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Chemical modification of chitosan 8: preparation of chitosan–dendrimer hybrids via short spacer

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Abstract

Polyamidoamine (PAMAM) dendrimers of various generations ($G = 0.5$–5) were prepared from commercial aminoacetaldehyde diethyl acetal. After transforming acetal to aldehyde, chitosan–dendrimer hybrids were prepared by reductive *N*-alkylation. The reactivity of dendrimer to primary amino group of chitosan was decreased at $G = 3.5$ or above MW > 6305. Chitosan–sialodendrimer hybrid ($G = 3$) was also prepared under the same conditions. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Chitosan; Dendrimer; Polyamide amine; Aldehyde; Sialic acid; Short spacer

1. Introduction

Chitosan is a polysaccharide formed primarily of repeating units of β(1-4)-2-amino-2-deoxy-D-glucopyranose, although it includes small amount of *N*-acetyl-D-glucosamine (generally below 20%). Chitosan shows interesting biological properties such as immunological (Nishimura, Nishimura, Nishi, Saiki, Tokura & Azuma, 1984), antibacterial (Tanigawa, Tanaka, Sashiwa, Saimoto & Shigemasa, 1992), and wound healing activity (Minami, Okamoto, Matsuhashi, Sashiwa, Saimoto, Shigemasa et al., 1992). Moreover, chitosan has also been showed to be non-toxic and biodegradable (Sashiwa, Saimoto, Shigemasa, Ogawa & Tokura, 1990; Shigemasa, Saito, Sashiwa & Saimoto, 1994) in vivo experiments. Dendrimers offer several possibilities in molecular design owing to their multifunctional properties such as neoglycoconjugates (Zanini and Roy, 1997), host-guest chemistry, dendritic catalysts, and so on (Bosman, Janssen & Meijer, 1999; Frechet, 1994). Most recently, we prepared chitosan–dendrimer hybrid via tetra(ethylene glycol) spacer (Sashiwa, Shigemasa & Roy, 2000). However, several steps were necessary to suitably functionalized 11-amino-3,6,9-trioxanedecanal as a starting material to build polyamidoamine (PAMAM)-based dendrimers. Since long spacer are not necessary to build chitosan–dendrimer hybrid, shorter commercially available reagents such as aminoacetaldehyde diethylacetal **1** should be more convenient to prepare chitosan attachable PAMAM dendrimers. Furthermore, it would still be interesting to explore the effect of spacers of different length to prepare chitosan–dendrimer hybrid on receptor binding interaction.

In this study, we report the preparation of chitosan–dendrimer hybrid via short spacer and high generation. Additionally, we also report the preparation of sialic acid bound chitosan–dendrimer hybrids.

2. Experimental section

2.1. Materials

Chitosan (Flonac C, NHAc = 0.2, DP = 140, FW of unit = 169) was purchased Kyowa Tecnos Co, Japan. Aminoacetaldehyde diethylacetal (FW = 133.2) and other reagents were also purchased from Aldrich Co., and used without further purification. Dialysis membrane (MW 12,000 cut off) was purchased from Sigma Co.

2.2. General methods

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz AMX NMR spectrometer. Proton chemical shifts (δ) are given relative to internal $CHCl_3$ for $CDCl_3$ or

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3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (water soluble TMS: 0 ppm) for D_2O or 0.5 M DCl in D_2O solution. Carbon chemical shifts are also given relative to $CDCl_3$ or water soluble TMS (0 ppm). The degree of substitution (DS) of the hybrid was determined by ^1H NMR. Degree of polymerization (DP) of chitosan was determined by GPC (Sashiwa & Shigemasa, 1999) with pullulan as standard on a Shimadzu LC-6A apparatus (column, Asahipak GS-220H, GS-310H, and GS-510 H; eluent, 0.1 M AcOH buffer (pH 4.7) containing 0.1 M NaCl; flow rate, 1.0 ml/min; temperature, 50°C).

2.3. Preparation of methyl ester **2** ($G = 0.5$)

The compound **2** was prepared via Michael reaction according to the reported procedure (Aoi, Itoh, Okada et al., 1997; Tomalia, Naylor & Goddard, 1990). Typical procedure is described as follows. To a solution of amine (**1**: 20 mmol) in MeOH (30 ml) was added methyl acrylate (60 mmol: 3 equiv). The mixture was stirred at 40°C and monitored by TLC (CH_2Cl_2/MeOH = 10/1 in addition with small amount of Et_3N). After five days, the mixture was evaporated to dryness and purified by column chromatography using a gradient from CH_2Cl_2/MeOH = 40/1 to 10/1 with Et_3N to give methyl ester **2** in qyantitative yield. Data for **2**: ^1H NMR ($CDCl_3$) δ 1.17 (t, $J = 7.1$ Hz, 6 H, C*H*$_3$ of Et), 2.44 (br, 4 H, NC*H*$_2$(3)), 2.58 (d, $J = 4.1$ Hz, 2 H, C*H*$_2$(2)N), 2.84 (br, 4 H, C*H*$_2$(4)CO_2Me), 3.49 and 3.52 (br, $J = 7.1$ Hz, 4 H, OC*H*$_2$ of Et), 3.63 (s, 6 H, CO_2*Me*), 4.62 (s, 1 H, C*H*(1) of acetal); ^{13}C NMR ($CDCl_3$) δ 15.3 (*C*H$_3$), 32.7 (*C*H$_2$(4)CO_2Me), 50.3 (N*C*H$_2$(3)), 51.5 (CO_2*Me*), 57.1 (*C*H$_2$(2)N), 62.5 (*C*H$_2$ of Et), 102.3 (*C*H(1) of acetal), 172.9 (*C*O$_2$Me); FAB-MS (pos) calcd for $C_{14}H_{27}N_1O_6$ 305, found 306.2 ($M^+ + 1$, 58% base peak). 1–4 means: $(EtO)_2CH(1)CH_2(2)N(CH_2(3)CH_2(4)CO_2Me)_2$

