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# Synthesis of organosoluble chitosan derivatives with polyphenolic side chains

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ABSTRACT

A one-pot synthesis was used to produce chitosan derivatives with polyphenolic side chains via a regioselective phenolic coupling reaction. Under Mannich reaction conditions, treatment of chitosan with formaldehyde and methyl 2,4-dihydroxybenzoate gave *N*-(2,6-dihydroxy-3-methoxycarbonylphenyl)methylated chitosan in good yield (87%). Formation of a C—C bond occurred regioselectively at the C(3) position of methyl 2,4-dihydroxybenzoate. Chitosan derivatives having various phenolic compounds as a side chain were easily synthesized in a similar manner. The chitosan derivatives showed good biodegradability and improved their solubility in methanol (9.8 mg $mL^{-1}$) and 2-methoxyethanol (> 10 mg $mL^{-1}$). The UV protection provided by the derivatives with phenolic benzophenone side chain was evaluated using UV spectra of polyethylene terephthalate and poly(vinyl butyral-co-vinyl alcohol-co-vinyl acetate) films coated with the derivatives and the derivatives absorbed effectively in the UV-A region (<60%). Self-aggregation of the chitosan derivatives with the phenolic side chain was observed by using a fluorescent probe in aqueous solution.



## 1. Introduction

Chitin and chitosan have attracted increasing attention as biodegradable and functional substrates for use in agriculture, food industry, and medicines (Muzzarelli et al., 2012; Ifuku et al., 2009; Okamoto et al., 2002). Chitosan shows unique chemical and biochemical properties because of the presence of an active amino group. Unfortunately, it is not soluble in common organic solvents and neutral water. The water solubility of chitosan can be improved by introducing carbohydrate branches into the amino group to produce branched polysaccharide analogs, which show good and new chemical and biological functions (Aggarwal & Matthew, 2007; Hall & Yalpan, 1980; Hashimoto, Morimoto, Saimoto, Shigemasa, & Sato, 2006; Li et al., 1999; Morimoto et al., 2001, 2002, 2011; Muslim et al., 2001; Strand, Issa, Christensen, Varum, & Artursson, 2008; Sugimoto, Morimoto, Sashiwa, Saimoto, and Shigemasa (1998)). However, these chitosan derivatives do not show good affinity to hydrophobic synthetic resins. Reductive *N*-benzylation of chitosan has been used to produce organosoluble chitosan derivatives, with good affinity to synthetic resins (Renbutsu et al., 2005, 2007). Organosoluble chitosan derivatives usually show low biodegradability, and their biodegradability should be compared to that of original chitosan.

Reductive *N*-benzylation of chitosan requires a benzaldehyde-type compound as a side chain precursor. However, these types of substrates are not commercially available, which limits use of this reaction. As alternative side chain precursors, commercially available phenolic compounds could be used to produce chitosan derivatives with polyphenolic side chains. One potential substrate is phenolic benzophenone, which is widely used as a UV protective agent because of its effective UV absorption and efficient radiationless decay of excited energy (Zayat, Garcia-Parejo, & Levy, 2007). Chitosan derivatives with side chains derived from benzophenone formulated as a master batch to coat or blend with polymer matrices to protect them from UV irradiation. Because polymers with hydrophobic side chains can self-aggregate though hydrophobic interactions, self-aggregation of these derivatives should be investigated.

In this research, the regioselective phenolic coupling reaction (Omura et al., 2001; Saimoto et al., 1996; Terada et al., 2003) was used in a one-pot synthesis of chitosan derivatives with polyphenolic side chains. Biodegradation of the derivatives was investigated using chitosanase RD, which is a crude product that contains mainly endo-type chitosanase. It is widely used for a biodegradation testing of chitosan or chitosan derivatives, and hydrolyzes them into oligomers and monomer units (D-glucosamine). The UV protection properties and

