*-succinylated chitosan.

stirred at rt. After 1 h, $NaCNBH_3$ (3 equiv/CHO) was added. After 1 day, the reaction mixture was quenched by precipitation with 5% NaOH (2 mL) and acetone (80 mL). The precipitate was collected by filtration, dispersed with 0.5 M NaOH for 2 h, dialyzed, and lyophilized to give chitosan–sialic acid conjugates **3** in 74–100% yields. The degree of substitution (DS) of conjugate **3** was determined by ^{1}H NMR from the peak ratio at δ 3.20 (0.96 H: H-2 of GlcN and *N*-alkylated GlcN) and 6.97 (Ar). The DS estimated from the ratio at δ 2.32 (H-3_{3eq} of Neu5Ac) and 3.20 (H-2) is also available. Data for **3** (DS = 0.53): ^{1}H NMR (0.2 M DCl in D_2O) δ 1.88 (t, 0.53 H, $J_{3ax-4eq}$ = 12.1 Hz, H-3_{3ax} of Neu5Ac), 2.06 (m, 1.71 H, NHAc), 2.32 (dd, 0.53 H, $J_{3eq-4eq}$ = 5.0 Hz, H-3_{3eq} of Neu5Ac), 3.20 (br, 0.96 H, H-2 of GlcN and *N*-alkylated GlcN in chitosan), 3.55–4.10 (m, H-4,5,6,7,8,9 of Neu5Ac, H-3,4,5,6 of GlcN), 6.97 (d, J = 7.44 Hz, 1.06 H, H-3,5 of Ar), 7.27 (d, J = 7.44 Hz, 1.06 H, H-2, 6 of Ar); ^{13}C NMR δ 25.0 (NHAc), 41.7 (C-3 of Neu5Ac), 53.3 (CH_2Ar), 55.0 (C-5 of Neu5Ac), 58.8 (C-2 of GlcN), 63.0 (C-6 of GlcN), 66.1 (C-9 of Neu5Ac), 69.5 (C-4 of Neu5Ac), 71.2 (C-7 of Neu5Ac), 73.2 (C-8 of Neu5Ac), 73.4 (C-6 of Neu5Ac and C-3 of GlcN), 77.7 (C-5 of GlcN), 79.4 (C-4 of GlcN), 98.1 (C-2 of Neu5Ac), 100.4 (C-1 of GlcN), 119.0 (C-2, 6 of Ar), 125.0 (C-4 of Ar), 135.0 (C-3, 5 of Ar), 159.8 (C-1 of Ar), 175.9 (NHCO), 177.9 (CO_2H of Neu5Ac).

N-succinylation of primary amino groups in conjugate **3** was performed according to the previous reports.[1a,d,20] Conjugate (**3**: 100 mg) was dispersed in H_2O (20 mL) and MeOH (60 mL) containing AcOH (100 mg). Succinic anhydride (3.0 mmol) was added in excess and the mixture was stirred at rt. After 1 day, the mixture was concentrated to ca. 10 mL, 1.0 M NaOH (10 mL) was added, and the mixture (in 0.5 M NaOH) was stirred at room temperature. After 2 h, the mixture was dialyzed for 2 days, and lyophilized to afford water-soluble *N*-succinylated conjugates **4** in 90–100% yields. Corresponding signals assigned as *N*-succinyl group in **4** were found in ^{1}H NMR (D_2O) at δ 2.54 (br, NHC(O)–CH_2) and 2.62 (br, CH_2–CO_2Na), and ^{13}C NMR at δ 35.5 and 35.8 (CH_2 of succinyl).

Chitosan–Sialic Acid Conjugate (6, 7). Chitosan (**1a**: 100 mg) was dissolved in H_2O (10 mL), AcOH (100 mg), and MeOH (40 mL). A suitable amount of reported *p*-isothiocyanatophenyl α-sialoside[22] **5** (Table 2) was added to the solution. The mixture was stirred at rt. for 1 day, and then quenched, dialyzed, and lyophilized to give chitosan–sialic acid conjugates **6** in 90–100% yields. The DS of **6** was determined by ^{1}H NMR as above. Data for **6** (DS = 0.38): ^{1}H NMR (0.2 M DCl in D_2O) δ 2.02 (m, 1.26 H, NHAc), 2.90 (br, 0.38 H, H-3_{3eq} of Neu5Ac), 3.20 (br, 0.58 H, H-2 of unsubstituted GlcN in chitosan), 3.50–4.10 (m, H-4,5,6,7,8,9 of Neu5Ac, H-3,4,5,6 of GlcN), 4.67 (s, 0.42 H, H-1 of GlcNAc and substituted GlcN), 7.38 (brs, 0.76 H, H-3,5 of Ar), 7.63 (brs, 0.76 H, H-2, 6 of Ar); ^{13}C NMR δ 25.0 (NHAc), 42.5 (C-3 of Neu5Ac), 54.5 (C-5 of Neu5Ac), 58.8 (C-2 of GlcN), 63.0 (C-6 of GlcN), 65.7 (C-9 of Neu5Ac), 70.8 (C-4 of Neu5Ac), 71.2 (C-7 of Neu5Ac), 73.0 (C-8 of Neu5Ac), 73.5 (C-3 of GlcN), 74.7 (C-6 of Neu5Ac), 77.7 (C-5 of GlcN), 79.5 (C-4 of GlcN), 89.4 (C-2 of Neu5Ac), 100.4 (C-1 of GlcN), 129.2 (C-2, 6 of Ar), 140.5 (C-3, 5 of Ar), 174.4 (NHCO), 178.0 (CO_2H of Neu5Ac). *N*-succinylation of primary amino groups in conjugate **6** was performed as above and gave conjugate **7** in 80–90% yield.