2.4. Preparation of diamine **3** ($G = 1$)

To a solution of methyl ester (**2**: 20 mmol) in MeOH was added ethylenediamine (400 mmol: 20 equiv), and the mixture was stirred at room temperature for 3 days. The mixture was evaporated and then dried completely using pump to remove excess amount of ethylenediamine, and obtained crude diamine **3** (***G* = 1**). Since it was difficult to purify diamine **3** by column chlomatography, it was used directly to the next step. Data for **3**: ^1H NMR ($CDCl_3$) δ 1.12 (t, $J = 7.1$ Hz, 6 H, C*H*$_3$ of Et), 2.29 (t, $J = 6.3$ Hz, 4 H, NC*H*$_2$(3)), 2.54 (d, $J = 4.9$ Hz, 2 H, CHC*H*$_2$(2)N), 2.73–2.75 (m, 8 H, C*H*$_2$(4)CONHC*H*$_2$(5)), 3.12 (br, 4 H, N*H*$_2$), 3.20 (dd, $J = 5.9$ Hz, 4 H, CH_2(6)NH_2), 3.44 and 3.59 (m, 4 H, OC*H*$_2$ of Et), 4.48 (t, $J = 4.9$ Hz, 1 H, C*H*(1) of acetal), 7.24 (br, 2 H, CON*H*(*a*)); ^{13}C NMR ($CDCl_3$) δ 15.3 (*C*H$_3$), 33.9 (*C*H$_2$(4)CONH) (N*C*H$_2$(3)), 41.1 (CONHC*H*$_2$(5)), 41.5 (*C*H$_2$(6)NH$_2$), 51.0 (N*C*H$_2$(3)), 56.6 (CHC*H*$_2$(2)N), 62.2 (*C*H$_2$ of Et), 101.2 (*C*H(1) of acetal), 172.9 (*C*ONH(a)); FAB-MS (pos) calcd for $C_{16}H_{35}N_5O_4$ 361, found 361.7 ($M^+ + 1$, 100% base peak). 1–6: $(EtO)_2CH(1)CH_2(2)N(CH_2(3)CH_2(4)CONH(a)CH_2(5)CH_2(6)NH_2)_2$

2.5. Preparation of dendrimers (***4*–*11*: *G* = 1.5–5**)

The preparation of dendrimers of methyl esters **4** ($G = 1.5$), **6** ($G = 2.5$), **8** ($G = 3.5$), **10** ($G = 4.5$) and amines **5** ($G = 2$), **7** ($G = 3$), **9** ($G = 4$), **11** ($G = 5$) were prepared in a similar manner as above. The yields of **4**, **6**, **8**, and **10** were 100, 84, 44 and 60%, respectively. Amines **5**, **7**, **9**, **11** were obtained as quantitative yields, respectively. Data for **4** ($G = 1.5$): ^1H NMR ($CDCl_3$) δ 1.12 (t, $J = 7.1$ Hz, 6 H, C*H*$_3$), 2.40 (m, 4 H, NC*H*$_2$(3, 7)), 2.52 (t, $J = 6.1$ Hz, 4 H, C*H*$_2$(6)N), 2.64 (d, $J = 4.4$ Hz, 2 H, CHC*H*$_2$(2)N), 2.73 (t, $J = 6.7$ Hz, 8 H, C*H*$_2$(8)CO_2Me), 2.86 (br, 4 H, C*H*$_2$(4)CO), 3.25 (dd, $J = 5.8$ Hz, 4 H, CONHC*H*$_2$(5)), 3.50 and 3.6–3.7 (m, 4 H, C*H*$_2$ of Et), 3.64 (s, 12 H, CO_2*Me*), 4.57 (br, 1 H, C*H*(1) of acetal), 7.10 (br, 2 H, CON*H*(*a*)); ^{13}C NMR ($CDCl_3$) δ 15.3 (*C*H$_3$), 32.6 (*C*H$_2$(8)CO_2Me), 36.2 (*C*H$_2$(4)CONH), 37.1 (CONHC*H*$_2$(5)), 49.2 (N*C*H$_2$(7)), 50.5 (N*C*H$_2$(3)), 51.6 (CO_2*Me*), 52.9 (*C*H$_2$(4)N), 57.5 (CH*C*H$_2$(2)N), 62.2 (*C*H$_2$ of Et), 102.4 (*C*H(1) of acetal); FAB-MS (pos) calcd for $C_{32}H_{59}N_5O_{12}$ 705, found 706.3 (M^+, 13% base peak). CH_2(1–2), see compound **2**; CH_2(3–8): N[CH_2(3)CH_2(4)CONH(a)CH_2(5)CH_2(6)N(CH_2(7)CH_2(8)CO_2Me)$_2$]$_2$.

Data for **5** ($G = 2$): ^1H NMR ($CDCl_3$) δ 1.18 (t, $J = 7.1$ Hz, 6 H, C*H*$_3$), 2.36 (br, 16 H, NC*H*$_2$(7) and N*H*$_2$), 2.54 (br, 4 H, NC*H*$_2$(3)), 2.62 (br, 2 H, CHC*H*$_2$(2)N), 2.74–2.84 (m, 28 H, C*H*$_2$(4,5,6,8,9)), 3.26 (br, 8 H, C*H*$_2$(10)NH$_2$), 3.52 and 3.65 (m, 4 H, C*H*$_2$ of Et), 4.57 (br, 1 H, C*H*(1) of acetal), 7.96 (br, 4 H, CON*H*(*b*)), 8.00 (br, 2 H, CON*H*(*a*)); ^{13}C NMR ($CDCl_3$) δ 15.0 (*C*H$_3$), 33.3 (*C*H$_2$(4)CO), 33.6 (*C*H$_2$(8)CO), 39.6 (NH*C*H$_2$(5)), 40.8 (NH*C*H$_2$(9)), 41.4 (*C*H$_2$(6)N), 41.5 (*C*H$_2$(10)NH$_2$), 50.1 (N*C*H$_2$(7)), 52.3 (N*C*H$_2$(3)), 56.0 (*C*H$_2$(2)N), 61.6 (*C*H$_2$ of Et), 100.9 (*C*H(1) of acetal), 172.0 (CO(a)), 172.4 (CO(b)); FAB-MS (pos) calcd for $C_{36}H_{75}N_{13}O_8$ 817, found 818.5 ($M^+ + 1$, 16% base peak). CH_2(7–10):[–N(CH_2(7)CH_2(8)-CONH(b) CH_2(9)CH_2(10)NH_2)$_2$]$_2$.

Data for **6** ($G = 2.5$): ^1H NMR ($CDCl_3$)δ 1.12 (t, $J = 7.1$ Hz, 6 H, C*H*$_3$), 2.36 (br, 16 H, NC*H*$_2$(7) and N*H*$_2$), 2.54 (br, 4 H, NC*H*$_2$(3)), 2.62 (br, 2 H, CHC*H*$_2$(2)N), 2.74–2.84 (m, 28 H, C*H*$_2$(4,5,6,8,9)), 3.26 (br, 8 H, C*H*$_2$(10)NH$_2$), 3.52 and 3.65 (m, 4 H, C*H*$_2$ of Et), 3.64 (s, 24 H, CO_2*Me*), 4.57 (br, 1 H, C*H*(1) of acetal), 7.96 (br, 4 H, CON*H*(*b*)), 8.00 (br, 2 H, CON*H*(*a*)); ^{13}C NMR ($CDCl_3$) δ 15.0 (*C*H$_3$), 33.3 (*C*H$_2$(4)CO), 33.6 (*C*H$_2$(8)CO), 39.6 (NH*C*H$_2$(5)), 40.8 (NH*C*H$_2$(9)), 41.4 (*C*H$_2$(6)N), 41.5 (*C*H$_2$(10)NH$_2$), 50.1 (N*C*H$_2$(7)), 51.6 (CO_2*Me*), 52.3 (N*C*H$_2$(3)), 56.0 (*C*H$_2$(2)N), 61.6 (*C*H$_2$ of Et), 100.9 (*C*H(1) of acetal), 172.0 (CO(a)), 172.4 (CO(b)); FAB-MS (pos) calcd for $C_{68}H_{123}N_{13}O_{24}$ 1505, found 1506.8 ($M^+ + 1.8$, 1.1% base peak). CH_2(7–12):[–N(CH_2(7)CH_2(8)CONH(b)CH_2(9)CH_2(10)N(CH_2(11)CH_2(12)CO_2Me)$_2$)$_2$]$_2$

