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Chemical modification of chitosan. Part 9: Reaction of *N*-carboxyethylchitosan methyl ester with diamines of acetal ending PAMAM dendrimers

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Abstract

N-carboxyethylchitosan methyl ester (degree of substitution (DS) = 1.2 per repeating residue of chitosan), which was prepared from chitosan and methyl acrylate by the Michael reaction, was reacted with diamine and gave a water-soluble product. The DS value of the diamine moiety was 0.64–0.94. Acetal ending poly(amidoamine) (PAMAM) dendrimers were successfully attached to the ester and gave an *N*-carboxyethylchitosan–dendrimer hybrid without any crosslinking. The DS values of the hybrids were gradually decreased from 0.61 to 0.04 with the increasing generation of dendrimer.

Keywords: Chitosan; *N*-carboxyethylchitosan methyl ester; Dendrimer

1. Introduction

Chitosan **1** is a polysaccharide mainly composed of D-glucosamine units. Chitosan shows some biological activities such as immunological (Nishimura, Nishimura, Seo, Nishi, Tokura & Azuma, 1986), antibacterial (Tanigawa, Tanaka, Sashiwa, Saimoto & Shigemasa, 1992; Tokura, Ueno, Miyazaki & Nishi, 1997), and wound healing activity (Minami, Okamoto, Tanioka, Sashiwa, Saimoto, Matsuhashi et al., 1993). Moreover, chitosan itself is non-toxic and biodegradable (Sashiwa, Saimoto, Shigemasa, Ogawa & Tokura, 1990; Shigemasa, Saito, Sashiwa & Saimoto, 1994). Therefore, chitosan is an appealing bioactive polymer for further development. Since chitosan itself is insoluble in water at neutral pH, the application of chitosan is quite limited. Therefore, chemical modification of chitosan to provide water-soluble materials is of prime interest to generate novel biomaterials.

The Michael type reaction is a well-known process to add various amines onto α,β-unsaturated carbonyl compounds. Using this reaction, poly(amidoamine)(PAMAM) dendrimers were prepared successfully (Tomalia, Baker, Dewald, Hall, Kallos, Martin et al., 1985; Tomalia, Naylor & Goddard, 1990). Although various *N*-alkylation of chitosan were prepared with aldehyde (Muzzarelli & Tanfani, 1985; Muzzarelli, Tanfani, Emanuell & Mariotti, 1982; Muzzarelli, Tanfani, Mariotti & Emanuell, 1982; Sashiwa & Shigemasa, 1999; Yalpani & Hall, 1984), there was no report of *N*-alkylation by 1,4-conjugate additions. Recently, we found that the *N*-alkylation of chitosan with methyl acrylate by 1,4-conjugate additions proceeded successfully with albeit long reaction time (e.g. 5 or 10 days) at 40°C and gave *N*-carboxyethylchitosan methyl ester **2** (Sashiwa, Shigemasa & Roy, 2000). In the synthesis of PAMAM dendrimer, methyl ester reacts at the half-side of the amine in ethylenediamine and gives an amidoamine moiety. Furthermore, it was reported that the surface amines of the PAMAM dendrimer could be attached to methyl esters of other PAMAM dendrimers and obtained "core-shell *tecto*-(dendrimer) molecules" (Li, Swanson, Qin, Brothers, Piehler, Tomalia et al., 1999). We report herein the reaction of *N*-carboxyethyl chitosan methyl ester **2** with various diamines or acetal ending PAMAM dendrimers ($G = 1$–5).