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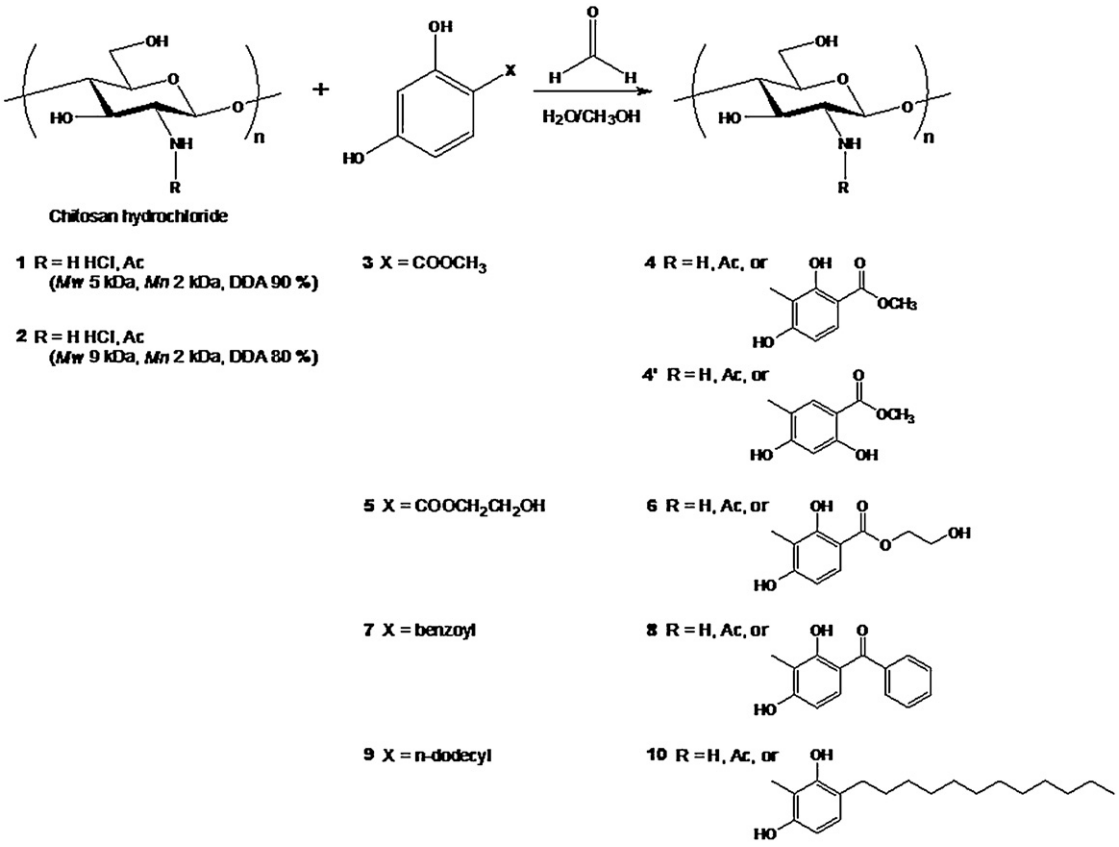


**Fig. 1.** Synthesis of chitosan derivatives.

self-aggregation behavior of the chitosan derivatives were also investigated.

## 2. Experimental

### 2.1. Materials and general methods

The following chitosan samples were from Koyo Chemical Co., Ltd. (Osaka, Japan): **1** (L0221-20FD, degree of deacetylation (DDA) 90%, $M_w$ 5 kDa, $M_n$ 2 kDa, hydrochloride); **2** (SC12181, DDA 80%, $M_w$ 9 kDa, $M_n$ 2 kDa, hydrochloride). Chitosanase RD was from Seikagaku Kogyo (Tokyo, Japan). Poly(vinyl butyral-co-vinyl alcohol-co-vinyl acetate) (PVB) was provided by Denki Kagaku Kogyo (Tokyo, Japan). Phenolic compounds and other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used as received. The DDA values were determined by $^1$H NMR analysis (JEOL JNM-ECP500) and elemental analysis (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996). Chitosan derivatives **8** and **10** were prepared according to a reported procedure (Saimoto et al., 2009).

