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Synthesis of novel chitosan with chitosan side chains

Minoru Morimoto[a], Masaru Nakao[b], Naoya Ishibashi[b], Yoshihiro Shigemasa[b], Shinsuke Ifuku[b], Hiroyuki Saimoto[b,*]

[a] Research Center for Bioscience and Technology, Tottori University, Koyama, Tottori 680-8550, Japan
[b] Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, 4-101 Minami, Koyama, Tottori 680-8552, Japan

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ABSTRACT

Although extensive research is in progress on the use of chitin and chitosan, poor solubility has been one of the main obstacles to their effective utilization. Introduction of carbohydrate branches into chitin and chitosans gave branched polysaccharide analogs, which showed good water solubility and induced new chemical and biological functions. In this study, hyperbranched chitosan derivatives were synthesized by introducing chitosan branches to the amino group of chitosan by the reductive *N*-alkylation method. Compared with the linear chitosan, the hyperbranched derivatives exhibited improved water solubility in the physiological conditions (pH 6.8). Although the viscosity of starting chitosans for main chain increased with the increase in their weight average molecular weights (80–190 kDa), the corresponding hyperbranched chitosan derivatives showed relatively low viscosities.

1. Introduction

Chitin and chitosan, natural biopolymers that are the second most abundant biopolysaccharides after cellulose, have attracted growing attention in the agricultural, food, industrial and medicinal fields as functional substances (Ifuku et al., 2009; Morimoto, Saimoto, & Shigemasa, 2002; Okamoto et al., 2002; Renbutsu et al., 2005). However, poor solubility has been one of the main obstacles to their effective utilization, and to improve the solubility, we have synthesized various derivatives by chemical modification (Muslim et al., 2001; Renbutsu et al., 2008; Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998; Terada et al., 2003). Branched polysaccharides exist in nature and some of them are known to show biological activities including intercellular recognition and adhesion. Introduction of carbohydrate branches into chitin and chitosans gave branched polysaccharide analogs, which showed good water solubility and induced new chemical and biological functions (Hashimoto, Morimoto, Saimoto, Shigemasa, & Sato, 2006; Li et al., 1999; Li et al., 2000; Omura et al., 2001). As nitrous acid depolymerization of chitosan is well known to give low molecular weight chitosan derivatives with a 2,5-dehydro-D-mannofuranose unit at the reducing end, the chitosan–mannofuranose chains thus obtained were introduced to the amino group of chitosan to give branched chitosan derivatives that showed new biological functionalities (Aggarwal & Matthew, 2007; Aggarwal & Matthew, 2009; Strand, Issa, Christensen, Varum, & Artursson, 2008). Recently, the chemistry of designed dendrimers and hyperbranched polymers has been extensively studied (Arce et al., 2003; De Jesus, Ihre, Gagne, Frechet, & Szoka, 2002). In this study, hyperbranched chitosan derivatives were synthesized by introducing chitosan branches to the amino group of chitosan by the reductive *N*-alkylation method.

2. Experimental

2.1. Materials and general methods

The following chitosans were obtained from Koyo Chemical Co., Ltd., Japan: **1** (FH-80, degree of deacetylation (DDA) 87%, *M*w 650 kDa, *M*n 73 kDa); **2** (FM-80L, DDA 86%, *M*w 450 kDa, *M*n 82 kDa); **3** (Lot No. 107311, DDA 45%, *M*w 190 kDa, *M*n 88 kDa); **4** (SK-10 Lot No. 1023-10, DDA 84%, *M*w 140 kDa, *M*n 31 kDa); **5** (Lot No. L05261, DDA 95%, *M*w 80 kDa, *M*n 21 kDa); **6** (Lot No. L04171, DDA 97%, *M*w 71 kDa, *M*n 20 kDa); **7** (HCl salt, Lot No. 1101-13T, purified by dialysis, DDA 71%, *M*w 8 kDa, *M*n 2 kDa); **8** (HCl salt, Lot No. L0221-20FD, DDA 90%, *M*w 5 kDa, *M*n 2 kDa). Chitosan (Flonac C, **9**, DDA 84%, *M*w 160 kDa, *M*n 37 kDa) was procured from Kyowa Technos Co., Japan. Chitosan (Chitosans **5** and **10**, Lot No. TSQ4638, DDA 86%, *M*w 100 kDa, *M*n 20 kDa) and other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan and used without further purification. The DDA values were determined by ^{1}H NMR analysis and elemental analysis (Shigemasa, Matsuura,

* Corresponding author. Tel.: +81 857 31 5693; fax: +81 857 31 5693.
E-mail address: saimoto@chem.tottori-u.ac.jp (H. Saimoto).