2. Results and discussion

2.1. Reaction of methyl ester **2** with diamine

Yin, Zhu and Tomalia (1998) reported the preparation of

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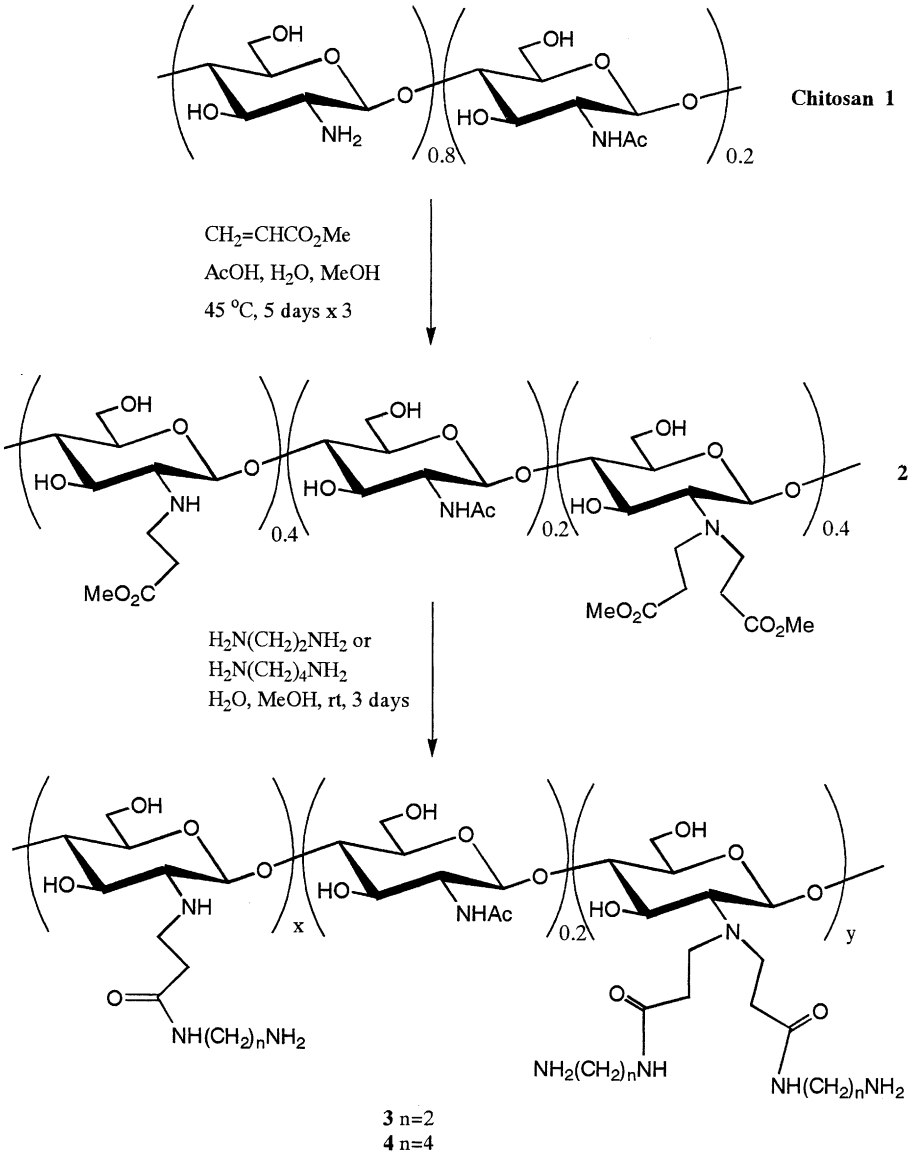
rod-shaped, cylindrical dendrimers based on secondary amines of poly(ethyleneimine) cores without any side reactions like crosslinking. This report suggests that the reaction of esters with diamines could be achieved on the polymer chain without any crosslinking. On the other hand, *N*-carboxyethyl chitosan methyl ester **2** was prepared successfully by 1,4-conjugate additions (Sashiwa et al., 2000). At first, we tested the reaction of methyl ester **2** (degree of substitution, DS = 1.2) with diamines such as ethylenediamine or 1,4-diaminobutane (Scheme 1). The results are summarized in Table 1. In spite of methyl ester **2** being insoluble in methanol and aqueous medium (solvent A: H_2O/MeOH = 1:10), the diamine moiety could be attached to methyl ester **2**. The unreacted diamine in the mixture was removed perfectly by dialysis (MW = 12,000 cutoff). The DS of the diamine moiety were around 0.64–0.67 for 3 days' reaction. The DS was increased to 0.94 with increasing reaction time (7 days). Although the reactivity is slightly low (DS = 0.77), this reaction could be achieved even in methanol as medium. It is noteworthy that all of compounds **3** and **4** were dissolved in neutral water. From the ^{1}H NMR spectra, typical methylene proton signals at δ 1.60–1.77 in addition to the *N*-carboxyethylchitosan backbone signals for the 1,4-diaminobutane substituted product **4**.

2.2. Preparation of PAMAM dendrimer from aminoacetaldehyde diethylacetal

Using commercial aminoacetaldehyde diethyl acetal **5** as an amine source, the generation of the PAMAM dendrimer was performed according to the authorized procedure (Tomalia et al., 1985, 1990). These dendrimers of each generation **6**–**15** (Scheme 2) were well defined by ^{1}H and



Scheme 1.

Table 1
Reaction of *N*-carboxyethylchitosan methyl ester **2** with diamines

Diamine[a]	Solvent[b]	Time (days)	Product	DS	Solubility[c] in H_2O
Ethylenediamine	A	3	**3**	0.64	Yes
1,4-Diaminobutane	A	3	**4**	0.67	Yes
	A	7	**4**	0.94	Yes
	MeOH	7	**4**	0.77	Yes

[a] 10 equiv/COOMe.
[b] A, H_2O (10 ml) and MeOH (100 ml).
[c] 10 mg of compound was suspended in 2 ml of solvent at rt and observed after 1 day.

