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Aggregation phenomenon of PEG-grafted chitosan in aqueous solution

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Chitosan (deacetylated chitin), which is a naturally occurring polysaccharide having primary amino groups, is known to be insoluble in water because of its strong intermolecular hydrogen bonds. We prepared water-soluble poly(ethylene glycol) grafted chitosan (PEG-g-chitosan) through the chemical modification of chitosan and investigated its aggregation phenomenon in aqueous solutions. The solution properties of PEG-g-chitosans differ depending on the degree of introduction of PEG in aqueous solution, and were studied by measuring transmittance and light scattering. The PEG-g-chitosans could form aggregates spontaneously by means of their intermolecular hydrogen bonds in the aqueous solution. The PEG-g-chitosan aggregates could also uptake *N*-phenyl-1-naphthylamine (PNA) as a hydrophobic substance in neutral conditions and this PNA could be released from the aggregates in acidic conditions. © 1998 Elsevier Science Ltd. All rights reserved.

(Keywords: chitosan; poly(ethylene glycol); graft)

INTRODUCTION

Chitosan is a deacetylated product of chitin, which is a structural polysaccharide commonly found in nature. Since chitosan is one of basic polysaccharides which are rare in nature, and have relatively good biocompatibility, many applications of chitosan as biomaterials were investigated. However, the quite low solubility of chitosan due to its highly crystallized structure[1] presents a problem to its application. In order to overcome the poor solubility of chitosan in water or organic solvents, chemical modification of chitosan has been carried out. For example, *N*-carboxymethylation[2], sulfonation[3], quaternarization[4–6] and *N*- and *O*-hydroxyalkylation[7] of chitosan were carried out to obtain water-soluble chitosan derivatives. However, to obtain good solubility in water, a large number of hydrophilic groups must be introduced to provide chitosan derivatives where most of the glucosamine units are modified. To obtain a highly water-soluble chitosan derivative by a low degree of substitution, a high-molecular weight hydrophilic modifier is preferable. However, there are almost no reports on the modification of chitosan using polymers. Poly(ethylene glycol) (PEG) is a highly water-soluble amphipathic polymer and frequently used for chemical modification of natural and artificial macromolecules for biomedical applications. Grafting PEG onto chitosan should be a promising approach to obtain water-soluble chitosan derivatives. In fact, one of the authors previously synthesized chitosan oligoethyleneglycol derivatives for use as a drug carrier of anticancer agents[8]. However, the solution properties of the oligoethyleneglycol derivatives of chitosan were not studied.

Recently, block-copolymer micelles[9–11] or self-aggregates of hydrophobized water-soluble polymers[12–14] have been extensively studied as novel types of carriers for drug delivery systems (DDS). These polymers consisting of hydrophilic and hydrophobic segments can form micelle structures with the hydrophobic inner core and the hydrophilic outer shell in aqueous media. Such polymeric micelles have been proposed as drug delivery vehicles for poorly water-soluble drugs.

The present paper is concerned with the preparation of chitosan modified chemically by grafting PEG. The chitosan modified chemically by grafting of poly(ethylene glycol) (PEG-g-chitosan) (*Figure 1*) can be soluble not only in water, but also in organic solvents. Although the introduction of PEG chains onto chitosan dissolve the modified chitosan molecule by interaction with water or organic solvents, the unmodified glucosamine units of PEG-g-chitosan should still possess strong inter- or intra-molecular interaction with other unmodified glucosamine units by way of hydrogen bonds as found in native chitosan. So, our attention is focused on the formation of a new type of PEG-g-chitosan aggregate due to its intermolecular hydrogen bonds in aqueous media (*Figure 2*). We investigated the aggregation phenomena in aqueous solution by measuring transmittance, light scattering, and uptake and release behaviour of *N*-phenyl-1-naphthylamine (PNA) by the aggregates.

