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Facile preparation and inclusion ability of a chitosan derivative bearing carboxymethyl-β-cyclodextrin

Etsuko Furusaki, Yoshiharu Ueno, Nobuo Sakairi*, Norio Nishi & Seiichi Tokura

Graduate School of Environmental Earth Science, Hokkaido University, Nishi-5, Kita-ku, Sapporo 060, Japan

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Coupling carboxymethylated β-cyclodextrin and partially deacetylated chitin (Mw = 7300) afforded a new type of functional chitosan derivative having an ability to form an inclusion complex. The property to form an inclusion complex was studied using a fluorescent dye, 6-(*p*-toluidino)-2-naphthalene-6-sulfonate (TNS), as the guest molecule. It was found that the presence of the cyclodextrin bonded to chitosan enhanced the relative intensity of TNS fluorescence significantly (3.6 times as much as that of β-cyclodextrin). Fluorometric titration revealed that the 1:1 stoichiometrical complex was formed and that the equilibrium constant was 1.13–1.68 × 10^3 M^{-1}. Ionic interaction between the sulfonic acid moiety of TNS and the amino group of chitosan was suggested from experiments which involved changing the pH and ionic strength of the buffer solution as well as adding a surface-active agent. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Chitosan obtained by basic hydrolysis of a natural mucopolysaccharide chitin is a straight-chain polymer consisting of β-(1→4)-linked D-glucosamine. Chitosan has three kinds of reactive functional groups: an amino group, and primary and secondary hydroxyl groups, at the C-2, C-3 and C-6 positions. Chemical modifications of these groups have provided numerous useful materials (Kurita, 1986). However, little attention has been given to development of a separating system utilizing hydrophobic interaction between modified chitin and organic compounds.

Cyclodextrins (CDs) are cyclic oligomers of α-D-glucopyranose, which exhibit unique characteristics to form inclusion complexes with various organic compounds. The inclusion compounds have been widely used in basic research and industrial processes for the microencapsulation of unstable or volatile substances (Saenger, 1980).

Recently, immobilized CDs and CD polymers have been successfully used as stationary phases in gas, liquid and affinity chromatography (Li & Purdy, 1992), resulting in a highly selective system for the separation of structural isomers or chiral compounds. These successes prompted us to synthesize a new class of CD polymers which have an optically active backbone, and to examine their physical properties. In this paper, we describe the preparation of a β-CD bonded chitosan (β-CD–chitosan) by coupling carboxymethylated β-CD (CM-β-CD) and a partially deacetylated chitin oligomer. We also examined the inclusion properties of the β-CD–chitosan by means of fluorescence techniques.

