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Slow-release system of pegylated lysozyme utilizing formation of polypseudorotaxanes with cyclodextrins

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ABSTRACT

Poly(ethylene glycol) (PEG, MW 2200) chains were introduced into lysozyme molecule. The resulting pegylayed lysozyme formed polypseudorotaxanes with α- and γ-cyclodextrins (α- and γ-CyDs, respectively), by inserting one PEG chain in the α-CyD cavity and two PEG chains in the γ-CyD cavity. The pegylated lysozyme/CyD polypseudorotaxanes were less soluble in water and the release rate of the pegylated protein decreased in the order of the pegylated lysozyme > the γ-CyD polypseudorotaxane > the α-CyD polypseudorotaxane. The enzymatic activity of the pegylated lysozyme released from the polypseudorotaxanes was the same as that of the pegylated protein alone, indicating no decrease in the activity through the polypseudorotaxane formation. The results indicate that the pegylated lysozyme/CyD polypseudorotaxanes can work as a slow-release system, and the polypseudorotaxane formation with CyDs may serve as a new strategy for the preparation of slow-release system of pegylated proteins and peptides.

1. Introduction

Bioconjugate technology has been widely used to improve therapeutic efficacies and pharmaceutical properties of protein drugs, for example, by conjugating the drugs with polymers such as acrylic polymers, cellulosic polymers or poly(ethylene glycol) (PEG) (Chen et al., 1997; Baudys et al., 1998; Hinds and Kim, 2002; Harris and Chess, 2003; Fee and Van Alstine, 2006). In particular, when PEG is covalently attached to a protein, it transfers many of the polymer's favorable characteristics to the resulting conjugate, such as increased circulating half-life in blood, enhanced proteolytic resistance, reduced antigenicity and immunogenicity, reduced aggregation, and improved bioavailability. There are many examples of pegylation of proteins such as adenosine deamidase, insulin, interferon-α2, β-lactoglobulin, α-chymotrypsin, lipase, bovine liver catalase, asparaginase, and superoxide dismutase, of which the first three conjugates are on the market (Hinds and Kim, 2002; Harris and Chess, 2003; Fee and Van Alstine, 2006).

Supramolecular assemblies have attracted a great amount of attention, due to its intriguing topologies and its application in various fields such as nanodevices, sensors, molecular switches, and drug delivery systems (Harada, 2001; Wenz et al., 2006). Macrocyclic compounds are most often used as host molecules in supramolecular chemistry, of which cyclodextrins (CyDs) have been widely applied to drug delivery system because of their good bioadaptability (Uekama et al., 1998; Uekama, 1999; Saenger, 1980). Harada et al. first reported the supramolecular assemblies of PEG and α-CyD, in which a number of the cyclic molecules are spontaneously threaded onto the polymer chain (Harada and Kamachi, 1990; Harada et al., 1992). These complexes are called polypseudorotaxanes, because the CyD can be dethreaded from the polymer chain when dissolved in water. This complexation shows the size-dependency, i.e. the small cavity of α-CyD forms a polypseudorotaxane with PEG, while the large cavity of β-CyD with poly(propylene glycol). A number of applications of polypseudorotaxanes as a biomaterial are reported, e.g. gene delivery carrier (Yamashita et al., 2006; Li et al., 2006a,b; Yang et al., 2007), biodegradable hydrogel (Li et al., 2006a,b; Li and Loh, 2008) and galectin binding material (Nelson et al., 2004). Li et al. (2003) reported the controlled release system utilizing the gel formation of CyD polypseudorotaxanes. In spite of many studies on the formation and application of polypseudorotaxanes reported so far, little is known about the combination of pegylated drugs and CyDs and their application to drug release controls. In previous studies (Higashi et al., 2007, 2008), we found that the pegylated insulin forms polypseudorotaxanes with α- and γ-CyDs in a similar manner as PEG does. In this study, we investigated the formation of polypseudorotaxanes with a pegylated protein, lysozyme, having

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of a molecular weight (14.3 kDa) about twice larger than that of insulin (6 kDa), and report here that it forms polypseudorotaxanes with CyDs and the resulting supramolecular assemblies can work as a slow-release system.