EXPERIMENTAL

Materials

Chitosan with a degree of deacetylation of 100 mol% sugar unit^{-1} and a number-average molecular weight of 1.5×10^5 was provided by Kimitsu Chemical Industries, Ltd. Methoxy poly(ethylene glycol) acid (MeO-PEG acid; $M_n = 5000$) was purchased from Sigma Chemical Co. and used without further purification. Organic solvents and water were purified by usual distillation and reverse-osmotic membrane filtration methods before use, respectively. The other materials were of commercial grade and were used without further purification.

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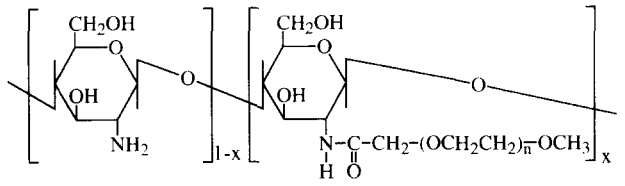

Figure 1 Structure of PEG-grafted chitosan

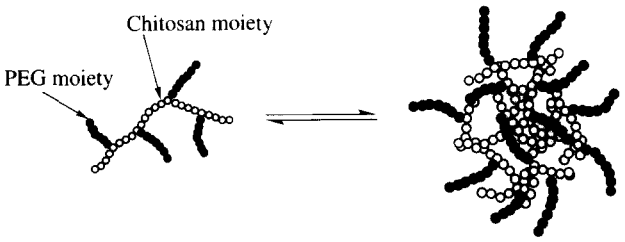

Figure 2 Formation of intermolecular aggregate of PEG-g-chitosans by hydrogen bonds in aqueous solution

Synthesis of PEG-g-chitosan

6-*O*-triphenylmethyl-chitosan was prepared from chitosan by the method reported by Kurita *et al.*[15] PEG-g-chitosan was prepared according to the following methods. The coupling reaction of 6-*O*-triphenylmethyl-chitosan with MeO-PEG acid was carried out by using the water-soluble carbodiimide (WSC)–hydroxybenzotriazole (HOBt) method in *N,N*-dimethylformamide (DMF) to give PEG-grafted-6-*O*-triphenylmethyl-chitosan. The reaction mixture was subjected to gel-filtration chromatography (column: Sephadex LH-60, 2 × 100 cm, eluent: DMF) monitored by u.v. detector at 265 nm to afford the conjugate. The high molecular weight fraction was separated and evaporated under reduced pressure. The yellow solid obtained was treated by 50% (v/v) acetic acid for 2 h to deprotect its triphenylmethyl groups. Then the acidic solution was neutralized with triethylamine (TEA), and dialyzed in cellulose tube (cut-off $M_W = 1.0 \times 10^4$) against water, and freeze-dried to give the objective PEG-g-chitosan. Deprotection of triphenylmethyl groups was confirmed by disappearance of absorption at 265 nm using u.v. spectrophotometer. The purity of PEG-g-chitosan obtained was confirmed by gel-permeation chromatography (GPC) (column: Shodex OH pack SB-803, Showa Denko, eluent: 1/15 M phosphate buffer, standard: pullulan) monitored by refractive index (r.i.) detector. We synthesized PEG-g-chitosan conjugates with varying degrees of PEG introduction (DPEG) by changing the feed ratio of MeO-PEG acid to 6-*O*-triphenylmethyl-chitosan.

DPEG values were estimated by colloidal titration method[16], where a negative colloid solution of PEG-g-chitosan was titrated with 1/400 N polyanionic solution of potassium polyvinyl sulfate to a conductometric end-point by the conventional toluidine blue indicator method. The characterization of PEG-g-chitosan was carried out by measurements of *FT*i.r. Following spectral data shows the typical example for PEG-g-chitosan having 25% of DPEG, CP25.

IR(KBr): 3441 (broad, OH, NH and NH_2), 2882 (CH_2), 1651 (C=O), 1108 cm^{-1} (C–O–C).