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

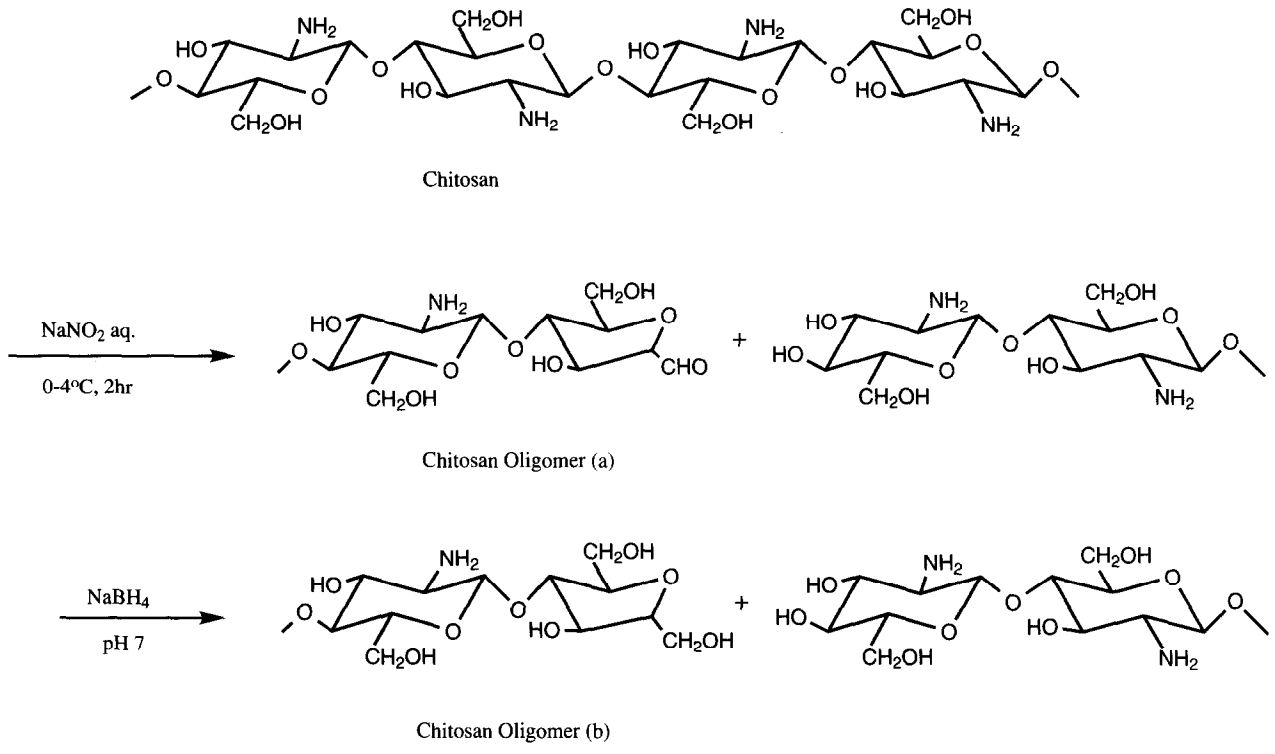
Materials

Chitosan (degree of *N*-deacetylation was 0.80) and β-CD were purchased from Kyowa Technos Co. Ltd (Chiba, Japan) and Nihon Shokuhin Kako Co. Ltd (Tokyo, Japan), respectively. 6-(*p*-Toluidino)-2-naphthalene-6-sulfonate (TNS) was purchased from Fluka Chemie AG (Buchs, Switzerland). Reagents for synthesis were supplied from Wako Pure Chemical Co. Ltd (Osaka, Japan) and used without further purification.

Methods of analyses

Depolymerization of chitosan by nitrous acid

A solution of $NaNO_2$ (4.88 g, 70.8 mmol) in water (50 ml) at 0–4°C was added dropwise to a solution of chitosan (50 g) in 5% aqueous acetic acid (1000 ml) with constant stirring. The mixture was stirred for 9 h at 0–



Scheme 1. Depolymerization of chitosan by nitrous acid.

4°C, and neutralized with concentrated aqueous ammonia. To the resulting mixture, portions of $NaBH_4$ (total, 5.35 g, 149 mmol) were added while maintaining a constant temperature of 4°C. Following the addition of $NaBH_4$, the solution was stirred overnight at room temperature (23°C) (Scheme 1).

Insoluble material was removed by filtration. The filtrate was concentrated to 250 ml and a precipitate was formed by adding methanol (750 ml). The precipitate (F-1 PPT) was collected by centrifugation at 6000 rpm and washed successively by methanol, acetone and diethyl ether several times, and air-dried at room temperature. The supernatant and the washings were combined, concentrated to 120 ml, and precipitated with methanol (1200 ml) as previously described for preparation of F-1 PPT, giving the second fraction (F-2 PPT). The supernatant was concentrated to 50 ml, diluted with methanol (400 ml), and precipitated with acetone (2250 ml). The precipitate was filtered, washed successively with acetone and diethyl ether, and air-dried to give the third fraction (F-3 PPT). The yields, molecular weights, and degrees of deacetylation of the low molecular weight chitosan derivatives are summarized in Table 1.

Table 1. Products of nitrous acid depolymerization of chitosan

PPT	Solvent	Yield (g)	Mw	Degree of deacetylation
F-1	H_2O:MeOH = 1:3	10.54	12,700	0.74
F-2	H_2O:MeOH = 1:10	13.58	7300	0.72
F-3	MeOH:acetone = 1:5	17.00	2700	0.23

Preparation of carboxymethylated β-CD

According to the method in the literature (Satomura *et al.*, 1988), a mixture of β-CD (100 g) and sodium hydroxide (93 g) in water (370 ml) was treated with a 16.3% monochloroacetic acid solution (270 ml), precipitated with methanol, and dried at 40°C under vacuum to give the carboxymethylated β-CD (CM-β-CD; 60 g).