^{13}C NMR spectra, and the acetal ending PAMAM dendrimers were useful for the preparation of different type chitosan–dendrimer hybrids through the deprotection of acetal, followed by reductive *N*-alkylation of chitosan (Scheme 3: Sashiwa, Shigemasa & Roy, submitted for publication). In this study, the surface amines in these dendrimers were used to attach the chitosan backbone.

2.3. Attachment of dendrimer to methyl ester 2

Scheme 4 and Table 2 show the reaction of *N*-carboxyethylchitosan methyl ester **2** with acetal ending amine dendrimers (**7**, **9**, **11**, **13**, and **15**). Compound **7** as diamine was attached to ester **2** in a similar manner as compounds **3** and **4**. The DS value (which is defined as the number of dendrimers per repeating residue of product) was increased from 0.61 to 0.74 with an increasing amount of **7**. It is noteworthy that polyamine dendrimers such as **9** ($G = 2$: $NH_2 = 4$), **11** ($G = 3$: $NH_2 = 8$), **13** ($G = 4$: $NH_2 = 16$), and **15** ($G = 5$: $NH_2 = 32$) could attach to ester **2** without any crosslinking. All compounds were soluble in 0.2 M HCl and a part of compounds **16** and **17** were soluble even in neutral water (10 mg of cmpound/2 ml of solvent; rt, observed after 1 day). The viscometric property of hybrids **16**–**20** in 0.2 M HCl were almost the same as ester **2**, which suggests that no intramolecular crosslinking or aggregation occurs in this reaction. The DS values were decreased gradually from 0.61 to 0.04 with increasing the generation, especially over $G = 3$ which would be caused by the steric hindrance of the high molecular weight of the dendrimer. Table 3 and Scheme 5 show the possible chemical structures of compounds **4**, **16**, and **17** from the ^1H NMR analysis and colorimetric determination of primary amine with ninhydrin. From the amount of DS, it is without meaning that a part of the primary amino groups at the end of branching should form the amido linkage with other methyl esters intermolecularly, so that a macrocyclic ring should be formed. The presence of unreacted methyl esters (which was observed in ^1H NMR but difficult to count owing to overlapping other signals) would be caused also by the steric hindrance in the polymer chain. The ninhydrin analysis for hybrids **18**–**20** ($G = 3$–5) could not be carried out owing to the insolubility in neutral water.

In conclusion, we successfully prepared a novel type of chitosan dendrimer hybrid. Detailed molecular conformation and biological aspects of these hybrids will be studied in the near future.

3. Experimental section

3.1. Materials

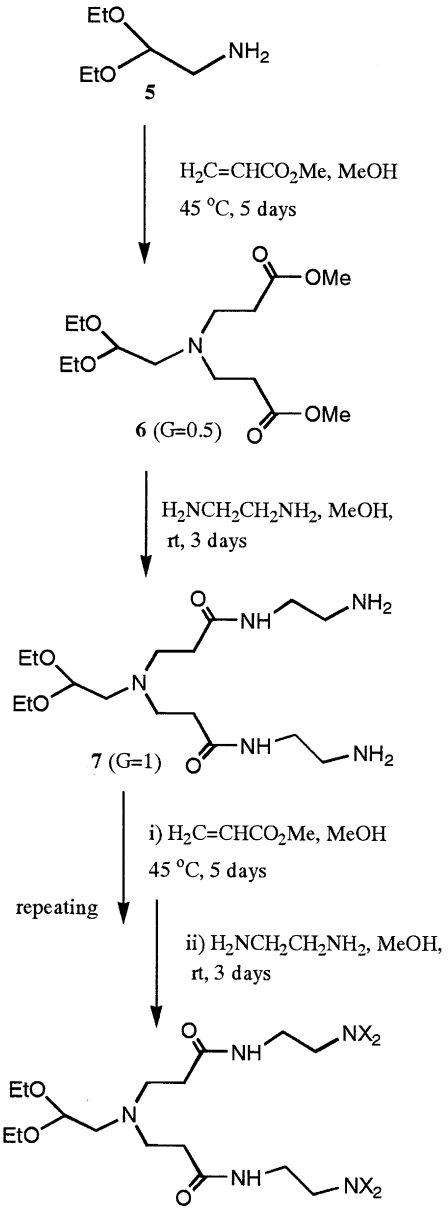
Chitosan **1** (Flonac C, NHAc = 0.2, DP = 140, FW of unit = 169) was purchased from Kyowa Tecnos Co., Japan. Aminoacetaldehyde diethyl acetal **5** and other reagents were also purchased from Aldrich Co., and used without further purification. Dialysis membrane (MW 12,000 cutoff) was purchased from Sigma Co. Acetal ending PAMAM dendrimers (**6**–**15**) were prepared and structurally characterized in the previous paper (Sashiwa et al., submitted for publication).