Transmittance measurement

To investigate the solution property of PEG-g-chitosan, the optical transmittance of aqueous solutions of PEG-g-chitosan having various DPEG values was measured at 500 nm (UV-2500PC; Shimadzu). Since PEG-g-chitosan could not dissolve easily in aqueous solution, we prepared the PEG-g-chitosan aqueous solution by the following method. The PEG-g-chitosan was stirred in water for 10 min to give a milky suspension. The milky suspension obtained was further sonicated at room temperature by using a probe-type sonicator (UD-200; Tomy Seiko Co.) with 200 W until the transmittance change could not be observed. Typically the sonication time was 2 min. The obtained PEG-g-chitosan was incubated at room temperature for 1 day before the measurement. The concentration of PEG-g-chitosan aqueous solution was adjusted to 4 mg ml^{-1} for this experiment.

Light scattering measurement

To investigate the aggregation phenomena and to measure the hydrodynamic radius of PEG-g-chitosan aggregates, the dynamic light scattering (DLS) measurement was carried out on a DLS-700 (Otsuka Electronics) at 30°C. PEG-g-chitosans, CP22, CP25 and CP55, whose DPEG values were 22, 25 and 55 mol% sugar $unit^{-1}$, respectively, were used for the DLS measurement. The PEG-g-chitosan was stirred in water for 10 min to give a milky suspension. The milky suspension obtained was further sonicated at room temperature by using a probe type of sonicator. The obtained solution was filtered through disposable syringe filter (nominal pore size: 450 nm; Iwaki Co.) into a measuring cell and incubated at room temperature for 1 day. The concentration of PEG-g-chitosan aqueous solution was fixed at 2.0 mg ml^{-1} for this experiment. To confirm the reversibility of the aggregation and the self-aggregation process, we evaluated the particle size distribution of PEG-g-chitosan aggregates in water, in dimethylsulfoxide (DMSO), which is good solvent of PEG-g-chitosan, and in water after dialyzed from DMSO solution against water using cellulose tube (cut-off $M_W = 1.0 \times 10^4$). The dynamic light scattering was carried out with fixing the scattering angle at 90°.

The molecular weight, the second virial coefficient and the aggregation number of PEG-g-chitosan aggregates in water were estimated by static light scattering (SLS) measurement at 30°C. The PEG-g-chitosan solutions were prepared by the same method described above using CP25 and CP55. The angular range of SLS measurement was from 30° to 130°. The concentration of PEG-g-chitosan was varied in the range 0.1–2.0 mg ml^{-1}. The molecular weight of the PEG-g-chitosan aggregates was estimated by Zimm plot.

Uptake behaviour of PNA into PEG-g-chitosan aggregates

The interaction of PEG-g-chitosan aggregates with PNA as a hydrophobic substance was evaluated by transmittance measurement. The PEG-g-chitosans solutions were prepared by the same method as shown in light scattering measurement section using CP25 and CP55. The concentration of CP25 and CP55 was fixed at 1.5×10^{-6} M, which meant 2.0 and 4.0 mg ml^{-1}, respectively. PNA was dissolved in methanol solution (5.0×10^{-3} M). Small aliquot of PNA methanol solution (0.01 ml) was added dropwise onto 1 ml of PEG-g-chitosan aqueous solution, water or PEG aqueous solution. After 2 min stirring, the transmittance of resulting mixture (T) was measured at 500 nm (UV-2500PC; Shimadzu). Then, the relative transparency (T/T_0) value was calculated, where T_0 meant the transmittance of each solution without the addition of

PNA. The uptake behaviour of PNA into PEG-g-chitosan aggregates was also investigated as a function of PEG-g-chitosan concentration to estimate their critical micelle concentration (CMC) using CP25 and CP55. The concentration of PEG-g-chitosan was varied in the range from 3.0 $\times 10^{-2}$ to 4.0 mg ml^{-1}. The experiments were carried out by the same precedures descried above. The saturated amount of PNA uptaken into PEG-g-chitosan aggregates was defined as the amount of added PNA at which the transparency was changed drastically.