### 2.2. N-(2,6-dihydroxy-3-methoxycarbonylphenyl)methylation of chitosan (a typical procedure)

An aqueous formaldehyde solution (mass fraction 37%, 405 mg, 5.0 mmol as HCHO) was added to an aqueous solution (50 mL) of chitosan hydrochloride **2** (1.29 g, 5.0 mmol as amino group). After stirring at 65 °C for 1 h, a methanol solution (50 mL) of methyl 2,4-dihydroxybenzoate (**3**) (841 mg, 5.0 mmol) was added to the reaction mixture. The solution was then stirred at 65 °C for 24 h. After cooling, the reaction mixture was poured into acetone (1000 mL), which produced a precipitate that was separated by centrifugation. The precipitate was dissolved in water (40 mL), and lyophilized to give chitosan derivative **4** (1.27 g, degree of substitution (DS) 0.2, 87% yield). The DS was calculated from the ratio of N% to C%, which was estimated by elemental analysis. $^1$H NMR ($D_2O$) $\delta$ 2.09 (*N*-Ac), 3.0–3.1 (C(2)-H of GlcN unit), 3.5–4.1 (C(3), C(4), C(5), C(6)-H of chitosan chain, C(2)-H of GlcNAc unit, $COOCH_3$, and Ar-$CH_2$N), 4.6–4.9 (C(1)-H of chitosan chain), 6.57 (d, $J$ = 7.3 Hz, C(5)-H of benzene ring), 7.84 (d, $J$ = 7.3 Hz, C(4)-H of benzene ring); IR (KBr) 3100–3600, 1668, 1622, 1439, 1281, and 1070 cm$^{-1}$.

*N*-(3-(2-hydroxyethoxycarbonyl)-2,6-dihydroxyphenyl)methylated chitosan (**6**) was prepared from 2-hydroxyethyl 2,4-dihydroxybenzoate. $^1$H NMR ($D_2O$) $\delta$ 2.06 (*N*-Ac), 3.1–3.3 (C(2)-H of GlcN unit), 3.5–4.1 (C(3), C(4), C(5), C(6)-H of chitosan chain, C(2)-H of GlcNAc unit, $OCH_2CH_2O$, and Ar-$CH_2$N), 4.4–4.9 (C(1)-H of chitosan chain), 6.63 (d, $J$ = 9.0 Hz, C(5)-H of benzene ring), 7.96 (d, $J$ = 9.0 Hz, C(4)-H of benzene ring); IR (KBr) 3100–3600, 1655, 1624, 1452, 1278, and 1069 cm$^{-1}$.

### 2.3. Biodegradation

Each chitosan derivatives (0.75 mg mL$^{-1}$) was treated with chitosanase RD (0.012 mg mL$^{-1}$) in an acetate buffer solution (0.1 mol L$^{-1}$, pH 4.8) at 40 °C for 0–120 min. The amount of reducing end produced by depolymerization at the anhydroglycosidic bonds of chitosan was determined using a modified Schales' method with D-glucosamie hydrochloride as a standard (Imoto & Yagishita, 1971).

### 2.4. Solubility

Water, 5% hydrochloric acid, methanol, and 2-methoxyethanol were used for the solubility test. Each chitosan derivative (10 mg mL$^{-1}$) was soaked in each solvent at 20–25 °C for 24 h and

then centrifuged (9000 rpm, 25 min). The solubility was evaluated using the mass of recovered chitosan derivative (mg) from the soluble (supernatant) and insoluble (precipitate) fractions.

### 2.5. UV protection properties

A PVB film (diameter, 30 mm; thickness ca. 0.15 mm) was prepared by a casting method from a methanol solution of PVB (20 mg mL$^{-1}$, 2 mL). To evaluate the UV absorption, a methanol solution of **8b** was cast on a polyethylene terephthalate (PET) film (Lumirror, thickness 0.21 mm, Toray Industries Inc., Tokyo, Japan) and the PVB film. The amount of the 3-benzoyl-2,6-dihydroxyphenyl group of **8b** on the films was adjusted to 0.47 μmol cm$^{-2}$. After drying, the coating thickness with the **8b** was ca. 0.01 mm. UV spectra (percent transmittance) of the films were recorded with a Shimadzu (Kyoto, Japan) UV-1600 spectrophotometer from 200 to 600 nm.