Data for **7**($G = 3$): ^{1}H NMR (D_2O)δ1.26 (t, $J = 7.1$ Hz, 6 H, CH_3), 2.50 (t, $J = 7.1$ Hz, 28 H, NCH_2(3,7,11)), 2.70 (t, $J = 7.3$ Hz, 14 H, CH_2N(2,6,10)), 2.76–2.92 (m, 28 H, CH_2CO(4,8,12)), 3.30–3.40 (m, 44 H, CONHCH_2(5,9,13) and CH_2NH_2(14)), 3.68 and 3.82 (m, 4 H, CH_2 of Et), 4.74 (t, $J = 5.1$ Hz, 1 H, C*H*(1) of acetal), 8.14 (br,CON*H(a)*), 8.15 (br, CON*H(b)*), 8.51 (br, CON*H(c)*); ^{13}C NMR (D_2O) δ 14.05 (*C*H_3), 32.1 (*C*H_2CO(4)), 32.3 (*C*H_2(8)CO), 32.4 (*C*H_2CO(12)), 36.3 (CONHCH_2(5,9,13)), 39.3 (*C*H_2N (6,10,)), 41.0 (*C*H_2(14)NH_2), 48.6 (N*C*H_2(3, 11)), 50.8 (N*C*H_2(7)), 55.1 (*C*H_2(2)N), 63.0 (*C*H_2 of Et), 101.0 (*C*H(1) of acetal), 174.2 (NH*C*O(*a*)), 174.3 (NH*C*O(*b*)), 174.6(NH*C*O(*c*)); CH_2(11–14):[N(CH_2(11)CH_2(12)CONH (c)CH_2(13)CH_2(14)NH_2)$_2$)$_2$]$_2$.

Data for **8** ($G = 3.5$): ^{1}H NMR ($CDCl_3$) δ1.12 (t, $J =$ 7.1 Hz, 6 H, CH_3), 2.34–2.65 (br, 90 H, NCH_2 (2,3,6,7,10,11,14,15)), 2.70–2.90 (m, 60 H, CH_2 CO(4,8,12,16)), 3.23 (br, 28 H, CONHCH_2(5,9,13)), 3.45 and 3.65 (m, 4 H, CH_2 of Et), 3.62 (s, 48 H, CO_2*Me*), 4.57 (br, 1 H, C*H*(1) of acetal), 7.00 (br, 8 H, CON*H(c)*), 7.70 (br, 4 H, CON*H(b)*), 7.80 (br, 2 H, CON*H(a)*); ^{13}C NMR ($CDCl_3$) δ 15.3 (*C*H_3), 32.2 (*C*H_2(4)CO), 32.5 (N*C*H_2 (11)), 32.6 (*C*H_2CO_2Me(16)), 33.7 and 36.7 (*C*H_2 (8,12)CO), 37.4 (CONH*C*H_2(5)), 37.8 (CONH*C*H_2(9)), 49.2 (N*C*H_2(15)), 49.7 (CONH*C*H_2(7,13)), 50.2 (N*C*H_2 (3)), 51.5 (CO_2*Me*), 52.2 (*C*H_2N(10)), 52.9 (*C*H_2N(14)), 53. 8 (*C*H_2N(6)), 56.8 (*C*H_2N(2)), 62.0 (*C*H_2 of Et), 100.6 (*C*H(1) of acetal), 172.3 (*C*ONH(c)), 172.4 (*C*ONH(b, a)), 173.0 (*C*O_2Me). CH_2(11–16):[N(CH_2(11)CH_2(12)CONH (c)CH_2(13)–CH_2(14)N(CH_2(15)CH_2(16)CO_2Me)$_2$)$_2$]$_2$.

Data for **9** ($G = 4$): ^{1}H NMR (D_2O); δ1.23 (t, $J = 7.1$ Hz, 6 H, CH_3), 2.45–2.55 (m, 60 H, NCH_2(3,7,11,15)), 2.68 (m, 30 H, CH_2N(2,6,10,14)), 2.88 (m, 60 H, CH_2 CO(4,8,12,16)), 3.30–3.40 (m, 92 H, CONHCH_2(5,9,13,17) and CH_2NH_2(18)), 3.68 and 3.82 (m, 4 H, CH_2 of Et), 4.42 (br, 1 H, C*H*(1) of acetal), 8.11 (br,CON*H(a)*), 8.12 (br, CON*H(b)*), 8.15 (br, CON*H(c)*), 8.51 (br, CON*H(d)*); ^{13}C NMR (D_2O) δ 14.05 (*C*H_3), 32.3 (*C*H_2CO(4,8,12,16)), 36.3 (CONH*C*H_2(5,9,13,17)), 39.2–40.5 (*C*H_2N(6,10,14)), 43.9 (*C*H_2(18)NH_2), 48.6–51.4 (N*C*H_2(3,7,11,15)), 57.0 (*C*H_2(2)N), 63.3 (*C*H_2 of Et), 101.0 (*C*H(1) of acetal), 174.2 (NH*C*O(*a,b*)), 174.5 (NH*C*O(*c*)), 174.7 (NH*C*O(*d*)); CH_2(15–18):[N(CH_2(15)CH_2(16)CONH(d)CH_2(17)CH_2(18) NH_2)$_2$)$_2$]$_2$.