The amount of PNA uptaken into PEG-g-chitosan aggregates was investigated under various pH conditions. The preparation of PEG-g-chitosan solution having various pH values was carried out by the same methods descried above using phosphate buffer (pH 3.2–9.2). The saturated amount of PNA uptaken into PEG-g-chitosan aggregates was defined as the amount of added PNA at which the transparency was changed drastically.

The release behaviour of PNA from PEG-g-chitosan aggregates

The transmittance measurement was applied to evaluate the release behaviour of PNA molecules from PEG-g-chitosans aggregates. The solution of PEG-g-chitosans was prepared by the same method as shown in the dynamic light scattering measurement section using CP25. The concentration of CP25 was adjusted to 1.5 $\times 10^{-6}$ M, which meant 2.0 mg ml^{-1}. 0.08 ml of PNA methanol solution (5.0 $\times 10^{-3}$ M) was added to 1 ml of PEG-g-chitosan aqueous solution and incubated for at room temperature for 1 day. After the addition of 0.01 ml of acetic acid, the time course of transmittance change of the solution was monitored by a u.v. spectrophotometer (UV-2500PC; Shimadzu) at 500 nm.

RESULTS AND DISCUSSION

Preparation of PEG-g-chitosan

PEG-g-chitosans having various DPEG values (2, 7, 18, 22, 25, 30, 38, 47 or 55 mol% sugar unit^{-1}) could be obtained by varying the feed ratio of MeO-PEG acid to 6-*O*-triphenylmethyl-chitosan in the coupling reaction. The appearance of stronger i.r. absorptions at 2882 (CH_2) and 1171 cm^{-1} (C–O–C) showed the existence of PEG chains. The absorption at 1651 cm^{-1} showed the formation of amide bond in the product. No contamination of free MeO-PEG acid was confirmed by GPC (column: Shodex OH pack SB-803, Showa Denko, eluent: 1/15 M phosphate buffer) after purification by gel-filtration chromatography (column: Sephadex LH-60, 2 cm $\times$ 100 cm, eluent: DMF). All of the obtained PEG-g-chitosans were soluble in DMF and DMSO. PEG-g-chitosans having DPEG values over 22 mol% sugar unit^{-1} were soluble in chloroform. PEG-g-chitosans having DPEG values over 10 mol% sugar unit^{-1} were soluble in water after sonication. It was difficult to introduce PEG chains over 55 mol% sugar unit^{-1} because of steric hindrance of PEG chains on chitosan.

Aggregation phenomenon of PEG-g-chitosan in aqueous solution

The solution properties of PEG-g-chitosan were investigated by transmittance measurements. The results of optical transmittance change of PEG-g-chitosan aqueous solution as a function of DPEG are shown in *Figure 3*. The transmittance of PEG-g-chitosan aqueous solutions was drastically changed with increasing in DPEG value around

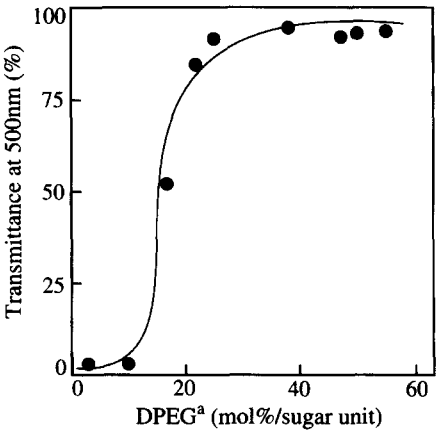

Figure 3 Dependence of DPEG value on the transmittance of PEG-g-chitosan aqueous solution. Concentration of PEG-g-chitosan = 4 mg ml^{-1}. [a] Degree of introduction of PEG per sugar unit