### 2.6. Self-aggregation behavior

Self-aggregation behavior was evaluated using fluorescence spectra changes with 8-anilinonaphthalene-1-sulfonic acid (ANS) as a fluorescent probe. The fluorescence spectra of an aqueous solution of ANS ($1.2 \times 10^{-6}$ mol L$^{-1}$, $\lambda_{ex}$ 350 nm) were recorded with a Shimadzu RF-5300 spectrofluorophotometer in the presence of each chitosan derivative (**2**, **3**, **4d**, **7**, **8a**, **9**, **10**; 0–0.9 mg mL$^{-1}$).

## 3. Results and discussion

### 3.1. Convenient method for the synthesis of N-phenylmethylated chitosan derivatives

Mannich-type reaction of methyl 2,4-dihydroxybenzoate (**3**) with primary amines and formaldehyde may proceed at C(3) and C(5) positions of **3**. In this study, $^1$H NMR spectra of the reaction products showed two doublet signals (6.57 and 7.84 ppm) corresponding to the aromatic protons with an *ortho*-coupling constant ($J$ = 7.3 Hz). This means that the reaction of the amino group of chitosan with formaldehyde and **3** proceeded at only the C(3) position of **3** to give *N*-(2,6-dihydroxyphenyl)methylated chitosan derivative **4** as shown (Fig. 1). No singlet signals for the aromatic protons of product **4′** were observed. In addition, $^{13}$C NMR analysis confirmed the signal corresponded to the methyl ester and Ar-$CH_2$N of **4**. Among all the signals for **4** shown in Fig. 2a, $^{13}$C NMR analysis in DEPT mode show selected signals corresponding to the methylene carbons (59–61 ppm) (Fig. 2b). Fig. 3 shows magnified spectra of the methylene carbon area. Compared with the C(6) methylene carbon signals of the starting chitosan (Fig. 3b), a new methylene carbon signal (60.8 ppm) appeared in the $^{13}$C NMR spectrum of **4** (Fig. 3a). Formation of a new link between the amino group of the chitosan and the aromatic ring of **3** was confirmed by this new signal.

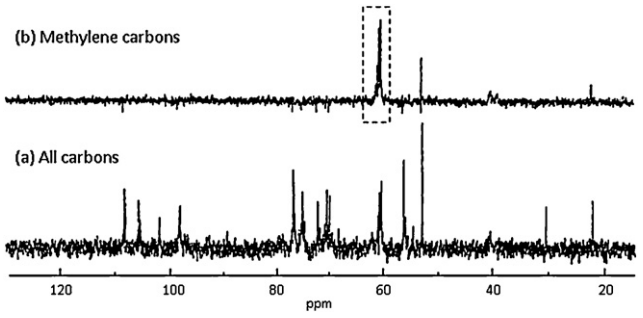


**Fig. 2.** $^{13}$C NMR analysis of chitosan derivative **4** in $D_2O$. (a) Complete decupling spectrum (all carbons) and (b) DEPT-135 spectrum (methylene carbons).

Entries 1–3 in Table 1 show that the DS of coupling products **4a**–**c** obtained from chitosan **1** increased as the amounts of added formaldehyde and **3** increased. Similarly, the DS of **4d**–**f** obtained from chitosan **2** increased from 0.2 to 0.5 (entries 4–6, Table 1).