Coupling of depolymerized chitosan and CM-β-CD

A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC, water-soluble carbodiimide 0.96 g) in water (1.5 ml) was added dropwise to a solution of depolymerized chitosan (F-2 PPT, 0.87 g) and CM-β-CD (4.02 g) in water (8 ml) maintained at 0–5°C. After being stirred for 40 h at 0–5°C, the mixture was dialyzed in Spectrapor membrane tubing (Arthur H. Thomas Co., Mw cut-off 3500) for 9 days with 10 changes of water. The resulting solution was concentrated and subsequently lyophilized to give β-CD–chitosan (1.23 g).

Determination of equilibrium constants

The equilibrium constants (K) of β-CD–chitosan–TNS complex were determined in a manner similar to that for a β-CD–TNS complex reported by Kondo *et al.* (1976). The concentration of β-CD–chitosan was estimated on the basis of β-CD units on the polymer.

RESULTS AND DISCUSSION

Preparation of low molecular weight chitosan

Although high molecular weight chitosan ($Mw = 10^4$–10^6) is soluble in water when it forms a salt with an organic acid, such as formic acid and acetic acid, the high viscosity and the acidity of the solution have restricted its chemical modification. In order to carry out the coupling in aqueous solution under neutral conditions, we chose low molecular weight chitosan, which is soluble in water with low viscosity as a substrate for the reaction.

Several methods have so far been reported for the preparation of the low molecular weight chitin or chitosan. Chemical degradations using hydrochloric acid (Rupley, 1964; Horowitz *et al.*, 1957) or hydrogen fluoride (Bosso *et al.*, 1986) often result in a complex mixture due to drastic reaction conditions and to side reactions such as the Maillard reaction through Schiff base formation. Although enzymatic degradations using chitinase (Takiguchi & Shimahara, 1989) and chitosanases (Izumi & Ohtakara, 1987) are reported to proceed selectively under mild conditions, they were only applicable to small-scale preparations.

Recently, we have succeeded (Nakao, 1992) in a large-scale preparation of low molecular weight chitosan using a modified procedure for nitrous acid degradation of chitosan (Peniston & Johnson, 1975; Yaku *et al.*, 1977). Our procedure is summarized in Scheme 1. Thus, partially deacetylated chitin was treated with sodium nitrite in aqueous acetic acid at ice-cold temperature. The reduced oligomers (a) that had an unstable aldehyde group of a 2,5-anhydro-D-mannose residue at the reducing ends were reduced with sodium borohydride directly without extraction, giving stable 2,5-anhydro-D-mannitol derivatives (b). The series of reactions could be carried out without either an extensive change in the color of the reaction mixture or the formation of a large amount of by-products. Furthermore, the chitosan oligomer produced was found to be readily obtained by fractional precipitation, using solvent systems of water–methanol–acetone.

Preparation of β-CD–chitosan

The next problem in our synthesis was the coupling reaction of a cyclodextrin derivative and the chitosan oligomer. As we expected, formation of peptide linkage between the low molecular weight chitosan and CM-β-CD could be carried out in water, employing water-soluble carbodiimide (WSC) as the condensing agent. In our preliminary experiments, unreacted compounds such as CD, chitosan oligomer and WSC could be removed by dialysis. The degree of substitution (DS) of the product was estimated from the area ratio of H-1 signals between CM-β-CD (δ 5.59, 5.78) and chitosan (δ 5.18) in the ^{1}H-NMR spectrum recorded in deuterium oxide (Fig. 1). As a result, the ratio of glucosamine residues to β-CD units was calculated to be about 4:1 (Fig. 2).