2. Materials and methods

2.1. Materials

Hen egg lysozyme (58,100 U/mg, MW 14.3 kDa) was purchased from Sigma (St. Louis, MO). α-Succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (MW about 2300) was obtained from NOF Co. (Tokyo, Japan). PEG-bis amine was donated by Kawaken Fine Chemicals Co. (Tokyo, Japan). Succinimidyl-benzyloxycarbonyl L-phenylalanine (Zphe-OSu) was obtained from Kawaken Fine Chemical Co. (Tokyo, Japan). CyDs were donated by Nihon Shokuhin Kako (Tokyo, Japan). All other materials were of reagent grade, and deionized double distilled water was used.

2.2. Preparation of pegylated lysozyme

Pegylated lysozyme was synthesized according to the method of Nodake and Yamasaki (2000). Briefly, lysozyme (molecular weight 14.3 kDa, 40 mg) was incubated with α-succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (20 mg) in 4 mL of phosphate buffer solution (pH 8) at 4 °C for 12 h. The reaction solution was dialyzed using a membrane filter (Spectra/Por® membrane MWCO: 3500) and lyophilized. The pegylation of lysozyme molecule was confirmed by MALDI-TOF mass-spectrometry, and no contamination of free PEG in the pegylated lysozyme was confirmed by TLC and FAB mass-spectrometry. TLC analysis was conducted, with using silica-gel 60 F254 obtained from Merck (Darmstadt, Germany) and an eluent of chloroform/methanol = 5:1 (indicator: iodine).

2.3. Preparation of polypseudorotaxanes of pegylated lysozyme with CyDs

Pegylated lysozyme/CyD polypseudorotaxanes were prepared by adding 0.5 mL of aqueous pegylated lysozyme solution (10 μmol, 192 mg) in 1.48 mL of aqueous α-CyD (145 mg/mL) or 0.62 mL of aqueous γ-CyD (232 mg/mL) solution and then standing the solutions for 12 h at 4 °C. The resulting precipitates of the polypseudorotaxanes were filtered and dried under reduced pressure.

2.4. Preparation of CyD polypseudorotaxane with PEG 2000 or PEG 400 in different volumes of γ-CyD solutions

PEG (molecular weight 400 or 2000 Da, 20 mg) was added to 0.7 mL or 7.0 mL of aqueous γ-CyD solutions of the same concentration (γ-CyD 232 mg/mL), but of the different amounts of γ-CyD between the 0.7 and 7.0 mL solutions. The solutions were stand for 30 min at room temperature. The resulting precipitates of the polypseudorotaxanes were filtered, washed with water and dried under reduced pressure.

2.5. Preparation of end-capped conjugate of PEG with bis-benzyloxycarbonyl L-phenylalanine ($Zphe_2$-PEG) and its CyD polypseudorotaxanes

The end-capped PEG conjugate was prepared according the modified method of Ooya and Yui (1999). Briefly, PEG-bis amine (molecular weight 2 kDa, 200 mg) was incubated with an excessive amount of Zphe-OSu (198 mg) in 2 mL DMF at room temperature for 24 h. The reaction solution was washed with ethyl ether and dried under reduced pressure. No contamination of free PEG in the conjugate was confirmed by TLC as described above and FAB mass-spectrometry (Fig. S4). The polypseudorotaxane of the end-capped PEG conjugate was prepared by the method described above, i.e. the resulting conjugate (5.5 mg) was dissolved in water and added to 0.3 mL of α-CyD (145 mg/mL) or 0.15 mL of γ-CyD (232 mg/mL) solutions, and then standing the solutions for 30 min at room temperature.

2.6. Structures of pegylated lysozyme/CyD polypseudorotaxanes

Powder X-ray diffraction patterns of CyD polypseudorotaxanes were measured using a powder X-ray diffractometer (Rigaku RINT 2500, Tokyo, Japan) under the following conditions: Ni-filtered Cu Kα radiation (1.542 Å), 40 kV, 40 mA, divergent slit of 1.74 mm (1°), scanning slit of 0.94 mm (1°), receiving slit of 0.15 mm, and goniometer angular increment of 1°/min. ^{1}H NMR spectra were taken at 25 °C on a JEOL JNM-R500 spectrometer (Tokyo, Japan), operating at 500 MHz, using a 5-mm sample tube.

2.7. Release of pegylated lysozyme from CyD polypseudorotaxanes

The release rate of the pegylated protein was measured by the modified dispersed-amount method (Higashi et al., 2008), i.e. 1.0 mL of pH 7.4 phosphate buffer was added in the polypseudorotaxane suspensions in slurry state (containing 0.1 μmol) at 37 °C. At appropriate intervals, an aliquot of the dissolution medium was withdrawn, centrifuged at 10,000 rpm for 5 min, and analyzed for the pegylated lysozyme by HPLC (YMC Pack C18 AP-type column, 4.6 mm i.d. × 150 mm), using a mobile phase of acetonitrile/water/trifluoroacetic acid (30:69.9:0.1) and acetonitrile/water/trifluoroacetic acid (95:4.9:0.1) and a gradient flow increasing the ratio of the latter solution from 0 to 100% in 60 min, with a flow rate of 1.0 mL/min, and detection at 280 nm.