Data for **10** ($G = 4.5$): ^{1}H NMR ($CDCl_3$) δ 1.12 (br, 6 H, CH_3), 2.20–2.55 (br, 186 H, NCH_2(2,3,6,7,10,11,14, (15,18,19)), 2.60–2.80 (m, 124 H, CH_2CO4,8, 12,16,20)), 3.20 (br, 60 H, CONHCH_2(5,9,13,17)), 3.45 and 3.65 (m, 4 H, CH_2 of Et), 3.57 (s, 96 H, CO_2*Me*), 4.53 (br, 1 H, C*H*(1) of acetal), 7.10 (s, 16 H, CON*H(d)*), 7.86 (s, 8 H, CON*H(c)*), 8.00 (s, 4 H, CON*H(b)*), 8.06 (s, 2 H, CON*H(a)*); ^{13}C NMR ($CDCl_3$) δ 15.3 (*C*H_3), 32.2 (*C*H_2(4)CO), 32.4–33.5 (*C*H_2(8,12,16)CO), 37.0 (CONH*C*H_2(13)), 37.1 (CONH*C*H_2 (5,17)), 37.7 (CONH*C*H_2(9)), 49.2 (N*C*H_2(7, 11, 15, 19)), 49.7 (*C*H_2CO_2Me(20)), 50.2 (N*C*H_2(3)), 51.3 (CO_2*Me*), 52.2 (*C*H_2N(18,14), 52.9 (*C*H_2N(10)), 54.3 (*C*H_2N(6), 56.8 (*C*H_2N(2)), 62.0 (*C*H_2 of Et), 100.6 (*C*H(1) of acetal), 172.0 (*C*ONH(a,b,c,d,e)), 172.8 (*C*OOMe); CH_2(17–20):[–CONH(d)CH_2(17)CH_2(18)N(CH_2(19)CH_2(20)CO_2Me)$_2$)$_2$)$_2$]$_2$.

Data for **11** ($G = 5$): ^{1}H NMR (D_2O) δ 1.27 (t, $J =$ 7.1 Hz, 6 H, CH_3), 2.50 (m, 124 H, NCH_2(3,7,11,15,19)), 2.68 (m, 62 H, CH_2N(2,6,10,14,18)),2.70–2.90 (m, 124 H, CH_2CO(4,8,12,16,20)), 3.3 (t, $J = 6.3$ Hz, 124 H, CONHCH_2(5,9,13,17,21)), 3.36 (m, 64 H, CH_2NH_2(22)), 4.42 (br, 1 H, C*H*(1) of acetal), 7.00 (br, 8 H, CON*H(c)*), 8.03 (br, CON*H(a,b)*), 8.06 (br, CON*H(c,d)*), 8.52 (br, CON*H(e)*); ^{13}C NMR (D_2O) δ32.3 (*C*H_2CO(4,8,12,16, 20)), 36.3 (CONH*C*H_2(5,9,13,17,21)), 39.3 (*C*H_2N(6,10, 14,18)), 41.9 (*C*H_2(22)NH_2), 48.7 (N*C*H_2(3,7,11,15,19)), 174.1 (NH*C*O(*a,b,c*)), 174.6 (NH*C*O(*d,e*)); CH_2(19–22):[N(CH_2(19)CH_2(20)CONH(e)CH_2(21)CH_2(22)NH_2)$_2$)$_2$]$_2$.

2.6. Removal of acetal and the preparation of chitosan–dendrimer hybrid

The removal of acetal from compond **2** was performed as follows. Compound **2** (350 mg: 1.15 mmol) was dissolved in trifluoroacetic acid (2.0 g) and 2 M HCl (0.5 g). After stirring at room temperature for 1 day, the mixture was evaporated and dried completely to give aldehyde **12**. The aldehyde **12** was used for the next step without any purifications. Chitosan (100 mg: 0.47 mmol of NH_2) was dissolved in water (10 ml) containing acetic acid (30 mg: 0.5 mmol). To a solution was added **12** (0.66 mmol: 1.4 equiv/NH_2) which dissolved in a mixed solvent of water (2 ml) and methanol (8 ml). The mixture was diluted with methanol (30 ml) and stirred. After 1 h, $NaBH_3CN$ (120 mg: 1.9 mmol: 2.9 equiv/aldehyde) was added to the mixture and stirred continuously for 1 day. The reaction was stopped by precipitation with sat. Na_2CO_3 (5 ml) and acetone (100 ml). The precipitate was collected by filtration, dispersed with water containing 1 M NaOH (5 ml), dialyzed for 2 days, and lyophilized to give chitosan–dendrimer hybrid **17** (100 mg: 100% of recovery). Hybrids **18**, **19**, **20**, **21** and **25** were also obtained in the same manner and 100% of recovery.

Data for **17** ($G = 0.5$, DS = 0.14): ^{1}H NMR (0.5 M DCl/D_2O) δ 2.06 (s, 0.6 H, NHAc), 3.03 (br, 1.68 H, NCH_2(1–3)), 3.19 (br, 0.66 H, H-2 of GlcN), 3.4 (br, 0.42 H, H-2 of GlcN-R and CH_2(2)N), 3.64 (s, 0.28 H, CH_2(4)CO_2H), 3.75–4.2 (m, 5.2 H, H-2 of GlcNAc (0.2 H), H-3,4,5,6 of chitosan (5 H)), 4.63 (br, 0.2 H, H-1 of GlcNAc), 4.89 (br, 0.66 H, H-1 of GlcN), 5.10 (0.14 H, H-1 of GlcN-R); ^{13}C NMR (0.5 M DCl/D_2O) δ 25.0 (NHAc), 31.1 (*C*H_2 (4)COOH), 50.0 (*C*H_2(2)), 53.0 (N*C*H_2(1, 3)), 58.7 (C-2 of chitosan), 63.2 (C-6), 72.0–73.0 (C-3), 77.6 (C-5), 79.3 (C-4 of GlcN, GlcN-R)), 81.4 (C-4 of NHAc), 99.4 (C-1 of GlcN-R), 100.4 (C-1 of GlcN), 104.1 (C-1 of GlcNAc), 177.0 (*C*OOH), 177.5 (NHAc). CH_2(1–4): GlcN-CH_2(1)CH_2(2)N(CH_2(3)CH_2(4)CO_2H)$_2$.

Data for **18** ($G = 1.5$, DS = 0.10): ^{1}H NMR (0.5 M DCl/D_2O) δ 2.09 (s, 0.6 H, NHAc), 2.81–2.91 (brm, 1.20 H,

NCH_2(1–3)), 3.01 (t, $J = 6.2$ Hz, 1.20 H, NCH_2(6,7)), 3.21 (br, H-2 of GlcN and CH_2(4)), 3.50 (t,CH_2(5)), 3.60 (t, $J =$ 6.2 Hz, 0.8 H, CH_2(8)COOH), 3.6–4.0 (m, 5.2 H, H-2 of GlcNAc, H-3,4,5,6 of chitosan), 4.59 (br, 0.2 H, H-1 of GlcNAc), 4.89 (br, 0.7 H, H-1 of GlcN); ^{13}C NMR (0.5 M DCl/D_2O) δ 25.1 (NHAc), 31.2 (CH_2(8)CO_2H 31.7 (CH_2(4)), 37.6 (CH_2(5)), 52.6 (NCH_2(7)), 53.2 (NCH_2 (1,3)), 56.0 (CH_2N(2,6)), 58.5 (C-2), 63.0 (C-6), 72.9 (C-3), 77.7 (C-5), 79.4 (C-4 of GlcN, GlcN-R)), 81.5 (C-4 of NHAc), 100.0 (C-1 of GlcN), 104.1 (C-1 of GlcNAc), 175.6 (NHCO(a)), 177.0 (CO_2H), 177.6 (NHAc). CH_2(3–8) and a: N(CH_2(3)CH_2(4)CONH(a)CH_2(5)CH_2(6)N(CH_2(7)CH_2(8)-$CO_2H)_2)_2$, 1–2: see compound **17**.