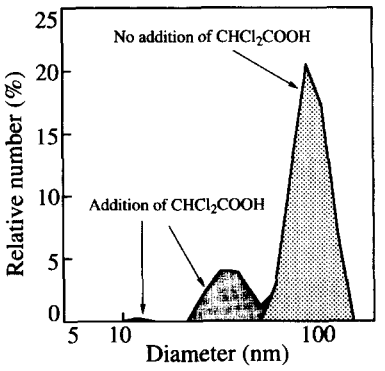

Figure 4 Particle size distribution of PEG-g-chitosan (DPEG = 22 mol% sugar unit^{-1}), CP22, aggregates in water with or without the addition of dichloroacetic acid measured by DLS

10–20 mol% sugar unit^{-1}. These results suggest that the solubilization effect of PEG chains became predominant and the hydrogen bonds between chitosan moieties were inhibited by static hindrance of PEG chain at the DPEG range over 20 mol% sugar unit^{-1}.

Figure 4 shows the particle size distribution of PEG-g-chitosan, CP22, in water with or without the addition of small amount of dichloroacetic acid. The aqueous solution of CP22 showed sharp single peak having about 90 nm of hydrodynamic diameter. However, after the addition of small amount of dichloroacetic acid as a hydrogen bond breaker, CP22 showed hydrodynamic diameters at 12 and 36 nm. Moreover, CP22 and other PEG-g-chitosans did not show the formation of particle having obvious hydrodynamic diameter in DMSO, which is a good solvent for PEG-g-chitosan. These results suggest that CP22 formed nanometer size particle in water by hydrogen bond formation at chitosan moiety. CP25 and CP55 also formed the nanometer size particles having 70 and 120 nm of hydrodynamic diameter in water, respectively. To confirm self-aggregate process, we investigated particle size distribution of PEG-g-chitosan, CP25, aqueous solution after dialysis from CP25 DMSO solution in water using cellulose tube. In DMSO solution, there was no aggregation formation, however, after dialysis in water the formation of nanometer size particle having 70 nm of average hydrodynamic diameter was observed (*Figure 5*). Such a phenomenon was also observed in the case of CP55. These

Table 1 The numerical values of PEG-g-chitosan aggregate obtained by light scattering method

Sample	Hydrodynamic radius [a] (nm)	Second virial coefficient [b] ($\times 10^{-5}$ mol ml^{-1} g^{-2})	Aggregation number [c]
CP25	70	6.0	7.0
CP55	120	8.0	1.5

[a]Measured by dynamic light scattering method.
[b]Measured by static light scattering method.
[c]Estimated from second virial coefficient value.

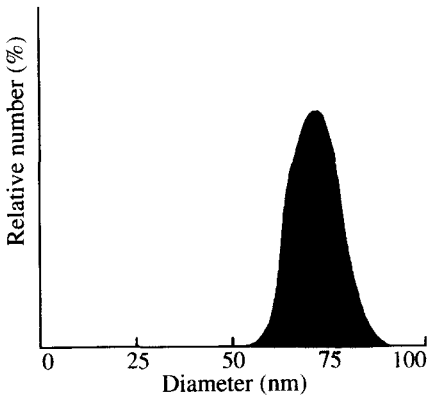

Figure 5 Particle size distribution of PEG-g-chitosan (DPEG = 25 mol% sugar unit^{-1}), CP25, aggregates in water measured by DLS after dialysis from DMSO solution against water

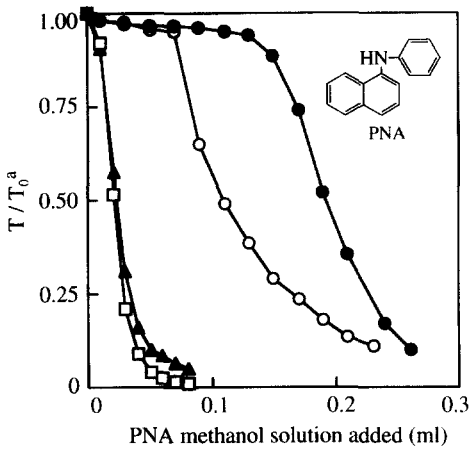

Figure 6 Transparency change of PEG-g-chitosan aggregate aqueous solution, PEG aqueous solution and water with the addition of PNA methanol solution. (●) aqueous solution of CP25; (○) aqueous solution of CP55; (▲) PEG aqueous solution; (□) water. The concentration of PNA methanol solution = 5.0×10^{-5} mol l^{-1}. [a] T/T_0: relative transparency, where T_0 is the transmittance of each aqueous solution without the addition of PNA methanol solution