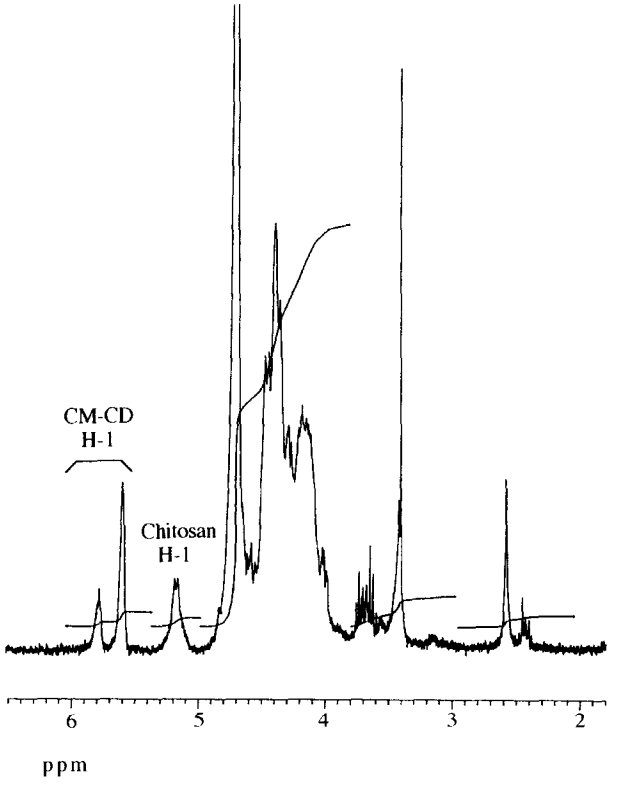


Fig. 1. ^{1}H-NMR spectrum of the β-CD–chitosan in D_2O, 80°C.

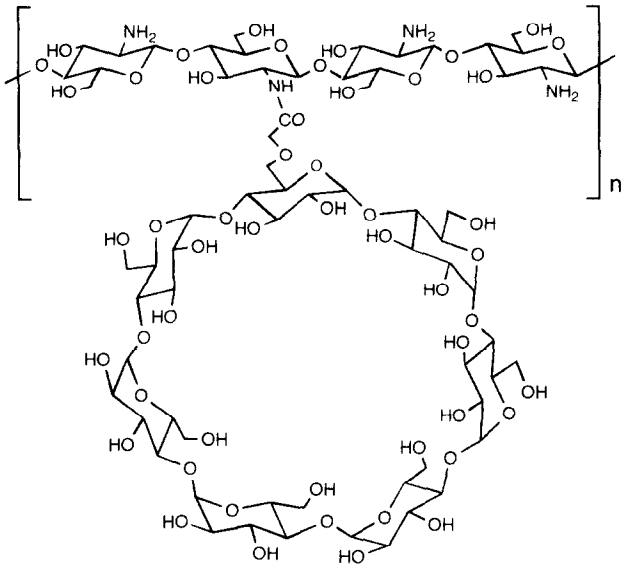


Fig. 2. Chemical structure of β-CD–chitosan.

Fluorescence studies with TNS

Having synthesized a new CD oligomer CD–chitosan, our interest was directed to the ability of this oligomer

to form inclusion complexes. TNS is one of a family of anilinonaphthalenesulfonates that have been extensively used as fluorescent probes for structural study on biopolymers, because the quantum yield increases as the microenvironmental polarity decreases and/or the probe's local viscosity increases (McClure & Edelman, 1966; Yanaoka, 1977).

The fluorescence spectra of TNS in the presence of β-CD or β-CD–chitosan in 0.1 M phosphate buffer (pH 3.5) are shown in Fig. 3. The relative fluorescence intensity of TNS in the buffer solution was very weak, while addition of β-CD to the buffer solution caused an increase of 25-fold in fluorescence intensity. Surprisingly, the fluorescence intensity was increased 90-fold when β-CD–chitosan was included in the buffer solution. With respect to the phenomenon of TNS fluorescent change by adding CDs, Kondo *et al.* (1976) previously reported that TNS showed pronounced fluorescence enhancement when α-, β-, and γ-CD was added to TNS solutions. One explanation may be that TNS may penetrate readily into the CD cavity that is relatively hydrophobic due to the high density of C–H and C–O–C groups.

In order to examine the effect of chitosan on the fluorescence intensity of TNS, the fluorescence spectrum was measured by adding chitosan (F-2 PPT) to the solution of β-CD–TNS (Fig. 3). The mixture, however, revealed almost the same spectrum as that of β-CD–TNS, suggesting that chitosan oligomer had no influence on the fluorescence enhancement. Actually, there was little enhancement of TNS fluorescence by chitosan alone.