Data for **19** ($G = 2.5$, DS = 0.13): ^{1}H NMR (0.5 M DCl/D_2O)δ δ 2.06 (s, 0.6 H, NHAc), 2.87 (t, $J = 5.4$ Hz, 1.56 H, NCH_2(1–3)), 2.97 (t, $J = 6.4$ Hz, 4.68 H, NCH_2(6,7,10,11)), 3.18 (br, H-2 of GlcN and CH_2(4,8)), 3.46 (t, 1.8 H, CH_2(5,9)), 3.60 (t, $J = 6.2$ Hz, 2.4 H, CH_2(12)CO_2H), 3.6–4.0 (m, 5.2 H, H-2 of GlcNAc, H-3,4,5,6 of chitosan), 4.60 (br, 0.2 H, H-1 of GlcNAc), 4.88 (d, $J = 8.5$ Hz, 0.67 H, H-1 of GlcN); ^{13}C NMR (0.5 M DCl/D_2O) δ 25.1 (NHAc), 31.2 (CH_2(12)CO_2H and CH_2(4,8)), 37.6 (CH_2(5,9)), 52.7 (NCH_2(1,3,7,11)), 56.1 (CH_2N(2,6,10)), 58.6 (C-2), 63.0 (C-6), 73.0 (C-3), 77.7 (C-5), 79.4 (C-4 of GlcN, GlcN-R)), 81.5 (C-4 of NHAc), 100.5 (C-1 of GlcN), 104.1 (C-1 of GlcNAc), 175.9 (NHCO(a,b)), 177.1 ($COOH$), 177.6 (NHAc). CH_2(9–12) and b: N((–CONH(b)CH_2(9)CH_2(10)N(CH_2(11)CH_2(12) $CO_2H)_2)_2$, 1–8, and a: see compounds **17**, **18**.

Data for **20** (DS = 0.03): ^{1}H NMR (0.5 M DCl/D_2O)δ 2.06 (s, 0.6 H, NHAc), 2.90 (m, 2.76 H, NCH_2(1,2,3,6,7,10,11,14,15)), 3.19 (br, 1.6 H, H-2 of GlcN and CH_2(4,8,12)), 3.44 (t, $J = 5.4$ Hz, 0.84 H, CH_2(5,9,13)), 3.53 (t, $J = 6.4$ Hz, 0.96 H, CH_2(16)CO_2H), 3.6–4.0 (m, 5.2 H, H-2 of GlcNAc, H-3,4,5,6), 4.60 (br, 0.2 H, H-1 of GlcNAc), 4.70 (br, H-1 of GlcN-R), 4.88 (d, $J =$ 7.4 Hz, 0.8 H, H-1 of GlcN); ^{13}C NMR (0.5 M DCl/D_2O) δ 25.1 (NHAc), 31.5 (CH_2(16)CO_2H), 31.7 (CH_2(4,8,12)), 37.2 (CH_2(5,9)), 37.6 (CH_2(13)), 52.5 (NCH_2(1,3,7,11)), 52.7 (NCH_2(15)), 54.9 (CH_2N(2,6,10)), 55.8 (CH_2N(14)), 58.6 (C-2), 63.0 (C-6), 73.0 (C-3), 77.7 (C-5), 79.4 (C-4 of GlcN, GlcN-R)), 81.5 (C-4 of NHAc), 100.5 (C-1 of GlcN), 104.1 (C-1 of GlcNAc), 175.1 (NHCO(a,b)), 175.8 (NHCO(c)), 177.9 (CO_2H and NHAc). 13–16 and c: N((–CONH(c)CH_2(13)CH_2(14)N(CH_2(15)CH_2(16)$CO_2H)_2)_2)_2$, 1–12, and a–b: see compounds **17**, **18**, **19**.

Data for **21** (DS = 0.005): ^{1}H NMR (0.5 M DCl/D_2O)δ 2.06 (s, 0.6 H, NHAc), 2.76–2.86 (m, 1.26 H, NCH_2), 3.19 (br, 1.1 H, H-2 of GlcN and CH_2(4,8,12,16)), 3.40 (t, $J =$ 5.4 Hz, 0.3 H, CH_2(5,9,13,17)), 3.45 (t, $J = 6.0$ Hz, 0.64 H, CH_2(20)CO_2H), 3.6–4.0 (m, 5.2 H, H-2 of GlcNAc, H-3,4,5,6), 4.60 (br, 0.2 H, H-1 of GlcNAc), 4.88 (br, 0.8 H, H-1 of GlcN); ^{13}C NMR (0.5 M DCl/D_2O) δ 25.1 (NHAc), 32.6 (CH_2(4,8,12,16) and CH_2(20)CO_2H), 37.5 (CH_2(5,9,13,17)), 50.7 (NCH_2(1,3,7,11,15,19)), 53.6 (CH_2N(2,6,10,14)), 55.1 (CH_2N(18)), 58.6 (C-2), 63.0 (C-6), 73.0 (C-3), 77.7 (C-5), 79.4 (C-4 of GlcN, GlcN-R)), 81.5 (C-4 of NHAc), 100.5 (C-1 of GlcN), 104.1 (C-1 of GlcNAc), 175.7 (NHCO(a,b,c,d)), 177.6 (NHAc), 179.4 (CO_2H). 17–20 and d: N((–CONH(d)CH_2(17)CH_2(18) N(CH_2(19)CH_2(20)$CO_2H)_2)_2)_2)_2$, 1–16, and a–c: see compounds **17**, **18**, **19**,**20**.