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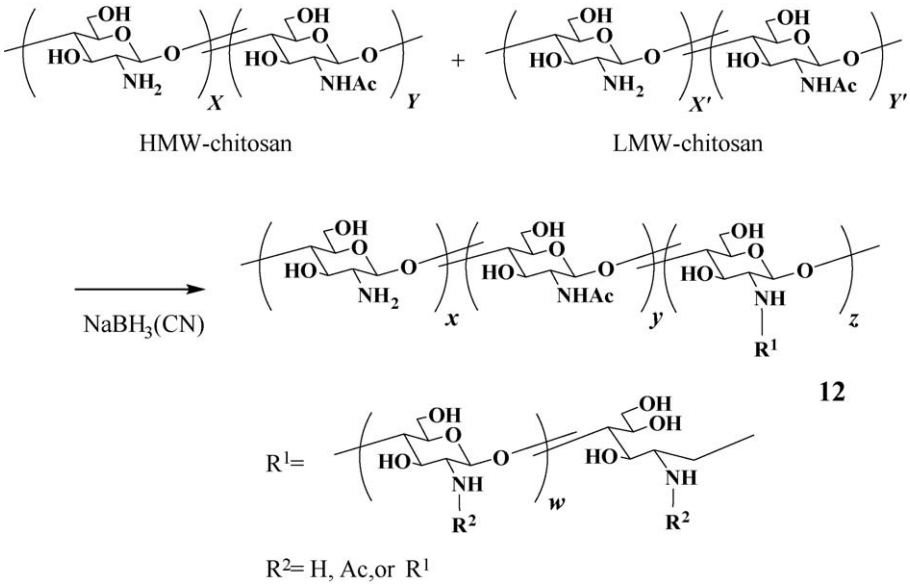

Fig. 1. Synthesis of hyperbranched chitosan derivatives.

Sashiwa, & Saimoto, 1996). Average molecular weights were estimated by the GPC method with pullulan as standard (column: two TSK-GEL GMPWXL or Shodex Asahipack GS520HQ, GS320HQ, GS220HQ; eluent: 0.1 M aq $NaNO_3$; flow rate: 0.5 ml/min; column temperature: 40 °C).

2.2. Preparation of hyperbranched chitosans (a typical procedure)

According to the reductive *N*-alkylation method (Hall & Yalpani, 1980; Muzzarelli, Tanfani, Mariotti, & Emanuelli, 1982), chitosan HCl salt **7** (6.33 g, ca. 3.17 mmol as reducing end) was added to a 1% aq AcOH solution of chitosan **5** (1.20 g, 6.99 mmol as amino group), and the mixture stirred at 20–25 °C for 12 h. After addition of $NaBH_3CN$ (0.40 g, 6.37 mmol), the mixture was stirred at 20–25 °C for a further 12 h. Dialysis of the reaction mixture with dialysis tube (14 kDa cut off) for 7 days followed by lyophilization gave the hyperbranched derivative (colorless sponge, 1.65 g, DDA 78%). The degree of hyperbranching (z), estimated to be 0.06 by ^{1}H NMR analysis and elemental analysis, was defined as follows:

$$z = \mathrm{BR/TR} = (\mathrm{HR} \times \mathrm{DS})/[\mathrm{HR} + \mathrm{LR} \times (\mathrm{HR} \times \mathrm{DS})] \quad (1)$$

where BR is the number of branched residues, TR the number of total residues, and HR is the average number of residues in HMW-chitosan, DS is conventional degree of substitution, and LR is the average number of residues in LMW-chitosan.