results suggested that PEG-g-chitosans could form the aggregates spontaneously by strong intermolecular hydrogen bonds between chitosan moiety in water. The values of hydrodynamic radius and average aggregation number of CP25 and CP55 aggregates in water are summarized in *Table 1*. From the results of SLS and Zimm plotting, molecular weights and second virial coefficients of the aggregates were estimated to be 9.3×10^6, 6.0×10^{-5} mol ml^{-1} g^{-2} for CP25 and 4.0×10^6, 8.0×10^{-5} mol ml^{-1} g^{-2} for CP55, respectively. The aggregation numbers of CP25 aggregate and CP55 aggregate were calculated to be 7.0 and 1.5, respectively. These results meant CP25 formed a compact aggregate, however, CP55 formed a looser aggregate or unimolecular micelle in aqueous solution.

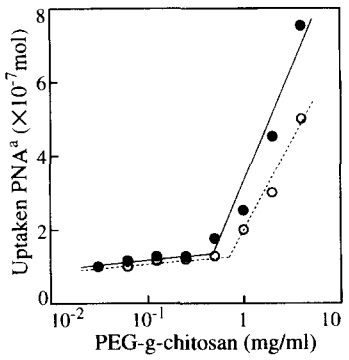

Figure 7 The amount of PNA uptaken into the PEG-g-chitosan aggregates as a function of the concentration of PEG-g-chitosan. (●) CP25 aggregate; (○) CP55 aggregate. [a] Estimated from the T/T_0 value

Uptake behaviour of PNA into PEG-g-chitosan aggregates

The interaction of PNA with CP25 and CP55 aggregates was investigated by transmittance measurement. *Figure 6* shows the change in transmittance of CP25, CP55 and PEG solution and water as a function of the amount of added PNA methanol solution. The transparency of water and PEG aqueous solution decreased drastically by the addition of small amount of PNA methanol solution because of suspended precipitation formation of PNA. On the other hand, the transparency of CP25 and CP55 aggregate solutions did not decrease until 15 drops and eight drops of PNA methanol solution, respectively. These results suggested that PEG-g-chitosan aggregates could dissolve a certain amount of PNA molecule and such an amphipathic hydrogel cross-linked by partial hydrogen bonds had a hydrophobic region which could uptake the hydrophobic PNA molecule. The CP25 aggregate could uptake a larger amount of the PNA molecule than the CP55 aggregate. Through the light scattering measurements, the CP55 aggregates was found to be a loose aggregate or unimolecular micelle. The CP55 aggregate was clarified as having less hydrogen bonds and a small volume hydrophobic region entrapping the hydrophobic small molecule, compared with the CP25 aggregate.

We also investigated the amount of PNA uptake into CP25 and CP55 aggregates as a function of PEG-g-chitosan concentration (*Figure 7*). The amounts of uptaken PNA into the CP25 aggregate and the CP55 aggregate were drastically increased around 0.5 mg ml^{-1} and 0.7 mg ml^{-1}, respectively. These bending points should corresponded to the CMCs where the intermolecular aggregation of PEG-g-chitosans occured. CP25 showed a slightly smaller CMC value and a larger slope over CMC than CP55. Such a difference meant that the intermolecular interaction of CP25 was stronger than that of CP55 and then CP25 have a larger capacity entrapping the hydrophobic PNA molecule.