2.7. Preparation of sialic acid bound dendrimer ($G = 3$)

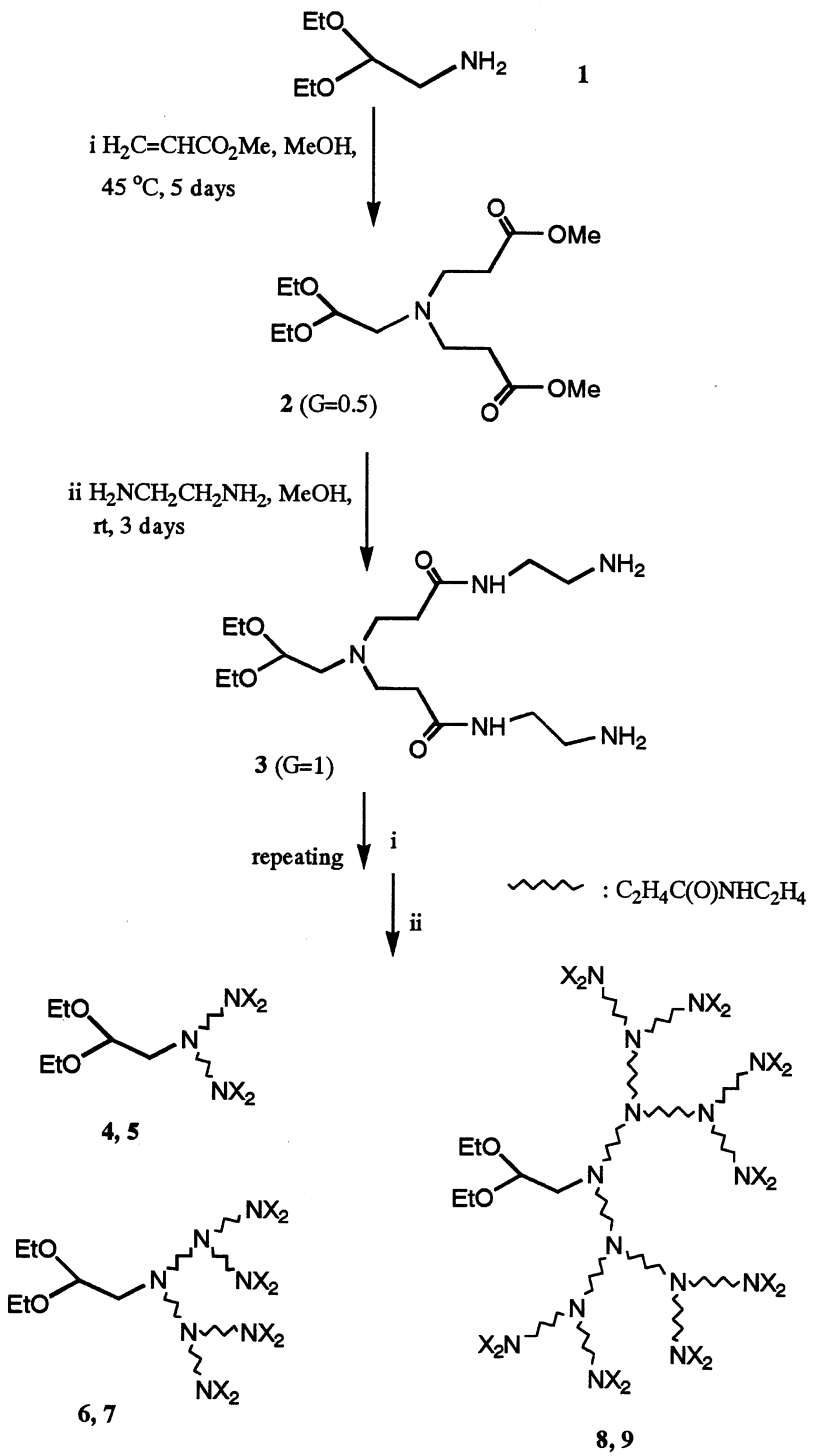
The compound **22** was prepared according to Roy, Tropper, Romanowska, Letellier, Cousineau, Meunier et al. (1991). To a solution of dendrimer **7** ($G = 3$, 0.1 mmol, 0.8 mmol of NH_2) in MeOH (20 ml) was added **22** (1.0 mmol, 1.25 equiv/NH_2) in MeOH (10 ml). After stirring at room temperature for 1 h, $NaBH_3CN$ (2.4 mmol) was added and continuously stirred at room temperature for 1 day. The mixture was evaporated and obtained crude product of **23**. The removal of acetal from compound **23** and attached to chitosan (200 mg) was performed as described above. The compound **24** was continuously treated with 0.1 M NaOH, dialyzed, lyophilized, and obtained compound **25** in a good recovery (210 mg).

Data for **25** (DS = 0.01): ^{1}H NMR (0.5 M DCl/D_2O)δ1.91 (t, $J = 12.4$ Hz, 0.08 H, H-3ax of Neu5Ac), 2.09 and 2.10 (d, 0.84 H, NHAc of chitosan and Neu5Ac), 2.35 (dd, $J = 5.0$ Hz, 0.08 H, H-3eq of Neu5Ac), 2.91 (br, 0.44 H, NCH_2(1,2,3,6,7,10,11)), 3.22 (br, 1.08 H, H-2 of GlcN and CH_2CO(4,8,12)), 3.46 (br, 0.44 H, CH_2(5,9,13,14)), 3.6–4.0 (m, H-2 of GlcNAc, H-3,4,5,6 of chitosan and H-4,7,8,9 of Neu5Ac), 4.09 (d, $J = 10.2$ Hz, H-5 of Neu5Ac), 4.41 (dd, $J = 7.0$ Hz, H-6 of Neu5Ac), 4.60 (br, 0.2 H, H-1 of GlcNAc), 4.91 (d, $J = 7.7$ Hz, 0.8 H, H-1 of GlcN), 7.00 (d, $J = 8.5$ Hz, 0.16H, H-ortho of C_6H_4-O), 7.41 (d, $J = 8.5$ Hz, 0.16H, H-meta of C_6H_4-O); ^{13}C NMR (0.5 M DCl/D_2O) δ 25.1 (NHAc of chitosan and Neu5Ac), 31.7 (CH_2(4,8,12)), 37.2 (CH_2(5,9)), 41.7 (C-3 of Neu5Ac), 52.7 (NCH_2(1,3,7,11), 53.5 ($CH_2C_6H_4$), 55.0 (CH_2N(2,6,10) and C-5 of Neu5Ac), 58.6 (C-2 of chitosan), 63.0 (C-6 of chitosan and C-9 of Neu5Ac), 66.1 (C-7 of Neu5Ac), 69.5 (C-4 of Neu5Ac), 71.2 (C-8 of Neu5Ac), 73.1 (C-3 of chitosan and C-6 of Neu5Ac), 77.7 (C-5 of chitosan), 79.4 (C-4 of GlcN), 81.5 (C-4 of NHAc), 100.1 (C-2 of Neu5Ac),100.4 (C-1 of GlcN), 104.1 (C-1 of GlcNAc), 118.9 (C-ortho), 134.8 (C-para, C-meta), 153.6 (C-ipso), 176.0 (NHCO(a,b,c)), 177.6 (NHAc). (11)–(14) and (c): N(CH_2(11)CH_2(12)CONH(c)CH_2(13)CH_2(14) $NHCH_2C_6H_4$–O–Neu5Ac)$_2)_2)_2$, (1)–(10), and (a,b): see above compounds.