It is well known that fluorometric titration is effective to analyze the stoichiometric relationship between host and guest molecules. It has been reported that the TNS molecule is too large to be included completely in a single β-CD cavity, and that β-CD forms both 1:1 and 2:1 complexes with TNS (Kondo *et al.*, 1976; Catena & Bright, 1989). Figure 4 shows the double reciprocal plots for titration of TNS with β-CD and β-CD–chitosan in phosphate buffer at pH 3.5. Linearity from the double reciprocal plots (Fig. 4) indicate that both β-CD and β-CD–chitosan form 1:1 complexes with TNS under our experimental conditions. Furthermore, equilibrium constants for β-CD–TNS and β-CD–chitosan–TNS at pH 3 were calculated to be 32.44×10^3 M^{-1} and 1.68×10^3 M^{-1}, respectively.

Since the toluidinyl moiety in the TNS molecule is considerably more hydrophobic than the naphthalenesulfonate moiety, the toluidinyl group should be incorporated in the cavity of β-CD, while the naphthalene moiety will still be exposed to solvent molecules. Enhancement of the fluorescence intensity by β-CD–chitosan is probably due to the difference of the environment around the naphthalenesulfonyl moiety of the TNS molecule. In order to clarify the mechanism of molecular recognition, the fluorescence changes of the complexes of β-CD–TNS and β-CD-chitosan–TNS were examined under various conditions described as follows.

The fluorescence intensities of the β-CD–chitosan–TNS complexes were first examined in phosphate

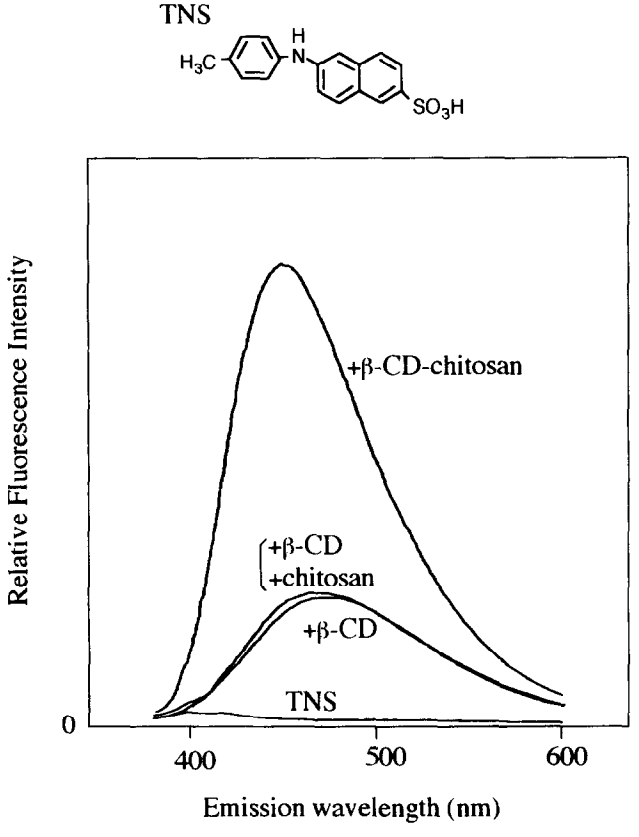


Fig. 3. Fluorescence spectra of TNS in the presence of β-CD, β-CD–chitosan and a mixture of β-CD and chitosan. [β-CD] = [β-CD– chitosan] = 10^3 M, [chitosan] = 5×10^{-3} M.

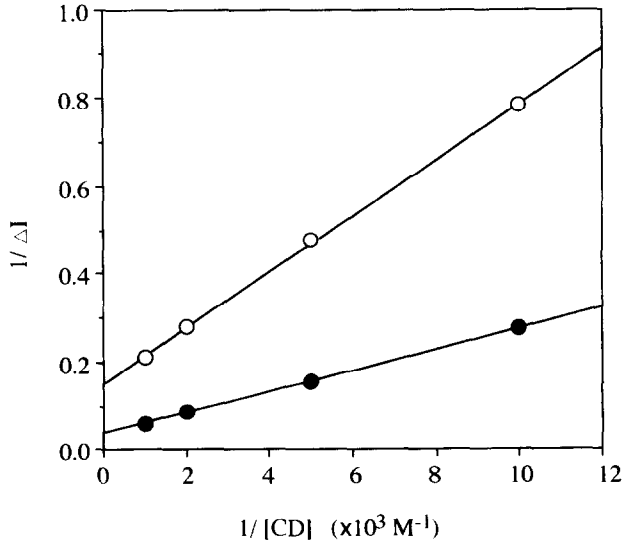


Fig. 4. Double reciprocal plots for titration of TNS by β-CD (○) and β-CD–chitosan (●). ΔI is the fluorescence intensity increment of TNS accompanying complex formation.