3.2. General methods

The degree of polymerization (DP) of the original 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-510H; eluent, 0.1 M AcOH buffer (pH 4.7) containing 0.1 M NaCl; flow rate, 1.0 ml/min; column temperature, 50°C). 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 $CHCl_3$ for $CDCl_3$ or 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 primary amino groups of products were quantitated by colorimetric determination using ninhydrin at 570 nm.

3.3. Reaction of methyl ester 2 with diamine or dendrimers

N-carboxyethylchitosan methyl ester (**2**, DS = 1.2, 100 mg, 0.54 mmol of CO_2Me) was prepared and structurally characterized in our previous report (Sashiwa et al., 2000). Methyl ester **2** was dispersed in H_2O (10 ml) and methanol (100 ml). To a suspension was added diamine (5.4 mmol: 10 equiv/CO_2Me) or dendrimers (1.0 equiv/CO_2Me) and stirred at room temperature for 3 days. The mixture was concentrated under 10 ml, diluted with H_2O

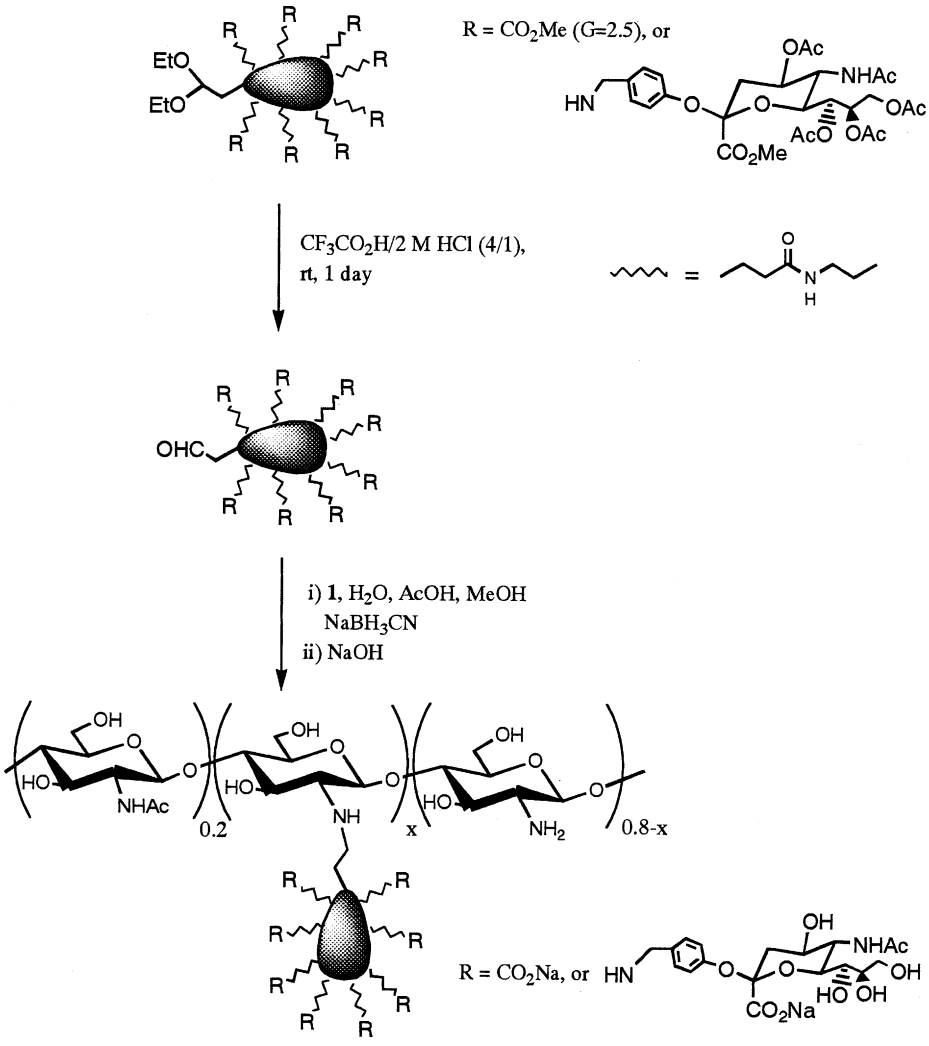


8 (G=1.5) X = $(CH_2)_2CO_2Me$; **9** (G=2) X = $(CH_2)_2C(O)NH(CH_2)_2NH_2$
10 (G=2.5) ; **11** (G=3)
12 (G=3.5) ; **13** (G=4)
14 (G=4.5) ; **15** (G=5)

Scheme 2.

(20 ml) containing 1 M NaOH (2 ml), dialyzed for 2 days, and lyophilized to give products in quantitative recovery. The degree of substitution of each product was estimated as follows. Compound **3**: peak ratio from methylene proton (3.23 ppm) and NHAc in chitosan (2.12 ppm, 0.60 H); Compound **4**: peak ratio from methylene proton (1.60–1.77 ppm) and NHAc in chitosan (2.06 ppm, 0.60 H): Compounds **16**–**20**: peak ratio from methyl proton (1.27–1.30 ppm) and NHAc in chitosan (2.06 ppm, 0.60 H).