3. Results and discussion

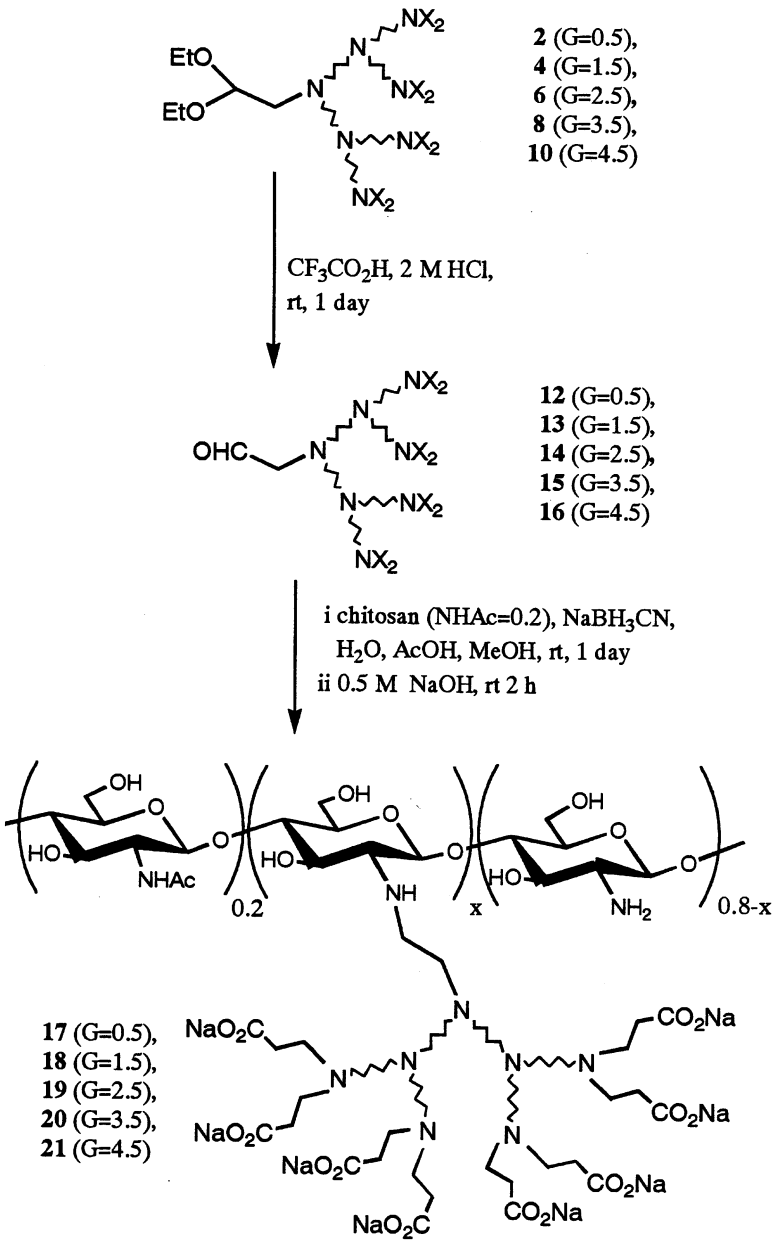
3.1. Preparation of PAMAM dendrimer

Using commercialized aminoacetaldehyde as an amine source, the generation of PAMAM dendrimer was performed according to reported procedure (Aoi et al.,



4 (G=1.5) X = $C_2H_4CO_2Me$; **5** (G=2) X = $C_2H_4C(O)NHC_2H_4NH_2$;
6 (G=2.5) ; **7** (G=3);
8 (G=3.5) ; **9** (G=4);
10 (G=4.5) ; **11** (G=5)

Scheme 1.