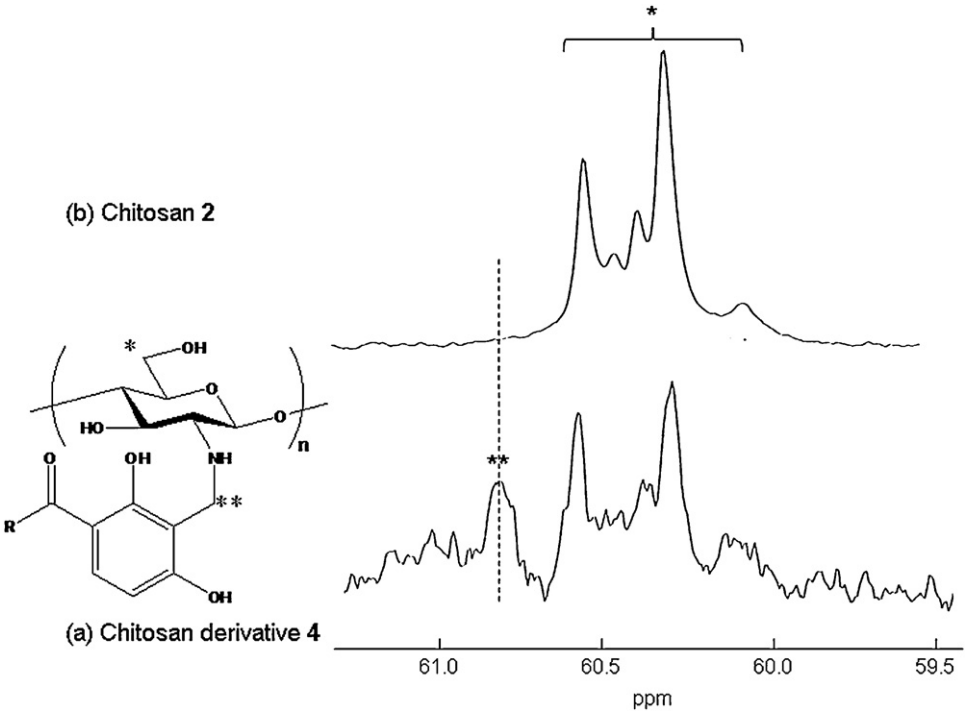


**Fig. 3.** Methylene carbons in DEPT-135 spectra of chitosan derivative **4** (a) and chitosan **2** (b).

**Table 1**
One-pot synthesis of chitosan derivatives.

| Entry | Chitosan (mmol as —$NH_2$) | HCHO (equiv.) | Phenol (equiv.) | Product (yield/%) | DS |
|---|---|---|---|---|---|
| 1 | **1** (5.0) | (1) | **3** (1) | **4a** (91) | 0.1 |
| 2 | **1** (5.0) | (2) | **3** (2) | **4b** (83) | 0.3 |
| 3 | **1** (5.0) | (3) | **3** (3) | **4c** (87) | 0.7 |
| 4 | **2** (5.0) | (1) | **3** (1) | **4d** (87) | 0.2 |
| 5 | **2** (5.0) | (2) | **3** (2) | **4e** (81) | 0.4 |
| 6 | **2** (5.0) | (3) | **3** (3) | **4f** (76) | 0.5 |
| 7 | **2** (5.0) | (1) | **5** (1) | **6a** (99) | 0.2 |
| 8 | **2** (5.0) | (3) | **5** (3) | **6b** (94) | 0.6 |
| 9 | **2** (5.0) | (1) | **7** (1) | **8a** (95) | 0.1 |
| 10 | **2** (5.0) | (3) | **7** (3) | **8b** (96) | 0.4 |
| 11 | **2** (5.0) | (1) | **9** (1) | **10** (95) | 0.03 |

DS: degree of substitution.

**Table 2**
Solubility of chitosan derivatives.

| Chitosan derivative | DS | Solubility (mg $mL^{-1}$) | | | |
|---|---|---|---|---|---|
| | | 5% HCl | Water | Methanol | 2-Methoxyethanol |
| **1** | | 10.0 | 10.0 | 8.2 | 3.1 |
| **4a** | 0.1 | 10.0 | 10.0 | 9.5 | 4.9 |
| **4b** | 0.3 | 10.0 | 10.0 | 9.7 | 7.2 |
| **4c** | 0.7 | 10.0 | 10.0 | 9.8 | 10.0 |
| **2** | | 10.0 | 10.0 | 5.8 | 1.2 |
| **4d** | 0.2 | 10.0 | 10.0 | 6.0 | 2.0 |
| **4e** | 0.4 | 10.0 | 10.0 | 7.9 | 5.3 |
| **4f** | 0.5 | 10.0 | 9.2 | 8.6 | 6.6 |
| **6a** | 0.2 | 10.0 | 10.0 | 6.0 | 2.5 |
| **6b** | 0.6 | 10.0 | 10.0 | 8.5 | 7.3 |
| **8a** | 0.1 | 10.0 | 10.0 | 6.0 | 3.8 |
| **8b** | 0.4 | 5.2 | 4.4 | 7.2 | 6.6 |

The solubility was evaluated after stirring for 24 h at the concentration of 10 mg $mL^{-1}$.

Chitosan derivatives **6**, **8**, and **10** were obtained from phenolic compounds **5**, **7**, and **9**, respectively (entries 7–11, Table 1).