Data for **3** (DS = 0.64): ^{1}H NMR (0.1 M DCl/D_2O) δ 2.12 (s, 0.6 H, NH*Ac*), 2.97 (S, 2.4 H, $-CH_2-$(b), 3.09 (s, *H*-2 of *N*-alkylated GlcN), 3.23 (s, 1.28 H, $-CH_2-$(c), 3.59 and 3.67 (m, $-CH_2-$(a,d)), 3.4–4.4 (m, *H*-2 of GlcNAc, and *H*-3,4,5,6 of hexosamine), 4.64 (br, H-1 of GlcNAc), 5.11 (br, *H*-1 of *N*-monoalkylated GlcN), 5.32 (br, *N*,*N*-dialkylated GlcN); ^{13}C NMR (0.1 M DCl/D_2O) δ 25.1 (NH*Ac*), 31.1, 32.7 (CH_2CO(b)), 39.4, 39.7, 42.0 ($-CH_2-$(a,d)), 58.7 (*C*-2 of GlcNAc), 63.5 (*C*-6), 64.8 (*C*-2 of *N*-mono and

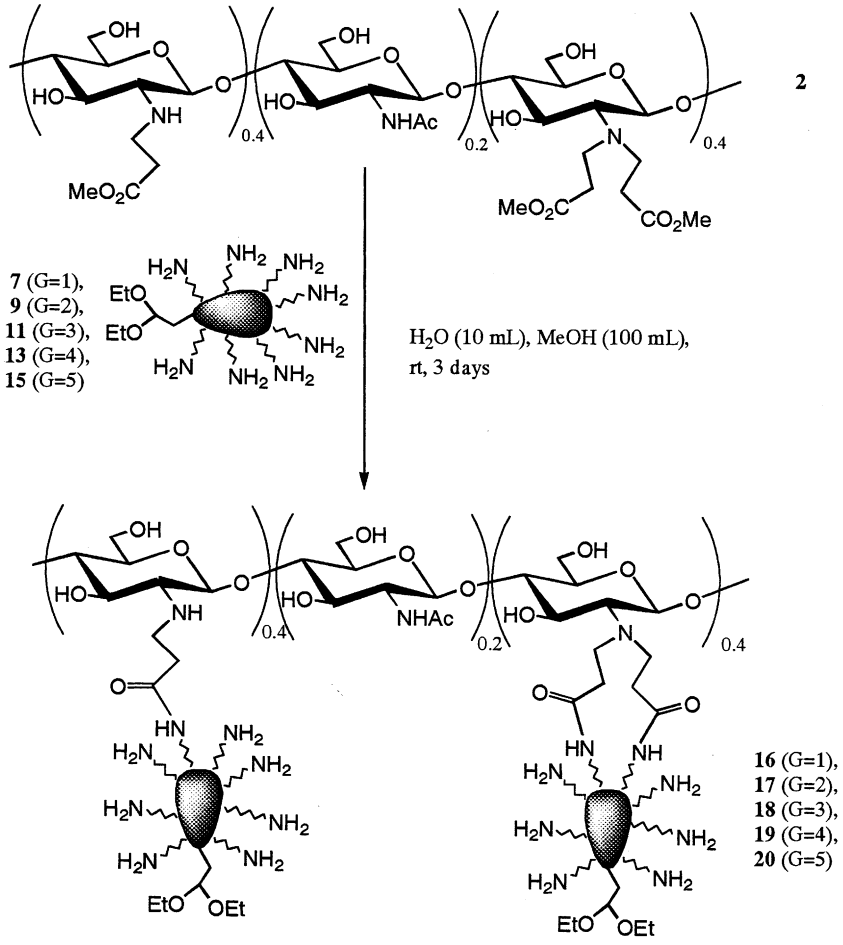


Scheme 3.

dialkylated GlcN), 68.7–72.0 (*C*-3), 77.5 (*C*-5), 79.3 (*C*-4), 97.7 (*C*-1 of *N*,*N*-dialkylated GlcN), 99.3 (*C*-1 of *N*-monoalkylated GlcN), 104.2 (*C*-1 of GlcNAc), 176.3 (NHAc), 177.5 (NH*C*O), 177.6 (*C*OOH). DS = 1.28/2 = 0.64. a–d: GlcN–CH_2(a)–CH_2(b)–CONH–CH_2(c)–CH_2(d)–NH_2.