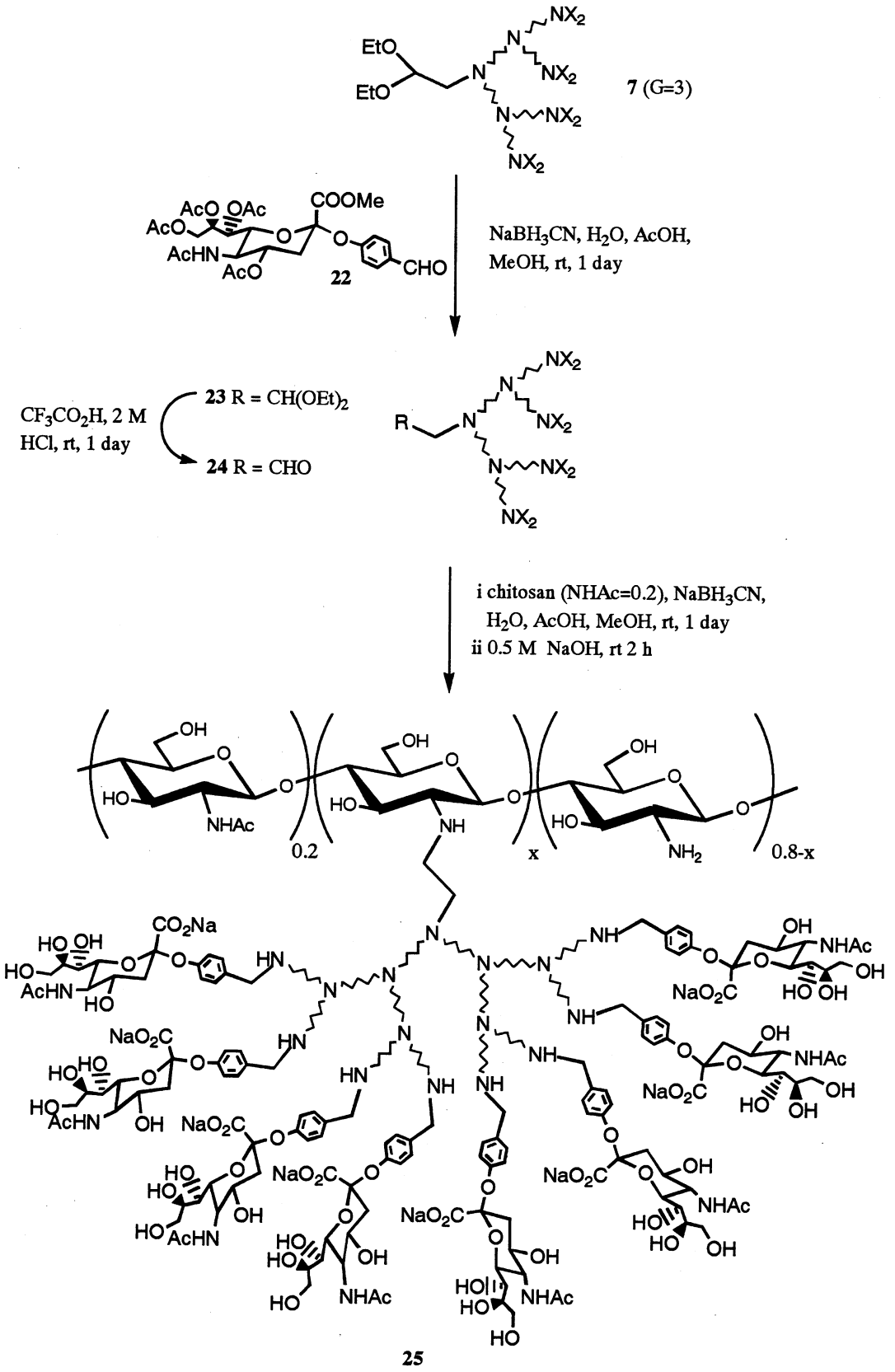


Scheme 2.

1997 and Tomalia et al., 1990: Scheme 1). Although the methyl esters could be purified with column chromatography and gave moderate or good yields (**2**, **4**, quant., **6** = 84%, **8** = 44%, **10** = 60%), it was difficult to purify amines (**3**, **5**, **7**, **9**, **11**) with column chromatography. Since the excess of e thylenediamine (bp = 118°C) could be removed with evaporation and drying procedure under high vacuum, these amines (**3**, **5**, **7**, **9**) were used for the next generation without further purification. From the ^{1}H and ^{13}C NMR spectra, there was no peaks of ethylenediamine in each spectra. Furthermore, the C=O signal in amide group were quite simple from the ^{13}C NMR spectra, and the mass spectra also suggested the corresponding structures.

3.2. Deprotection of acetal and attached dendrimer to chitosan

According to the previous study (Sashiwa et al., 2000), the deprotection of acetal **2** was carried out with 80% CF_3COOH in water at rt for 1 day. The deprotection, however, was not proceeded and only acetal was recovered under these conditions. Under the more drastic conditions, acetal was also recovered at 50°C for 1 day, moreover it was


7 (G=3)
22
NaBH3CN, H2O, AcOH,
MeOH, rt, 1 day
CF3CO2H, 2 M
HCl, rt, 1 day
23 R = CH(OEt)2
24 R = CHO
i chitosan (NHAc=0.2), NaBH3CN,
H2O, AcOH, MeOH, rt, 1 day
ii 0.5 M NaOH, rt 2 h
0.2
x
0.8-x
25

Scheme 3.

Table 1
Preparation of chitosan–dendrimer hybrid

Acetal		Molar ratio equiv/NH_2	Product	DS	Reactivity[a] (%)
Compd.	MW				
2	305	0.7	17	0.14	25
4	705	0.6	18	0.10	21
6	1505	0.7	19	0.13	23
8	3105	0.15	20	0.03	25
10	6305	0.07	21	0.005	9
23	6369	0.1	25	0.01	12.5

[a] Reactivity = [DS/(molar ratio × 0.8)] × 100.

decomposed at 85°C for 1 day. The addition of 2 M HCl (CF_3COOH/2M HCl = 4/1) was effective for the deprotection of acetal. Thus the deprotection of acetals (**2**, **4**, **6**, **8**, **10**) was carried out with the mixed solvent of CF_3COOH (2.0 g) and 2M HCl (0.5 g) at rt for 1 day, and then evaporated to dryness, followed by drying in vacuo for 1 day. Reductive *N*-alkylation of chitosan is a very convenient method for its chemical modification (Hall & Yalpani, 1980; Yalpani & Hall, 1984). So we selected these methods for the attachment dendrimers to chitosan. Since aldehydes (**12**–**16**) were difficult to purify with column because of the salt formation at tertiary amino groups of dendrimer, the following reductive *N*-alkylation of chitosan was carried out without any purification (Scheme 2). From the ^1H NMR spectra of **12**–**16** in D_2O, the CH_3 proton of diethyl acetal (1.17 ppm) and CH proton of acetal (5.1 ppm) were disappeared. The reductive *N*-alkylation of chitosan was performed according to our previous report (Sashiwa et al., 1999; Sashiwa et al., 2000) and purified by dialysis from the mixture in the presence of Na_2CO_3. From the ^1H and ^{13}C NMR spectra, the corresponding methylene signals of dendrimer were observed. The DS were estimated from the ratio of $(CO)CH_2$ signals at 2.87–2.96 ppm against H-1 proton of chitosan (4.6–5.1 ppm).

3.3. Sialic acid bound chitosan–dendrimer hybrid

To attach the functional molecule onto dendrimer, we selected sialic acid which is well known important carbohydrate to interact between cell and bacteria, virus, toxin, and another cells. We also selected *p*-formylphenylα-sialoside **22**, which was reported by Roy et al. (1991), to attach sialic acid moiety onto dendrimer (Scheme 3). The sialoside **22** was attached to dendrimer **7** ($G = 3$) with reductive *N*-alkylation. From the ^1H and ^{13}C NMR spectra, the absence of aldehyde **22** was confirmed and the chemical structure of **23** was also suggested from these spectra. Deprotection of acetal **23** was carried out under the same conditions described above, followed by attaching to chitosan by reductive *N*-alkylation as above. After deprotection of **24** with aq. NaOH, the chitosan–sialodendrimer hybrid **25** was given in a good yield (90%) from chitosan. The chemical structure of **25** was also suggested by ^1H and ^{13}C NMR spectra.

3.4. Reactivity of deprotected acetal to chitosan

Table 1 shows the molar ratio of acetal against amino group of chitosan and the DS of hybrids by reductive *N*-alkylation of chitosan. The reactivity of deprotected acetal (aldehyde) was estimated from the DS value of hybrid from the equation listed in Table 1. The acetals under the 3105 of molecular weight (MW) showed ca. 20–25% of reactivity. The reactivity was reasonable values compared with various aldehydes used in the previous study (Sashiwa et al., 1999; Sashiwa et al., 2000) owing to the simultaneous reduction of aldehyde to alcohol. The reactivity was obviously decreased around MW = 6305–6369 of dendrimers which would be caused by the steric hindrance owing to high MW of dendrimers.

In conclusion, we successfully prepared chitosan–dendrimer hybrid via short spacer. Highly generated chitosan–dendrimer hybrids and chitosan–sialodendrimer hybrid were also obtained. Now we are studying another molecular design of chitosan–dendrimer hybrid. Finally, we are looking forward that these chitosan–dendrimer hybrids are widely useful for not only biomedical region, but also some another field such as agriculture, electronics, life science, and so on.

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