2.8. Enzymatic activity of pegylated lysozyme

The enzymatic activity was measured according to the method of Kumagai et al. (1992), i.e. lysozyme or the pegylated lysozyme (250 U/mL, 0.36 μM) in phosphate buffer (pH 6.5, I = 0.03) were added to the suspension of *M. lysodeikticus* cells (0.1 mg/mL), and then incubated at 25 °C for 2 min. The turbidity of the suspension was measured at 540 nm using a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The enzymatic activity was estimated as a relative activity (% activity) based on the decrease of turbidity compared to the lysozyme alone system.

3. Results and discussion

3.1. Chemistry

The introduction of the PEG moiety to lysozyme molecule was carried out according to the method of Nodake and Yamasaki (2000), using α-succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene in a phosphate buffer solution (pH 8). The reaction solution was dialyzed using the membrane filter and lyophilized. The resulting product gave three peaks at the retention times of 12.0, 13.0 and 13.8 min in the HPLC chromatogram (Fig. S1) measured under the conditions described in Section 2 (release study). These peaks correspond to free lysozyme and the mono- and di-pegylated lysozymes, respectively. It was difficult to prepare exclusively the mono-substituted lysozyme under the reaction conditions because elongation of the reaction time resulted in a higher content of the di-substituted lysozyme whereas reduction of the time created higher contents of free lysozyme. Therefore, the pegylated lysozyme prepared under the present conditions

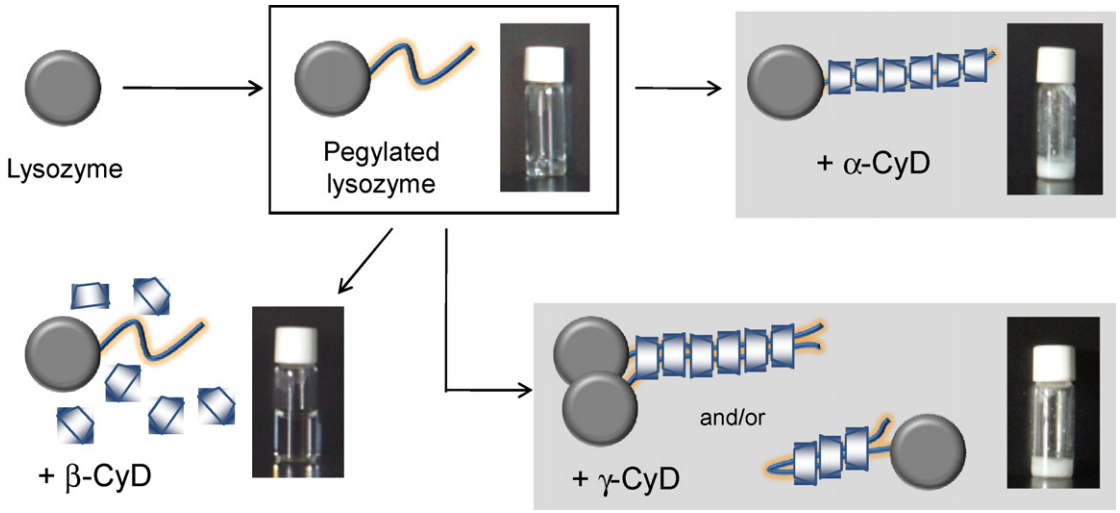

Fig. 1. Macroscopic photographs of precipitates of pegylated lysozyme/α-CyD and γ-CyD polypseudorotaxanes and their interaction modes.

contained 17% free lysozyme, 80% mono-pegylated lysozyme and 3% di-pegylated lysozyme (i.e. degree of average substitution of PEG = 0.85). In MALDI-TOF mass spectra, lysozyme gave a peak at about 14,300, and the mono- and di-substituted lysozyme gave dispersed peaks between 16,000–17,000 and 18,000–19,500 with a center at about 16,500 and 18,500, respectively, due to the dispersed molecular weights of PEG chains (Fig. S2). However, as described below, CyDs formed polypseudorotaxanes with pegylated lysozymes, precipitating the solid material, but not with free lysozyme that was in solution. Therefore, the solid pegylated lysozyme/CyD polypseudorotaxanes contained no free lysozyme.