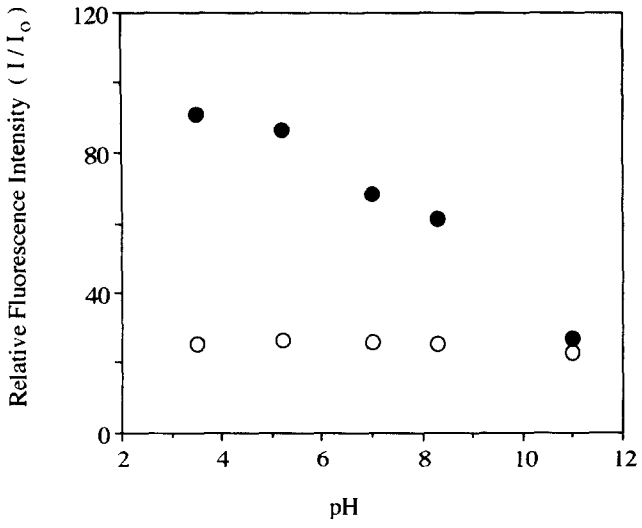


Fig. 5. Effects of pH on the fluorescence intensity of TNS in the presence of β-CD (○) and β-CD–chitosan (●), [β-CD] = [β-CD–chitosan] = 10^{-3} M. I and I_0 are the observed fluorescence intensity in the presence and absence of β-CD or β-CD–chitosan.

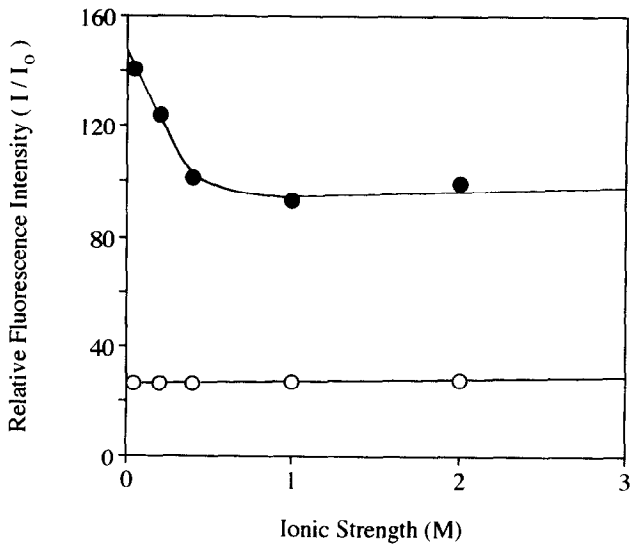


Fig. 6. Effects of ionic strength on the fluorescence intensity of TNS in the presence of β-CD (○) and β-CD–chitosan (●), [β-CD] = [β-CD–chitosan] = 10^3 M.

buffers of different pH. When the pH of the solution was increased, we observed a significant decrease in the fluorescence intensity of the β-CD–chitosan–TNS system (Fig. 5). On the other hand, the fluorescence intensity of the β-CD–TNS complex was almost constant in the range of pH 4–11 as reported by Kondo *et al.* (1976). However, the equilibrium constants of both β-CD–chitosan–TNS and β-CD–TNS complexes were not changed by pH as shown in Table 2. These facts suggest that the formation of an inclusion complex between β-CD–chitosan and TNS is relatively independent of pH but that the ionic interaction between β-CD–chitosan and TNS molecules may influence the structure of the complex.

We next investigated the effect of ionic strength upon the fluorescence spectra of the β-CD–chitosan–TNS complex. Although no significant changes were observed in the fluorescence intensity of the β-CD–TNS complex at pH 3.5, that of the β-CD–chitosan–TNS complex revealed a considerable decrease in intensity as the ionic strength reached 0.5 M as shown in Fig. 6. These results also supported a role for ionic interactions between TNS and β-CD–chitosan molecules.