In Eq. (1), HR and LR have known values corresponding to the chitosans. DS was obtained from the following equation:

$$(\mathrm{DDA_{HB}})/100 = \mathrm{GlcNR/TR} = [(\mathrm{HR} \times \mathrm{DDA_H}/100) + (\mathrm{LR} \times \mathrm{HR} \times \mathrm{DS} \times \mathrm{DDA_L}/100)]/\mathrm{TR} \quad (2)$$

where $\mathrm{DDA_{HB}}$ is DDA of hyperbranched chitosan, GlcNR is the number of GlcN residues in hyperbranched chitosan, $\mathrm{DDA_H}$ is DDA of HMW-chitosan, and $\mathrm{DDA_L}$ is DDA of LMW-chitosan. $\mathrm{DDA_{HB}}$ was obtained by ^{1}H NMR analysis and/or elemental analysis of hyperbranched chitosan derivatives.

2.3. Synthesis of labeled chitosans

Using a modification of the reported procedure (Morimoto et al., 2001), Rhodamine B isothiocyanate (45 mg, 0.087 mmol) was added to a 1% aq AcOH solution (50 ml) of chitosan **6** (2.00 g, 12.0 mmol as amino group), and the mixture stirred at 20–25 °C for 12 h. Dialysis of the reaction mixture followed by lyophilization gave the Rhodamine B labeled chitosan derivative **R-6** (1.78 g, 89% yield). Although ^{1}H NMR peaks corresponding to the Rhodamine B moiety were not observed, the absorption peak at ca. 530 nm in the UV–vis spectra and fluorescence peak at ca. 570 nm indicated formation of the desired product **R-6**.

Fluorescein isothiocyanate (FITC, 110 mg, 0.290 mmol) was added to an aqueous solution (30 ml) of chitosan HCl salt **8** (4.00 g, 18.3 mmol as amino group). After stirring at 20–25 °C for 12 h, the reaction mixture was poured into excess ethanol to produce a precipitate. Washing of the precipitate three times with ethanol

Table 1
Synthesis of hyperbranched chitosan derivatives.

Entry	HMW-chitosan				LMW-chitosan[a]		Product				
		g (mmol as $-NH_2$)	*M*w (kDa)	DDA (%)	g (mmol as reducing end)			g	DDA (%)	DS	*z*
1	**5**	1.20 (6.99)	80	95	**7**	6.33 (3.17)	**12a**	1.65	78	0.21	0.06
2	**10**	2.00 (10.3)	100	86	**7**	6.33 (3.17)	**12b**	2.56	77	0.13	0.05
3	**4**	2.00 (10.0)	140	84	**7**	5.00 (2.50)	**12c**	2.20	76	0.14	0.05
4	**9**	2.52 (10.0)	160	84	**7**	6.33 (3.17)	**12d**	3.20	78	0.19	0.06
5	**3**	2.52 (6.16)	190	45	**7**	6.33 (3.17)	**12e**	3.25	58	0.09	0.04
6	**2**	2.00 (10.3)	450	86	**7**	6.50 (3.25)	**12f**	2.25	75	0.24	0.06
7	**1**	2.00 (10.5)	650	87	**7**	6.50 (3.25)	**12g**	2.25	76	0.19	0.06

[a] Chitosan **7**: *M*w 8 kDa; DDA 71%.

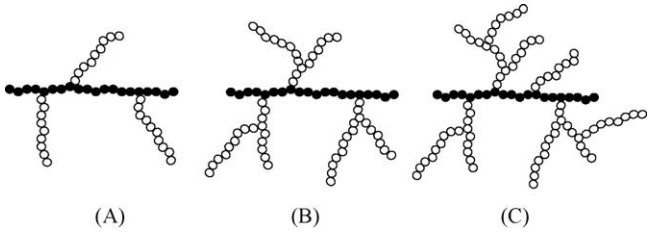

Fig. 2. Hyperbranched chitosan derivatives. Number of branched residues: (**A**) 3; (**B**) 6: (**C**) 9.

followed by lyophilization gave the FITC labeled chitosan derivative **FITC-8** (3.50 g, 88% yield). Although ^{1}H NMR peaks corresponding to the FITC moiety were not observed, the absorption peak at 494 nm in the UV–vis spectra and fluorescence peak at ca. 530 nm indicated formation of the desired product **FITC-8**.

The double-labeled chitosan **11**, which had both Rhodamine B and FITC moieties, was prepared from **R-6** and **FITC-8** by the reaction mentioned in a typical procedure.