3.2. Formation of polypseudorotaxanes of pegylated lysozyme with CyDs

Polypseudorotaxanes of the pegylated lysozyme with CyDs were prepared by mixing aqueous solutions of both components. Fig. 1 shows the solutions after mixing the conjugate and α-, β- and γ-CyD solutions and standing for 12 h at 4 °C. The α- and γ-CyD solutions gave white precipitates, whereas the β-CyD solution gave no precipitates, indicating the formation of polypseudorotaxanes of the pegylated lysozyme with α- and γ-CyDs, the same phenomenon as those observed for PEG/CyD systems reported by Harada et al. (1994). The solid polypseudorotaxanes were analyzed for lysozyme by the HPLC method described in Section 2, and the results indicated that the polypseudorotaxanes contain no free lysozyme, but contain only the mono- and di-pegylated lysozymes (ratio of the mono- and di-substituted proteins = about 95:5). This is due to the fact that pegylated lysozymes form polypseudorotaxanes with α- and γ-CyDs, precipitating the solid assemblies, whereas the unsubstituted lysozyme forms no such assembly, thus remaining in solution. Therefore, these results suggest that the formation of solid pegylated drug/CyD polypseudorotaxanes can be used as a tool for the isolation of pegylated drugs from reaction mixtures containing free and pegylated products.

Fig. 2 shows yields of the precipitated polypseudorotaxanes versus concentrations of CyDs employed in the polypseudorotaxane formation. The yield increased with increasing CyD concentration and reached to 75% (94% based on the content without free lysozyme) in using a 145 mg/mL α-CyD solution (saturated aqueous solution of α-CyD) and 84% (almost complete disposition of the pegylated lysozyme) in using a 232 mg/mL γ-CyD solution (saturated aqueous solution of γ-CyD). These results indicate that polypseudorotaxanes of pegylated lysozyme with CyDs can be prepared in high yields by using saturated CyD solutions.

Fig. 3 shows yields of precipitated polypseudorotaxanes versus the mixing molar ratios (CyDs/pegylated lysozyme). The yields increased with increasing CyD concentrations and leveled off at the molar ratios of about 20 and 10, suggesting the formation of

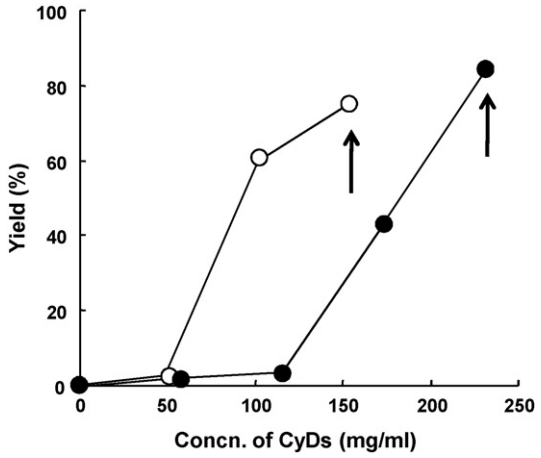

Fig. 2. Yields of pegylated lysozyme/α-CyD (○) and γ-CyD (●) polypseudorotaxanes prepared in different CyD concentrations. The polypseudorotaxanes were prepared at the CyD concentrations shown by an arrow and used in the following studies.

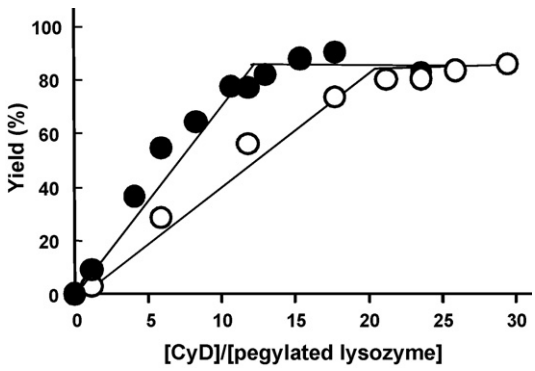

Fig. 3. Changes in yield of pegylated lysozyme/α-CyD (○) and γ-CyD (●) polypseudorotaxanes as a function of [CyD]/[pegylated lysozyme].

Table 1
Yields and compositions of pegylated lysozyme/CyD polypseudorotaxanes.