Chitosan–Melibiose Conjugate (9, 10). Chitosan (**1a**: 100 mg) was dissolved in H_2O (10 mL), AcOH (100 mg), and MeOH (40 mL). A suitable amount of commercial melibiose **8** (Table 4) was added to the solution. After 1 h, $NaCNBH_3$ (3 equiv/melibiose) was added and the mixture was stirred at rt. After 1 day, the mixture was quenched, dialyzed, and lyophilized in a similar manner to prepare conjugate **3**. The chitosan–melibiose conjugates **9** were obtained in 80–95% yields. The DS of **9** was determined by ^{1}H NMR from the peak ratio at δ 3.2–3.4 (H-2 of GlcN and *N*-alkylated GlcN) and 4.98 (H-1 of α-D-galactoside (Gal) in melibiose residue). Data for **9a** (DS = 0.58): ^{1}H NMR (0.2 M DCl in D_2O) δ 2.07 (s, 0.12 H, NHAc), 3.20–3.40 (br, 0.96 H, H-2 of GlcN and *N*-alkylated GlcN), 3.5–4.2 (br m, H-2,3,4,5,6 of β-D-glucoside (Glc) and α-D-Gal in melibiose, H-2 of GlcNAc, H-3,4,5,6 of GlcN and GlcNAc), 4.98 (br, H-1 of α-D-Gal); ^{13}C NMR δ 51.9 (NHCH_2), 63.5 (C-6 of GlcN), 64.2 (C-6 of β-D-Glc and α-D-Gal), 70.9–73.9 (C-2,3,4,5 of β-D-Glc and α-D-Gal, and C-3 of GlcN), 77.6 (C-5 of GlcN), 79.8 (C-4 of GlcN), 101.4 (C-1 of α-D-Gal). *N*-succinylations of conjugates **9** were performed as above and gave water-soluble conjugates **10** in 80–90% yields.

Chitosan–Melibiose Conjugate (12, 13). A suitable amount of reported *p*-formylphenyl β-melibioside **11** (Table 4) was used to prepare chitosan–melibiose conjugates **12** in a similar manner as above. The conjugates **12** were obtained in 89–95% yields. The DS of **12** was determined by ^{1}H NMR from the peak ratio at δ 3.20 (H-2 of GlcN and *N*-alkylated GlcN) and 7.22 (Ar). Data **12b** (DS = 0.26): ^{1}H NMR (0.2 M DCl in D_2O) δ 2.07 (s, 0.60 H, NH*Ac*), 3.20 (br m, 0.80 H, H-2 of GlcN and *N*-alkylated GlcN), 3.5–4.2 (br m, H-2,3,4,5,6 of β-D-Glc and α-D-Gal, H-2 of GlcNAc, H-3,4,5,6 of GlcN and GlcNAc), 4.60 (br, -NH-CH_2-Ph), 4.87 (br, H-1 of β-D-Glc), 5.25 (br, H-1 of α-D-Gal), 7.22 (br, 0.52 H, H-2,6 of Ar), 7.49 (br, 0.52 H, H-3,5 of Ar); ^{13}C NMR δ 25.1 (NHAc), 58.8 (C-2 of GlcN), 63.1 (C-6 of GlcN), 63.9 (C-6 of β-

D-Glc and α-D-Gal), 100.4 (C-1 of β-D-Glc), 101.6 (C-1 of α-D-Gal), 119.6 (C-2 and C-6 of Ar), 127.7 (C-4 of Ar), 135.4 (C-3 and C-5 Ar), 160.2 (C-1 Ar). *N*-succinylations of conjugates **12** were performed as above and gave water-soluble conjugates **13** in 80–90% yields.

Lectin Binding Assay. Agar gel diffusion experiments were performed in 1% agarose (BDH) containing 2% polyethylene glycol (MW = 8,000, Sigma) in phosphate buffered saline (PBS) according to a published report.[23] The concentration of conjugates were at 1 mg mL^{-1} in PBS, and that of the lectin (WGA or GSI-B_4) was 2 mg mL^{-1}. The precipitation bands were allowed to form overnight at 4 °C in a humid chamber.

We are indebted to Nippon Gaishi Co., Japan for a generous supply of *N*-acetylneuraminic acid.

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