Figure 8 shows the effect of pH on the amount of PNA uptaken into CP25 and CP55 aggregates in water. Under alkaline condition, the amount of PNA uptaken into each

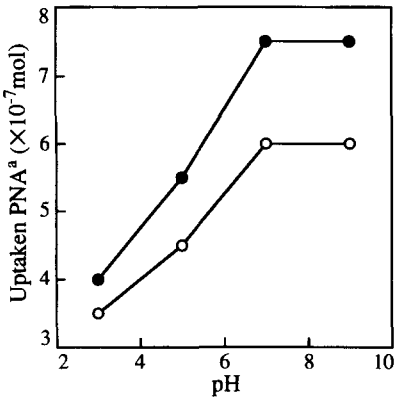

Figure 8 Dependence of pH on the amount of PNA uptaken into PEG-g-chitosan aggregates. (●) CP25 aggregate; (○) CP55 aggregate. [a]Estimated from the T/T_0 value

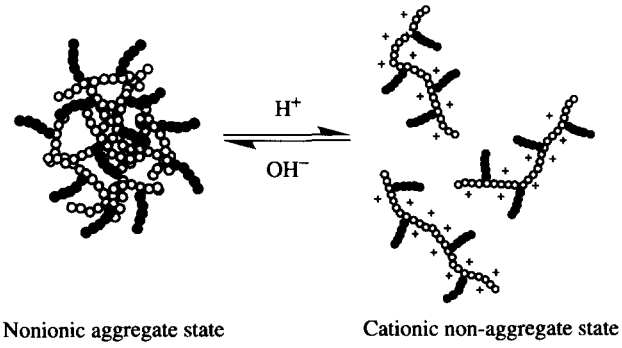

Figure 9 Schematic presentation for aggregation and dissociation behaviour of PEG-g-chitosan in aqueous solution in responce to pH of the solution

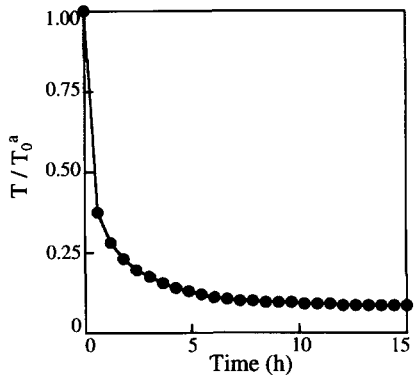

Figure 10 Release behaviour of PNA from PEG-g-chitosan, CP25, aggregate by addition of acetic acid. [a] T/T_0: relative transparency, where T_0 is the transmittance of PEG-g-chitosan aqueous solution without the addition of acetic acid

PEG-g-chitosan aggregate was not different from that under neutral conditions. However, in acidic conditions, the amount of PNA uptaken into both PEG-g-chitosan aggregates decreased gradually with decreasing pH value. These results suggest that PEG-g-chitosans could form intermolecular aggregates in neutral and alkaline conditions, while PEG-g-chitosans were dissociated in acidic conditions because of the electrostatic repulsion of protonated amino groups of chitosan moieties[17] (*Figure 9*).

The release of PNA from PEG-g-chitosan aggregates

The results of release behaviour of PNA from the CP25 aggregate as a function of time by the addition of acetic acid are shown in *Figure 10*. By the addition of acetic acid, the transparency of CP25 aqueous solution drastically decreased. These results meant that PEG-g-chitosan, CP25, was dissociated by intermolecular electrostatic repulsion of protonated amino groups of chitosan moieties under acidic conditions, and then the hydrophobic PNA was released from the inside of the aggregate.

CONCLUSIONS

We prepared water-soluble poly(ethylene glycol) grafted chitosan, PEG-g-chitosan, and investigated its aggregation phenomenon in aqueous solution. PEG-g-chitosans having a DPEG of 25 and 55 mol% sugar unit^{-1} were found to form aggregates due to intermolecular hydrogen bonds in aqueous solution. These PEG-g-chitosan aggregates could take up a small hydrophobic molecule such as PNA. PNA taken up into the aggregates could be released from the aggregates by changing the pH to an acidic condition. So, this PEG-g-chitosan aggregate can be expected to be used as a pH dependent material such as a drug carrier for drug delivery systems.

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