2.4. Fluorescence measurement

Fluorescence spectra and UV–vis spectra were recorded with a Shimadzu RF-5301 spectrofluorophotometer and a Shimadzu UV-1600 spectrophotometer, respectively. The labeled chitosan derivatives were dissolved in 1% aq AcOH solution and their concentrations adjusted to 0.1 mg ml^{-1}. The excitation wavelength was 510 nm for all spectra.

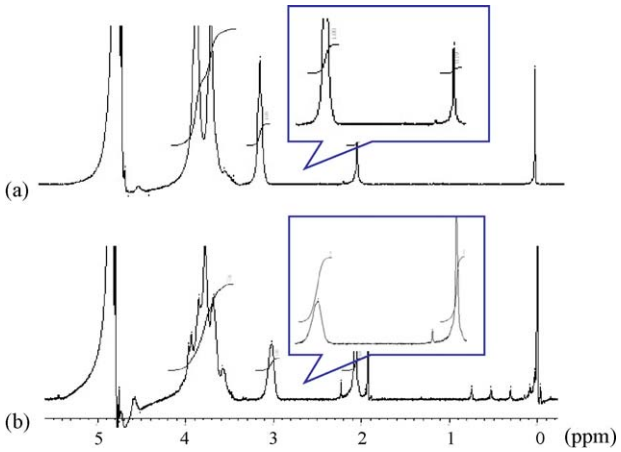

Fig. 3. ^{1}H NMR spectra (400 MHz, 1% DCl/D_2O) of (a) HMW-chitosan **5**, and (b) hyperbranched product **12a**.

2.5. Evaluation of solubility and viscosity

A sample was soaked in each solvent at a concentration in the range 0.5–15 mg ml^{-1} at 20–25 °C, and after 12 h the solubility was

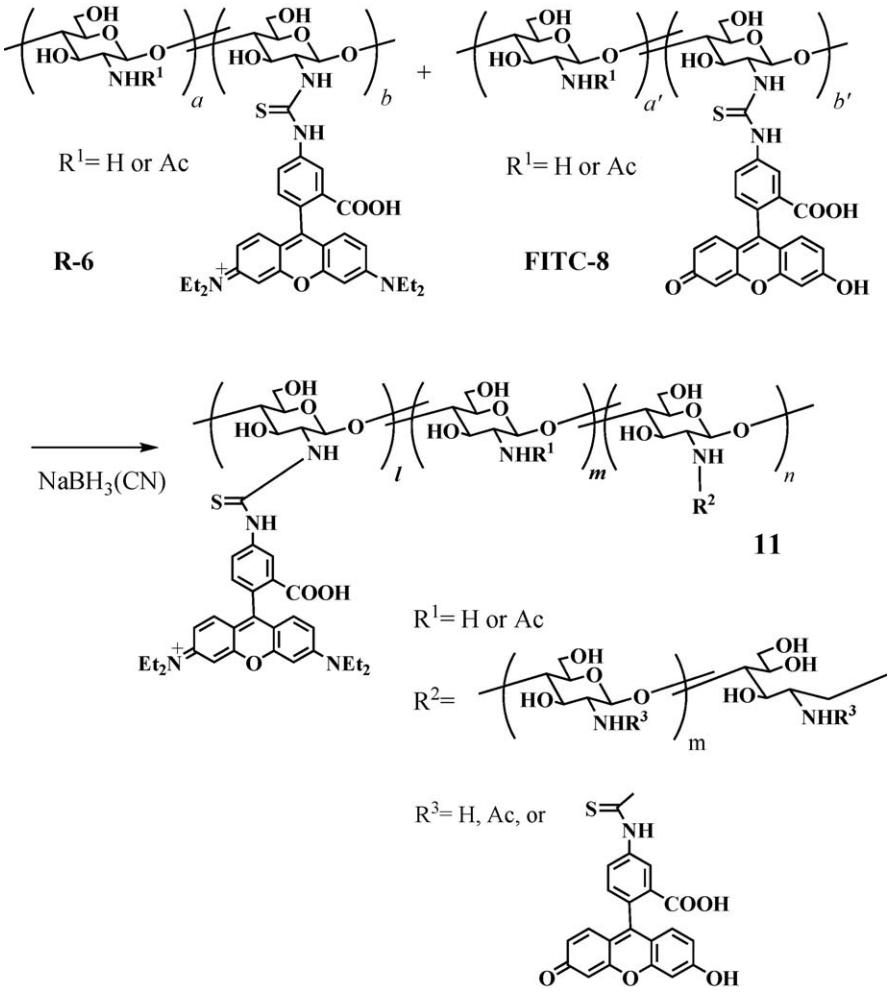

Fig. 4. Reaction of labeled HMW-chitosan and labeled LMW-chitosan.

