Chemical modification of chitosan: preparation of chitosan–sialic acid branched polysaccharide hybrids

Hitoshi Sashiwa,[a] Yutaka Makimura,[a] Yoshihiro Shigemasa[b] and René Roy*[a]

[a] *Department of Chemistry, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada. E-mail: rroy@science.uottowa.ca*
[b] *Faculty of Engineering, Tottori University, Tottori, 680-8552, Japan*

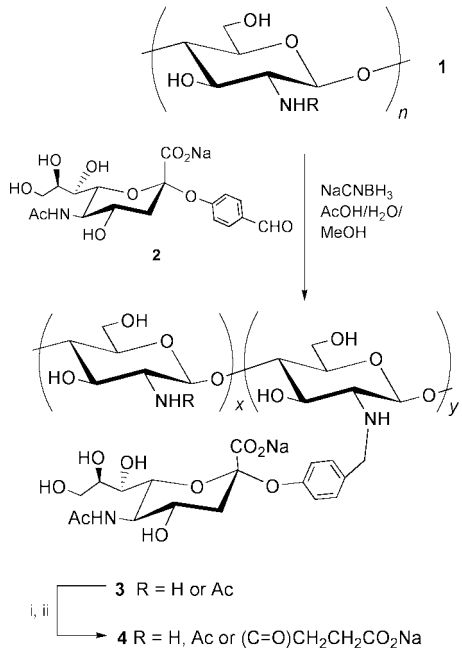
Received (in Corvallis, OR, USA) 6th March 2000, Accepted 11th April 2000
Published on the Web 9th May 2000

Water soluble hybrids of chitosan and sialic acid were prepared in good yields *via* reductive *N*-alkylation of *p*-formylphenyl α-sialoside 2 onto chitosan amine functionalities.

Chitosan **1** is a polysaccharide mainly composed of the β(1-4)-2-amino-2-deoxy-D-glucopyranose (D-glucosamine) repeating unit and includes a small amount (< 20%) of *N*-acetyl-D-glucosamine (GlcNAc) residues. At present, several interesting biological properties have been reported for chitosan alone, such as wound healing,[1] immunological activity,[2] and antibacterial effects.[3] Moreover, chitosan itself is non-toxic. Therefore, chitosan is an appealing bioactive polymer for further development. Additionally, sialic acid-containing polymers[4] have been shown to be potent inhibitors of hemagglutinination of human erythrocytes by *influenza* viruses.[4–6] *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.[7] We are now investigating the preparation and biological properties of sialic acid-bound chitosan (chitosan–sialic acid hybrid) as a new family of sialic acid-containing polymers. A noteworthy point is the effect of chitosan as a backbone polymer for investigating the biological properties of sialic acid, especially against infection by the *influenza* virus and for its immunological activity. We report herein the first preparation and chemical aspects of chitosan–sialic acid hybrids.

Reductive *N*-alkylation[8,9] of chitosan with aldehydes is a very convenient method for its chemical modification. Therefore, we attempted the direct reductive *N*-alkylation of chitosan with *p*-formylphenyl α-sialoside **2**.[10] Scheme 1 shows the preparation of the chitosan–sialic acid hybrid **3**† by reductive *N*-alkylation using sodium cyanoborohydride ($NaCNBH_3$). The degree of substitution (DS) of Neu5Ac can be controlled by increasing the amount of aldehyde **2** in the reaction mixture (Table 1). The reactivity level of **2** to chitosan was found to vary in the range 25–48%, which was caused by the simultaneous reduction of some of the aldehyde groups of **2** under the acidic reaction conditions. Water soluble material was obtained only at high DS (DS = 0.53) due to the high level of charged carboxyl groups.