Data for **4** (DS = 0.67): ^{1}H NMR (0.1 M DCl/D_2O) δ 1.60–1.77 (m, 2.68 H), CH_2(d)–NH_2 2.06 (s, 0.6 H, NH*Ac*), 3.04 (m, 5.08 H, –CH_2–(a,c,f), 3.30 (s, *H*-2 of *N*-alkylated GlcN), 3.4–4.4 (m, CH_2(b), *H*-2 of GlcNAc, and *H*-3,4,5,6 of hexosamine), 4.58 (br, H-1 of GlcNAc), 5.05 (br, *H*-1 of *N*-monoalkylated GlcN), 5.26 (br, *N*,*N*-dialkylated GlcN); ^{13}C NMR (0.1 M DCl/D_2O) δ 25.1 (NH*Ac*), 26.8, 27.1, 28.2 (*C*H_2(d,e)), 31.1, 33.1 (*C*H_2CO(b)), 41.6–42.1 (–*C*H_2–(a,c,f)), 58.9 (*C*-2 of GlcNAc), 63.5 (*C*-6), 64.8 (*C*-2 of *N*-mono and dialkylated GlcN), 68.8–72.0 (*C*-3), 77.7 (*C*-5), 79.7 (*C*-4), 97.7 (*C*-1 of *N*,*N*-dialkylated GlcN), 99.3 (*C*-1 of *N*-monoalkylated GlcN), 104.2 (*C*-1 of GlcNAc), 175.1, 175.2 (NHAc), 177.0 (NH*C*O), 177.4, 177.5 (*C*OOH). DS = 2.68/4 = 0.67. a–f: GlcN–CH_2(a)–CH_2(b)–CONH–CH_2(c)–CH_2(d)–CH_2(e)–CH_2(f)–NH_2.

Data for **16** (G = 1, DS = 0.61): ^{1}H NMR (0.1 M DCl/D_2O) δ 1.28 (t, J = 7.0 Hz, 3.66 H, CH_3 of OEt), 2.06 (s, 0.6 H, NH*Ac*), 2.94 (t, J = 6.4 Hz, –CH_2–(b,b′,e,e′)), 3.23 (t, J = 6.0 Hz, –CH_2–(d,d′))), 3.32 (br, *H*-2 of *N*-alkylated GlcN), 3.47 (d, J = 5.0 Hz, –CH_2–(g)), 3.6 (m, –*C*H_2–(a,a′,c,c′,f,f′)), 3.4–4.4 (m, *H*-2 of GlcNAc, and *H*-3,4,5,6 of hexosamine), 3.75 and 3.90 (m, –CH_2– of OEt), 4.58 (br, H-1 of GlcNAc), 5.05 (m, *H*-1 of *N*-monoalkylated GlcN and CH of acetal), 5.26 (br, *N*,*N*-dialkylated GlcN); ^{13}C NMR (0.1 M DCl/D_2O) δ 17.3 (CH_3), 25.1 (NH*Ac*), 31.5 (NHCO *C*H_2(b,b′,e,e′)), 39.7 (CONH*C*H_2(c)), 41.9 (CONH*C*H_2(d,d′)), 46.9 (*C*H_2NH_2(c′)), 54.3 (N–*C*H_2(a,a′,f,f′)), 58.3 (*C*H_2CHOEt$_2$(g)), 63.4 (*C*-6), 64.8 (*C*-2 of *N*-mono and dialkylated GlcN), 72.3 (*C*-3), 77.7 (*C*-5), 79.1 (*C*-4), 97.9 (*C*-1 of *N*,*N*-dialkylated GlcN), 99.4 (*C*-1 of *N*-monoalkylated GlcN), 100.2 (*C*H of acetal, 174.9, 175.5,



Scheme 4.

175.6 (*C*ONH of dendrimer), 179.0 (NH*C*O of GlcNAc). DS = 3.66/6 = 0.61. a–f: GlcN–CH_2(a)–CH_2(b)–CONH–CH_2(c)–CH_2(c)–CH_2(d)–NHCO–CH_2(e)–CH_2(f)–N–CH_2(g)–CH–OEt_2. a′, b′: GlcN–CH_2(a′)–CH_2(b′)–COOH; c′–f′:H_2N–CH_2(c′)–CH_2(d′)–NHCO–CH_2(e′)–CH_2(f′)–N.