Table 2
Solubility and viscosity of hyperbranched chitosan derivatives.

Entry	HMW-chitosan			Hyperbranched product[a]				
		*M*w (kDa)	DDA (%)		DDA (%)	*z*	Water solubility at pH 6.8 (mg ml^{-1})	Viscosity (cP)
1	**5**	80	95	**12a**	78	0.06	6	2.2
2	**10**	100	86	**12b**	77	0.05	10	3.3
3	**4**	140	84	**12c**	76	0.05	14	2.4
4	**9**	160	84	**12d**	78	0.06	12	5.6
5	**3**	190	45	**12e**	58	0.04	9	5.4
6	**2**	450	86	**12f**	75	0.06	3	30
7	**1**	650	87	**12g**	76	0.06	0.5	210

[a] Chitosan **7** (*M*w 8 kDa, DDA 71%) was used as LMW-chitosan.

evaluated. The viscosity of chitosan sample (10 mg ml^{-1}) in 1% aq AcOH was measured with a Visconic ELD rotational viscometer (Tokyo Keiki Co., Ltd., Japan).

3. Results and discussion

3.1. Synthesis of hyperbranched chitosan derivatives

Hyperbranched chitosan derivative **12** was synthesized from relatively high molecular weight chitosan (HMW-chitosan) and relatively low molecular weight chitosan (LMW-chitosan) by the reaction of the amino group of HMW-chitosan with the reducing end of LMW-chitosan as shown in Fig. 1 and Table 1.

Although the primary product of this reaction was branched chitosan (Fig. 2**A**), hyperbranched product **B** was formed in the next generation step, because the amino groups in both main chain and side chain in branched product **A** can be subjected to further reductive *N*-alkylation reaction. As the side chain moiety is not like a dendron prepared by stepwise branching, the chitosan derivative **12** is considered as hyperbranched compound **C** (Fig. 2), which has several generations of branches.

When we used the conventional degree of substituent (DS) in a preliminary study, the DS value in Entry 1 (Table 1) was 0.21 as a simple branched model **A** in Fig. 2. Subsequently we used the number of branched residues for the characterization of **12**. For example, models **A**, **B**, and **C** in Fig. 2 have 3, 6, and 9 branched residues, respectively. The degree of hyperbranching (*z*) was defined as the proportion of branched residues relative to the total number of residues. Under the experimental conditions shown in Table 1, all of the hyperbranched chitosan derivatives showed similar *z* values (0.04–0.06). When HMW-chitosan and LMW-chitosan with substantially different DDA values were employed, the proportional change of the peak area corresponding to the *N*-acetyl group could be easily estimated by ^{1}H NMR analysis as shown in Fig. 3.

3.2. Fluorescence study of hyperbranched chitosan derivatives

In a preliminary study, reductive *N*-alkylation reaction using only HMW-chitosan **5** did not give any water-soluble products, and reductive *N*-alkylation reaction using only LMW-chitosan **8** gave only a negligible amount of products after dialysis of the reaction mixture with dialysis tube (14 kDa cut off) for 7 days. However, the same reaction using both HMW-chitosan **5** and LMW-chitosan **8** gave the desired water-soluble products. Although these results suggested formation of hyperbranched products, it is not easy to obtain evidence corresponding to both components.

To confirm the formation of the hyperbranched chitosan derivative that consists of HMW-chitosan and LMW-chitosan, the double labeling method was applied. Rhodamine B isothiocyanate and FITC used in this study are convenient amine-reactive labeling agents with fluorescence peaks at 570 and 520 nm, respectively. Fluorescence spectra of the Rhodamine B labeled HMW-chitosan **R-6** and the FITC labeled LMW-chitosan **FITC-8** shown in Fig. 4 were clearly distinguishable. The fluorescence spectrum of the hyperbranched chitosan derivative **11** prepared using **R-6** and **FITC-8** gave two peaks at 570 nm and 530 nm arising from Rhodamine B and FITC, respectively (Fig. 5). That result indicates that this hyperbranched chitosan prepared in this study will be formed like the models shown in Fig. 2. A quantitative treatment for calculation of the degree of hyperbranching (*z*) by fluorescence intensity or absorbance was not attempted because of low peak intensities and large overlaps in the fluorescence and absorption spectra.