Hybrids **3** of low DS were insoluble in neutral water and thus would not be useful for biological evaluation. To improve the solubility, the remaining amino groups of the hybrids were transformed by succinylation with succinic anhydride (AcOH, H_2O, MeOH, room. temp., 1 day, neutralized with aq. NaOH then dialyzed and lyophilized) to give hybrids **4** in 90–100% yields. Under these mild aqueous conditions and basic workup, no lactones or cyclic imides were formed.[9] The chemical structures of the succinylated hybrids **4** are summarized in Table 2. Despite using a large excess of succinic anhydride, some unreactive amino groups (DS = 0.07–0.17) still remained. Complete succinylation was difficult owing to increasing steric hindrance of the polymer. High field ^1H NMR spectra also indicated that no succinylation had occurred at the *N*-glycosylation sites (δ H-1 of GclN-Neu5Ac). All succinylated products **4** were soluble in water. The protein binding properties of the novel sialylated polysaccharides were initially evaluated with wheat germ agglutinin (WGA: *Triticum vulgaris*) which is a plant lectin specific toward GlcNAc and Neu5Ac residues. Strong immunodiffusion bands were observed for water soluble hybrids **3** and **4** when compared to a negative control (*N*-succinylated chitosan), thus demonstrating the specificity of the binding of the Neu5Ac epitope in the hybrid to WGA lectin. The



Scheme 1 *Reagents and conditions*: (i) succinic anhydride (0.2 g, 2 mmol), AcOH (50 mg, 0.83 mmol), H_2O (8 mL), MeOH (32 mL); (ii) 1% aq. NaOH (20 mL), room temp., 2 h.

Table 1 Preparation of conjugates **3** with aldehyde **2**

Entry	**2** (equiv.)	DS[a]	Yield[b] (%)	Solubility in H_2O	MW[c]
1	0.2	0.06	100	No	27000
2	0.4	0.10	77	No	29000
3	0.6	0.29	76	No	39000
4	0.9	0.44	74	No	48000
5	1.2	0.53	84	Yes	53000

[a] DS was determined from the peak area of phenyl protons (δ 7.0–7.4) and H-2 of GlcN and *N*-alkylated GlcN residues (δ 3.2: 0.96 H). [b] Yield determined by weight recovery and accounting for changes in FW according to the substitution level determined by NMR spectroscopy. [c] MW calculated on the basis of the original chitosan MW of 23 061 (DP = 140) and accounting for FW changes based on NMR data (values rounded to the nearest thousand).

DOI: 10.1039/b001861i

Table 2 Chemical structures and binding assay of chitosan–Neu5Ac derivatives to WGA lectin

Compd.	Functional group (DS) –Sugar	–Suc	$–NH_2$	–NHAc	MW	Binding to lectin[a]
4	0.10	0.79	0.07	0.04	40000	++
4	0.29	0.53	0.14	0.04	47000	++
4	0.53	0.26	0.17	0.04	59000	++
3	0.53	0	0.43	0.04	53000	++
—[b]	0	0.50	0.46	0.04	30000	±

[a] ++, strong band; ±, very faint band. [b] *N*-succinylated chitosan.

very faint band shown in *N*-succinylated chitosan could be due to the small amount of GlcNAc residues already present in the initial polymer chain (DS = 96% GlcN; 4% GlcNAc).

In conclusion, water soluble and lectin binding chitosan–sialic acid hybrids have been successfully prepared. Further biological evaluation of these promising compounds will be investigated.

We are indebted to Nippon Gaishi Co., Japan for generously supplying sialic acid.

Notes and references

† *Materials and methods:* chitosan **1** [DS = 96% GlcN, 4% GlcNAc, DP = 140; molecular weight (MW) 23061] was used in this study. The degree of polymerization (DP) of the initial chitosan **1** was determined by GPC using pullulan as standard. The DS of the hybrids was determined by ^{1}H NMR spectroscopy (Bruker 500 MHz AMX). The remaining unmodified primary amino groups of the hybrids were quantitated by colorimetric determination using ninhydrin at 570 nm.

Typical procedure for the preparation of hybrid **3**: Chitosan (50 mg, 0.24 mmol NH_2) was dissolved in H_2O (8 mL) and MeOH (32 mL) containing AcOH (50 mg, 0.83 mmol). Various amounts of **2** (Table 1) were added to the solution which was stirred at room temperature. After 1 h, $NaCNBH_3$ (100 mg, 1.6 mmol) was added and after 1 day, the reaction mixture was quenched by precipitation with 5% aq. NaOH (2 mL, 2.5 mmol) and acetone (80 mL). The precipitate was collected by filtration, dispersed with H_2O containing NaOH (100 mg), dialyzed and lyophilized.