Data for **17** ($G = 2$, DS = 0.64): ^{1}H NMR (0.1 M DCl/D_2O) δ 1.28 (t, $J = 7.0$ Hz, 3.86 H, CH_3 of OEt), 2.07 (s, 0.6 H, NH*Ac*), 2.91 (t, $J = 6.3$ Hz, –CH_2–CO), 3.22 (t, $J = 6.0$ Hz, –CH_2–NHCO), 3.27 (br, *H*-2 of *N*-alkylated GlcN), 3.44 (m, –CH_2–$CHOEt_2$), 3.59 (t, $J = 5.8$ Hz, –N–CH_2–), 3.4–4.4 (m, *H*-2 of GlcNAc, and *H*-3,4,5,6 of hexosamine), 3.75 and 3.90 (m, –CH_2– of OE_t), 4.63 (br, H-1 of GlcNAc), 5.05 (m, *H*-1 of *N*-monoalkylated GlcN), 5.08 (t, $J = 5.0$ Hz, CH of acetal), 5.18 (br, *N*,*N*-dialkylated GlcN); ^{13}C NMR (0.1 M DCl/D_2O) δ 17.4 (CH_3), 25.1 (NH*Ac*), 31.8, 32.0 ($COCH_2$), 39.8 ($CONHCH_2$), 42.0 ($CONHCH_2$), 46.5 (CH_2NH_2), 51.9, 53.9, 54.8 (N–CH_2), 58.2 (CH_2CHOEt_2), 63.3 (*C*-6), 64.4 (*C*-2 of *N*-mono and dialkylated GlcN), 72.4 (*C*-3), 77.7 (*C*-5), 79.0 (*C*-4), 99.4 (*C*-1 of *N*-mono and dialkylated GlcN), 100.5 (*C*H of acetal), 104.2 (*C*-1 of GlcNAc), 175.6, 175.7, 177.0 (*C*ONH of dendrimer), 180.2 (NH*C*O of GlcNAc), 181.3 (COOH). DS = 3.86/6 = 0.64.

Data for **18** ($G = 3$, DS = 0.23): ^{1}H NMR (0.1 M DCl/D_2O) δ 1.27 (t, $J = 7.0$ Hz, 1.36 H, CH_3 of OEt), 2.08 (s, 0.6 H, NH*Ac*), 2.85–2.91 (m, –CH_2–CO), 3.22 (t, $J = 6.0$ Hz, –CH_2–NHCO), 3.27 (br, *H*-2 of *N*-alkylated GlcN), 3.44

Table 2
Reaction of *N*-carboxyethylchitosan methyl ester **2** with dendrimers (solvent: H_2O (10 ml) and MeOH (100 ml), rt, 3 days)

Dendrimer			Product	DS	Solubility[a]	
Compound	MW	Equiv/CO_2Me			H_2O	Acidic H_2O
7 ($G = 1$)	361	1	**16**	0.61	Yes	Yes
7 ($G = 1$)		4	**16**	0.74	Yes	Yes
9 ($G = 2$)	817	1	**17**	0.64	Yes	Yes
11 ($G = 3$)	1729	1	**18**	0.23	No	Yes
13 ($G = 4$)	3553	1	**19**	0.17	No	Yes
15 ($G = 5$)	7201	1	**20**	0.04	No	Yes

[a] 10 mg of compound was suspended in 2 ml of solvent at rt and observed after 1 day.

Table 3
Chemical structure of compounds **4**, **16**, and **17**

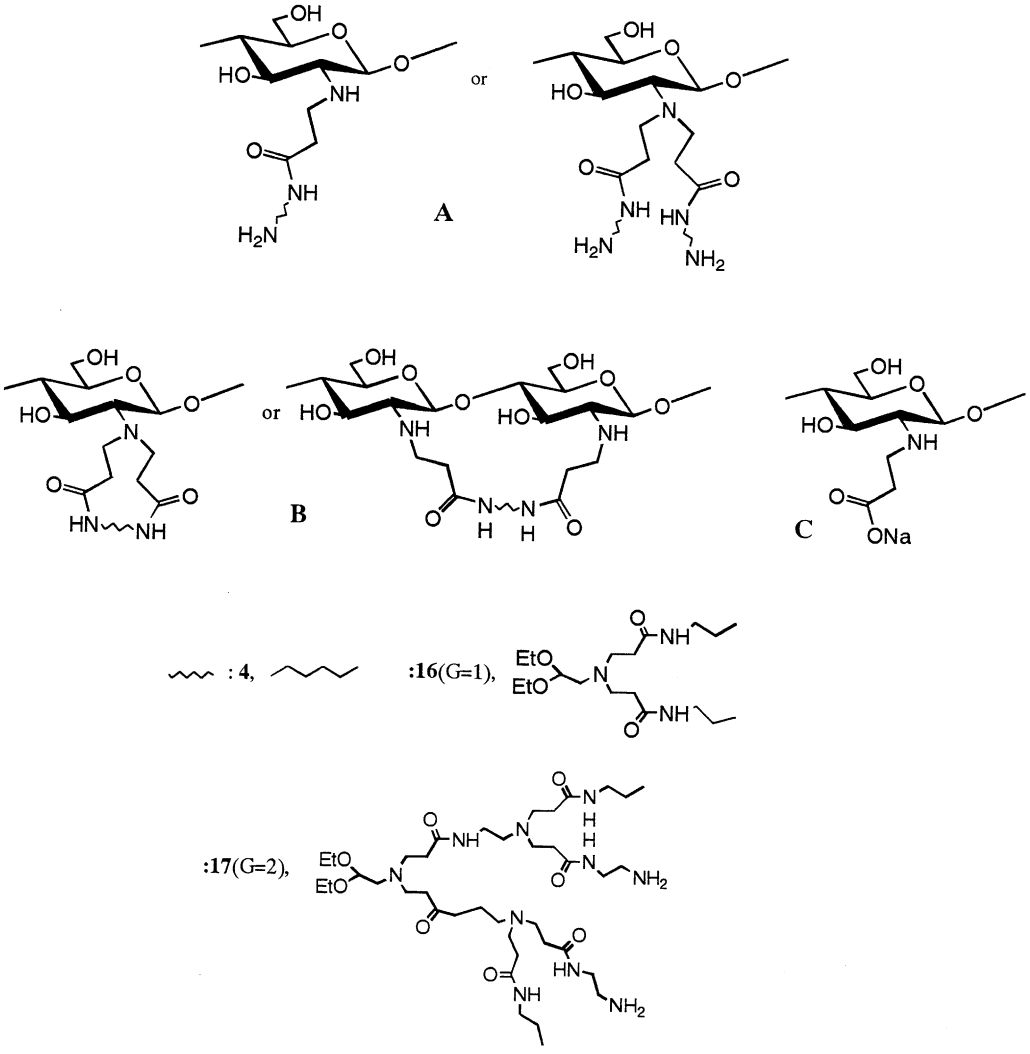
Compound	DS	Component[a]		
		A (NH_2)	*B* (ring)	*C* (CO_2Me)
4	0.94	0.84	0.10	0.16
16 ($G = 1$)	0.61	0.30	0.31	0.28
17 ($G = 2$)	0.64	0.33	0.31	0.25