3.3. Physical properties of hyperbranched chitosan derivatives

Although the starting linear HMW-chitosans were insoluble in water at pH 6.8, the hyperbranched chitosan derivatives showed good water solubility as shown in Entries 1–5 in Table 2. Because reductive *N*-alkylation reaction using only HMW-chitosan **5** did not give any water-soluble products, hyperbranching using HMW-chitosan and LMW-chitosan seems to be effective for obtaining water-soluble products. In the cases of HMW-chitosans **1** and **2**, whose weight average molecular weights were higher than 400 kDa, the water solubility was not much improved after hyperbranching. The water solubility of **12** was maximized in Entry 3. These results suggest that choice of the combination of HMW-chitosan with LMW-chitosan greatly affects the water solubility of the products. Good solubility in neutral water is advantageous for biomedical and industrial applications.

The viscosity of hyperbranched chitosan derivatives, featured in Fig. 6, is of particular interest. Although the viscosity of the linear HMW-chitosans **5**, **10**, **4**, **9**, and **3** increased with increase in their weight average molecular weights, the corresponding hyperbranched chitosan derivatives **12a**–**12e** showed similar viscosities (shown in Table 2). The implication is that the hyperbranched chi-

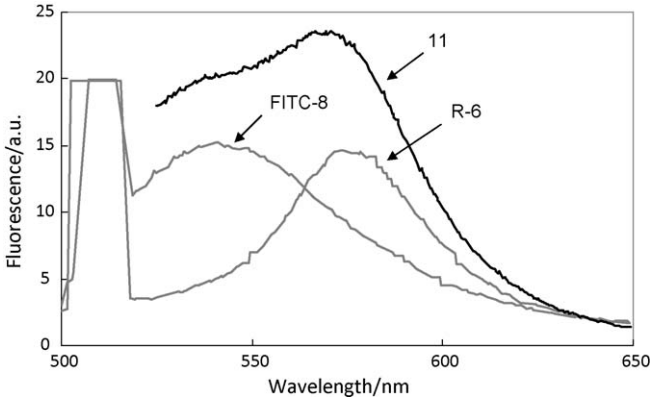

Fig. 5. Fluorescence spectra of double-labeled chitosan **11**, Rhodamine B labeled chitosan derivative **R-6**, and FITC labeled chitosan derivative **FITC-8**. Excitation wavelength = 510 nm.

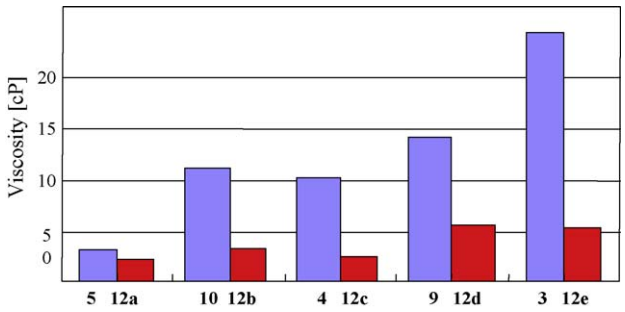

Fig. 6. Viscosity of HMW-chitosans and hyperbranched chitosan derivatives.

tosan derivatives are less intertwined compared with the linear chitosans. However, in the cases of HMW-chitosans **1** and **2** in Table 2, whose weight average molecular weights were higher than 400 kDa, the viscosity of the corresponding hyperbranched products **12f** and **12g** was relatively high.

4. Conclusion

Reductive *N*-alkylation reaction of HMW-chitosan with LMW-chitosan gave hyperbranched chitosan derivatives, which showed good water solubility. Incorporation of both HMW-chitosan and LMW-chitosan moieties in the hyperbranched products was confirmed by the double labeling method. The water solubility and viscosity of the hyperbranched products were affected by the choice of combination of HMW-chitosan with LMW-chitosan.

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