### 3.2. Biodegradability of the chitosan derivatives

The enzymatic degradation results for *N*-(2,6-dihydroxyphenyl)methylated chitosan derivatives **4d**, **4f**, and the original chitosan **2** with Chitosanase RD in acetate buffer solution are shown in Fig. 4. The change in concentration of the reducing end ($C-C_0$) for the original chitosan and its derivatives initially increased because of enzymatic depolymerization, and then leveled off. The time taken to reach this plateau decreased as the DS of the chitosan increased. This suggests the enzymatic depolymerization around the substituted residues could be inhibited by bulky aromatic side chains.

### 3.3. Solubility and UV protection properties of chitosan derivatives

The solubility results for the chitosan derivatives are shown in Table 2. While chitosans **1** and **2** had low solubility (approximately 1 mg $mL^{-1}$) in 2-methoxyethanol, the new derivatives **4a**–**4f** exhibited improved solubility in organic solvents such as methanol and 2-methoxyethanol. Chitosan derivatives **6** and **8** exhibited similar improvements in solubility in 2-methoxyethanol. By contrast, chitosan derivative **10**, which had a long hydrophobic alkyl chain, was sparingly soluble in 2-methoxyethanol.

Chitosan derivatives **8** with phenolic benzophenone side chains showed good solubility in some organic solvents. Therefore, they could be applied as UV-protective coatings or blending materials, formulated as a master batch. Fig. 5 shows the UV absorption spectra of PET and PVB coated films with chitosan derivative **8b**. The coated PET film effectively reduced transmittance of UV-A (315–400 nm) region compared with uncoated PET film. Transmittance of visible light region (400–750 nm) remained above 80%. Both UV and visible light passed through uncoated PVB film, while the coated PVB film completely blocked UV light (<400 nm). Because most of the UV radiation from sunlight that reaches the Earth's surface is UV-A, chitosan derivatives **8** will be useful as UV protection materials.

### 3.4. Self-aggregation behavior of the chitosan derivatives

Polymers with hydrophobic side chains, such as aromatic or alkyl groups, are known to self-aggregate through hydrophobic

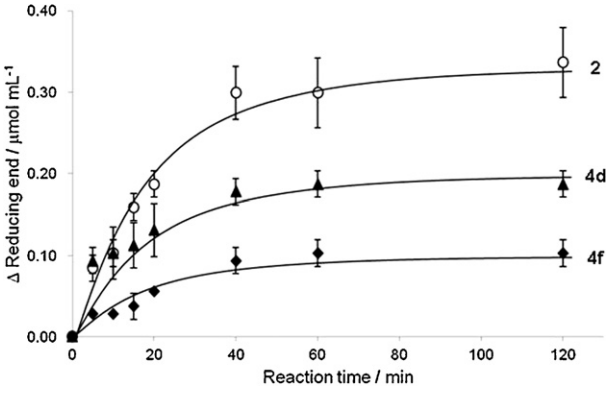


**Fig. 4.** Enzymatic degradation of chitosan and chitosan derivatives with chitosanase RD. Chitosan derivative: *N*-(2,6-dihydroxyphenyl)methylated chitosan with DS 0.2 (**4d**) and 0.5 (**4f**). Original chitosan with $M_w$ 9 kDa (**2**). Initial concentration of chitosan derivative: 0.75 mg $mL^{-1}$. Concentration of enzyme (chitosanase RD): 0.012 mg $mL^{-1}$. Acetate buffer solution (0.1 mol $L^{-1}$, pH 4.8), 40 °C. Δ reducing end: the increase in the concentration of reducing end was determined by a modified Schales' method.