Selected 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_{ax} of NeuNAc), 2.06 (m, 1.71 H, NHAc of Neu5Ac and GlcNAc in chitosan), 2.32 (dd, 0.53 H, $J_{3eq–4eq}$ = 5.0 Hz, H-3_{eq} of Neu5Ac), 3.20 (br, 0.96 H, H-2 of GlcN and *N*-alkylated GlcN), 3.55–4.10 (m, H-4,5,6,7,8,9 of Neu5Ac, H-2 of GlcNAc, H-3,4,5,6 of GlcN and GlcNAc), 6.97 (d, 1.06 H, J = 7.44 Hz, H-3 and H-5 of –OPh), 7.27 (d, 1.06 H, *J* 7.44 Hz, H-2 and H-6 of –OPh); ^{13}C NMR δ 25.0 (NAc), 41.7 (C-3 of Neu5Ac), 53.3 (CH_2Ph), 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), 77.7 (C-3 and 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 and C-6 of Ph), 125.0 (C-4 of Ph), 135.0 (C-3 and C-5 of Ph), 159.8 (C-1 of Ph), 175.9 (NHCO), 177.9 (CO_2H of Neu5Ac).

Succinylation of hybrid was performed as described previously.[9]

Agar gel diffusion experiments were performed in 1% agarose (BDH) containing 2% poly(ethylene glycol) (MW = 8000, Sigma) in phosphate-buffered saline (PBS) according to the method of Ouchterlony and Nilsson.[11] The concentration of conjugate **3** was 1 mg mL^{-1} in PBS, and that of WGA lectin was 2 mg mL^{-1}. The precipitation bands were allowed to form overnight at 4 °C in a humid chamber.

1 S. Minami, Y. Okamoto, A. Matsuhashi, H. Sashiwa, H. Saimoto, Y. Shigemasa, T. Tanigawa, T. Tanaka and S. Tokura, in *Advances in Chitin and Chitosan*, ed. C. J. Brine, P. A. Sandford and J. P. Zikakis, Elsevier, London, 1992, p. 61.

2 K. Nishimura, S. Nishimura, N. Nishi, I. Saiki, S. Tokura and I. Azuma, *Vaccine*, 1984, **2**, 93.

3 T. Tanigawa, Y. Tanaka, H. Sashiwa, H. Saimoto and Y. Shigemasa, in *Advances in Chitin and Chitosan*, ed. C. J. Brine, P. A. Sandford and J. P. Zikakis, Elsevier, London, 1992, p. 206.

4 R. Roy, C. A. Laferrière, A. Gamian, M. Chomik and H. J. Jennings, *J. Carbohydr. Chem.*, 1987, **6**, 161; R. Roy and C. A. Laferrière, *Carbohydr. Res.*, 1988, **177**, C1; A. Gamian, M. Chomik, C. A. Laferriere and R. Roy, *Can. J. Microbiol.*, 1991, **37**, 233; R. Roy, F. O. Andersson, G. Harm, S. Kelm and R. Schauer, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 1478.

5 N. E. Byramova, M. N. Mochalova, J. M. Belyanchikov, M. N. Matrosovich and N. V. Bovin, *J. Carbohydr. Chem.*, 1991, **10**, 691.

6 G. B. Sigal, M. Mammen, G. Dahmann and G. M. Whitesides, *J. Am. Chem. Soc.*, 1996, **118**, 3789 and references therein.

7 K. A. Karlsson, *Curr. Opin. Struct. Biol.*, 1995, **5**, 622.

8 R. A. A. Muzzarelli, F. Tanfani, M. Emanuell and S. Mariotti, *Carbohydr. Res.*, 1982, **107**, 199; R. A. A. Muzzarelli, F. Tanfani, S. Mariotti and M. Emanuell, *Carbohydr. Polym.*, 1982, **2**, 145.

9 H. Sashiwa and Y. Shigemasa, *Carbohydr. Polym.*, 1999, **39**, 127.

10 R. Roy, D. F. Tropper, A. Romanowska, M. Letellier, L. Cousineau, S. J. Meunier and J. Boratynski, *Glycoconjugate J.*, 1991, **8**, 75.

11 O. Ouchterlony and L. A. Nilsson, in *Handbook of Experimental Immunology*, ed. D. M. Weir, Blackwell Scientific Publications, Oxford, 1978, ch. 19.