[a] *A*, determined by ninhydrin analysis; $B = DS - A$; $C = 1.2 - (DS + B)$; *A*, *B*, and *C*, see Scheme 5.

(m, $-CH_2-CHOEt_2$), 3.59 (t, $J = 5.8$ Hz, $-N-CH_2-$), 3.4–4.4 (m, *H*-2 of GlcNAc, and *H*-3,4,5,6 of hexosamine), 4.64 (br, H-1 of GlcNAc), 4.98 (t, $J = 5.0$ Hz, CH of acetal), 5.04 (m, *H*-1 of *N*-monoalkylated GlcN), 4.98 (t, $J = 5.0$ Hz, CH of acetal), 5.19 (br, *N*,*N*-dialkylated GlcN); ^{13}C NMR (0.1 M DCl/D_2O) δ 17.5 (CH_3), 25.1 (NH*Ac*), 32.6–34.6 ($COCH_2$), 39.8 ($CONHCH_2$), 42.0 ($CONHCH_2$), 46.5 (CH_2NH_2), 52.0–54.8 ($N-CH_2$), 58.2 (CH_2CHOEt_2), 63.3 (*C*-6), 64.4 (*C*-2 of *N*-mono and dialkylated GlcN), 72.4 (*C*-3), 77.7 (*C*-5), 79.0 (*C*-4), 99.7 (*C*-1 of *N*-mono and dialkylated GlcN), 101.6 (*C*H of acetal), 176.2, 176.3 (*C*ONH of dendrimer), 180.7 (NH*C*O of GlcNAc), 181.5 (COOH). $DS = 1.36/6 = 0.23$.

Data for **19** ($G = 4$, $DS = 0.17$): ^1H NMR (0.1 M DCl/D_2O) δ 1.30 (t, $J = 7.0$ Hz, 1.02 H, CH_3 of OEt), 2.08 (s, 0.6 H, NH*Ac*), 2.95 (m, $-CH_2-CO$), 3.25 (m, $-CH_2-NHCO$), 3.63 (t, $J = 6.2$ Hz, $-N-CH_2-$), 3.2–4.4 (m, 2,3,4,5,6 of hexosamine), 4.64 (br, H-1 of GlcNAc), 5.13 (br, *H*-1 of *N*-monoalkylated GlcN), 5.30 (br, *N*,*N*-dialkylated GlcN). $DS = 1.02/6 = 0.17$.

Data for **20** ($G = 5$, $DS = 0.04$): ^1H NMR (0.1 M DCl/D_2O) δ 1.30 (br, 0.24 H, CH_3 of OEt), 2.08 (s, 0.6 H, NH*Ac*), 2.95 (br, $-CH_2-CO$), 3.25 (br, $-CH_2-NHCO$), 3.63 (br, $-N-CH_2-$), 3.2–4.4 (m, *H*-2,3,4,5,6 of hexosamine), 4.64 (br, H-1 of GlcNAc), 5.13 (br, *H*-1 of *N*-monoalkylated GlcN), 5.30 (br, *N*,*N*-dialkylated GlcN). $DS = 0.24/6 = 0.04$.



Scheme 5.

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