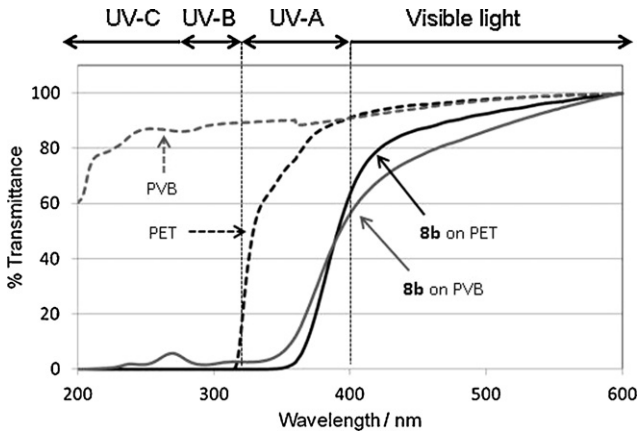


**Fig. 5.** UV protective properties of chitosan derivatives with phenolic benzophenone side chain. **8b** coated on PET (black line), PET (black dotted line), **8b** coated on PVB (gray line), PVB (gray dotted line).

interactions between the side chains. ANS is a well-known fluorescent probe that can be used to evaluate microscopic polarity (Akiyoshi, Yamaguchi, & Sunamoto, 1991). The fluorescence intensity of ANS in water is usually weak, but when it is incorporated into the hydrophobic environments such as micelles or cell membranes, the fluorescence intensity increases. In the present study, the fluorescence intensity of ANS in water increased as the concentration of chitosan derivatives (**2**, **4d**, and **10**) increased. No increase in fluorescence intensity was observed for the benzoyl derivative **8a** and 2,4-dihydroxybenzophenone, which is the side chain model of **8a** (data, not shown). This suggests that a side chain containing benzophenone structure, which induces effective radiationless decay, behaves as a fluorescence quencher. In Fig. 6, the relative maximum fluorescence intensities in the presence and absence of the chitosan derivative ($I/I_0$) were plotted as a function of the concentration of the samples (**2**, **4**, **8a**, and **10**). A linear correlation was observed for all samples except for **8a**. However, with the side chain models (**3** and **9**), the relative maximum fluorescence intensities ($I/I_0$) remained at almost zero (data not shown). These results suggested that hydrophobized chitosan derivatives such as **4d** and **10** form hydrophobic cores effectively by self-aggregation of the polysaccharides chains. The hydrophobicity of the core will depend on the hydrophobicity of the side chains (dodecylphenyl > methoxycarbonylphenyl). However, no critical micelle concentrations (cmc), bending point in Fig. 6, were observed in these experimental conditions. The cmc of the chitosan derivatives would be lower than 0.01 mg mL$^{-1}$.

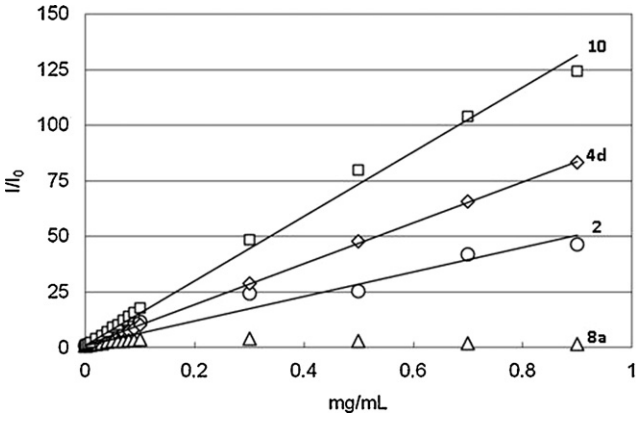


**Fig. 6.** Plot of fluorescence intensity ratio ($I/I_0$) for ANS versus concentration of the chitosan derivatives in water. $I$ and $I_0$ are the maximum fluorescence intensities in the presence and absence of the chitosan derivative, respectively. [Chitosan derivative]: 0–0.9 mg mL$^{-1}$; [ANS]: $1.2 \times 10^{-6}$ mol L$^{-1}$; $\lambda_{ex}$: 350 nm.

## 4. Conclusion

The chitosan derivatives with phenolic side chain were synthesized under Mannich reaction conditions by treatment of chitosan with formaldehyde and 2,4-dihydroxybenzoyl derivatives. The phenolic derivatives of chitosan were degraded by chitosanase RD, and showed improved solubility compared to original chitosan in methanol and 2-methoxyethanol. The chitosan derivatives with phenolic benzophenone side chains effectively absorbed in the UV-A region and could be used in UV protective coatings. The chitosan derivatives have various dihydroxybenzoyl groups as hydrophobic side chains self-aggregated in aqueous solution.

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