Table 2. Equilibrium constants (K) of complex of TNS with β-CD and β-CD–chitosan

pH	β-CD K ($\times 10^3$ M^{-1})	β-CD–chitosan K ($\times 10^3$ M^{-1})
3.5	2.44	1.68
5.2	2.61	1.16
7.0	2.54	1.13
8.3	2.67	1.30
11.0	2.62	1.51

Lastly, an inhibition experiment of the host–guest complexation of β-CD–chitosan–TNS was carried out using a surfactant, sodium dodecyl sulfate (SDS). It was reported that the surfactant was found to form a 1:1 complex with β-CD more strongly than with TNS (Jobe *et al.*, 1988). Figure 7 shows the fluorescence changes on the addition of SDS at pH 3.5. Although a slight decrease in the intensity of β-CD–TNS was observed, β-CD–chitosan was found to cause a significant decrease when SDS was added to the solution. Furthermore, when the concentration of SDS was more than 1.7 mM, a precipitate was formed in the β-CD–chitosan–TNS solution. These inhibition experiments also revealed that both β-CD and β-CD–chitosan form inclusion

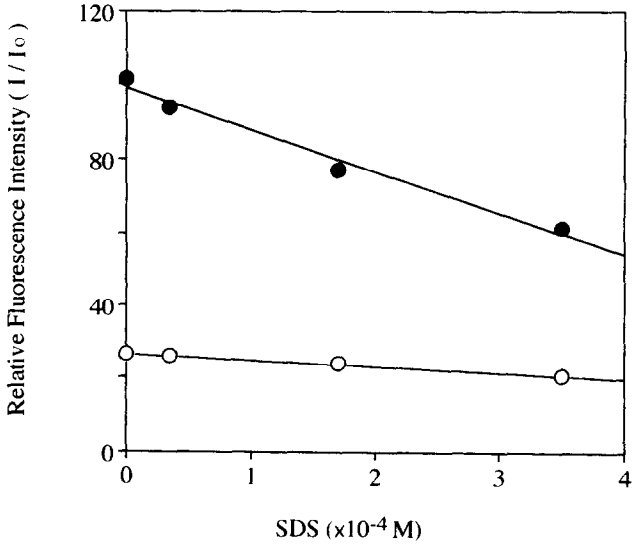


Fig. 7. Effects of SDS concentration on the fluorescence intensity of TNS in the presence of β-CD (○) and β-CD–chitosan (●), [β-CD] = [β-CD–chitosan] = 10^{-3} M.

complexes with TNS. However, since the decrease in the fluorescence intensity of the β-CD–chitosan–TNS system is much larger than that of the β-CD–TNS system, SDS might interfere with an ionic interaction between TNS and β-CD–chitosan.

The experiments described above demonstrate that the significant enhancement of the fluorescence intensity of the β-CD–chitosan–TNS complex was depressed by increasing the pH and ionic strength of the solution. One possible explanation for these findings may be that the TNS molecule is recognized by β-CD–chitosan through a hydrophobic interaction between the toluidinyl group and the CD moiety as well as the ionic interaction between the negatively charged naphthalenesulfonyl group and the positively charged amino group of β-CD–chitosan. As a consequence of these two interaction sites, the TNS molecule may be immobilized to the polymer guest, so that the relaxation and the vibrational deactivation (Ainsworth & Flanagan, 1969; Seliskar & Brand, 1971) of the excited TNS molecules on the polymer-guest are considerably decreased. On the other hand, amino group or neighboring CDs of β-CD–chitosan may interfere with the complex formation with TNS, so the complex of β-CD–chitosan–TNS is somewhat more unstable than the complex of β-CD–TNS as shown in Table 2. Nevertheless, since the immobilization of TNS in the case of β-CD–chitosan–TNS may contribute enormously to increasing the fluorescence intensity, β-CD–chitosan–TNS showed higher fluorescence intensity than β-CD–TNS. Indeed, an examination of CPK molecular models revealed that the toluidinyl group of the TNS molecule incorporated by β-CD–chitosan could form an ion pair between the sulfonate group and a free amino group of the glucosamine residue of the polymer-guest.

In conclusion, we have developed a facile synthesis of a novel chitosan–β-CD derivative formed from covalent modification of carboxymethyl-β-cyclodextrin with a low molecular weight chitosan oligomer. The polymer-guests having free amino groups on the chitosan backbone were found to recognize a TNS molecule through an ionic interaction as well as host–guest complexation.

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