Protein	CyDs	Yield (%)	$CyDs_{number}$ [a]	Coverage[b] (%)
Lysozyme	α-CyD	74.8	21.9	95.2
	γ-CyD	84.1	11.7	50.9

[a] Number CyDs units involved in the polypseudorotaxane formation with one PEG chain in the pegylated lysozyme.
[b] Coverage = 2(CyDs per PEG)/(PEG repeat units), assuming that CyDs include two PEG repeat units.

polypseudorotaxanes of 20/1 α-CyD/pegylated lysozyme and 10/1 γ-CyD/pegylated lysozyme, respectively (Choi et al., 2004).

The stoichiometry of the polypseudorotaxanes was accurately determined by measuring peak areas of the anomeric proton of CyDs and the ethylene protons of the pegylated lysozyme in ^{1}H NMR spectra after dissolving the solid polypseudorotaxanes in DMSO (Fig. S3). The results indicated that 22 and 12 moles of α- and γ-CyDs, respectively, are involved in the polypseudorotaxane formation, i.e. the coverage of the PEG chain by α- or γ-CyD is 95 or 51% when assumed that two (ethylene glycol) repeat units are included in one CyD cavity (Table 1) (Harada et al., 1994; Harada, 1996).

It has been reported that γ-CyD can include two chains of polymers such as PEG (Harada et al., 1994), poly(ε-caprolactone) or poly(ethyleneimine) (Jiao et al., 2002; Chan et al., 2007; Joung et al., 2007) in the large cavity, forming an intermolecular double-chain complexes. The above results clearly indicated that two PEG chains of the pegylated lysozyme were included in one γ-CyD cavity. However, in the case of PEG/γ-CyD polypseudorotaxanes, two conformational isomers may exist due to the extended and bent conformations of PEG chain, i.e. as shown in Figs. 1 and 4, one is the intermolecular double-chain complex that two PEG molecules are included in the γ-CyD channel in a molar ratio of 2:2n (PEG:CyD, n: number of γ-CyD) and the another is the intramolecular double-chain complex that one PEG molecule with the bent conformation is included in the γ-CyD channel in a molar ratio of 1:n. To confirm the formation of two conformationally different polypseudorotaxanes, we investigated the complex formation of PEGs 2000 (MW 2000) and 400 (MW 400) with γ-CyD, i.e. the former having the long chain can adopt both extended and bent conformations, while the latter having the short chain is difficult to adopt the bent conformation. Fig. 4 shows the yields of the polypseudorotaxanes, when PEGs 2000 and 400 were added in the different volumes (0.7 mL or 7.0 mL) of γ-CyD solutions containing the same concentrations (232 mg/mL). The yield of the PEG 2000 polypseudorotaxane did not decrease with increasing the volume (0.7–7.0 mL) of γ-CyD solution (Fig. 4A), whereas that of the PEG 400 polypseudorotaxane significantly decreased (Fig. 4B). These results suggest that PEG 2000 forms predominantly the intermolecular double-chain complex in the concentrated PEG solution (0.7 mL), while it forms the intramolecular complex of the bent conformation, in addition of the intermolecular extended-chain complex, in the diluted PEG solution (7.0 mL) leading no decrease in the yield of the polypseudorotaxane. On the other hand, PEG 400 forms predominantly the intermolecular double-chain complex in the concentrated PEG solution (0.7 mL), but it is difficult for PEG 400 to form the intramolecular complex of the bent conformation in the diluted PEG solution (7.0 mL) because it cannot adopt the bent conformation, leading the decrease in the yield of the polypseudorotaxane. These results suggested that both polypseudorotaxanes with the extended and bent conformations of PEG co-exist in the solid.

Further, we investigated the formation of polypseudorotaxanes of the PEG derivatives ($Zphe_2$-PEG) end-capped by bis-benzyloxycarbonyl L-phenylalanine with α- and γ-CyDs, as shown in Fig. 5. We assumed that $Zphe_2$-PEG cannot form polypseudorotaxanes with γ-CyD, if it adopts the extended conformation, because of the presence of the bulky group at both edges of PEG, or it forms polypseudorotaxanes in very slow rates, even if possible. However, if $ZpPhe_2$-PEG adopts the bent conformation, it forms polypseudorotaxane with γ-CyD. Fig. 5 shows the solutions after mixing the $Zphe_2$-PEG conjugate with α- or γ-CyD solutions and standing for 30 min at room temperature. The α-CyD solutions gave no precipitates, because the bulky end-cap inhibits the threading

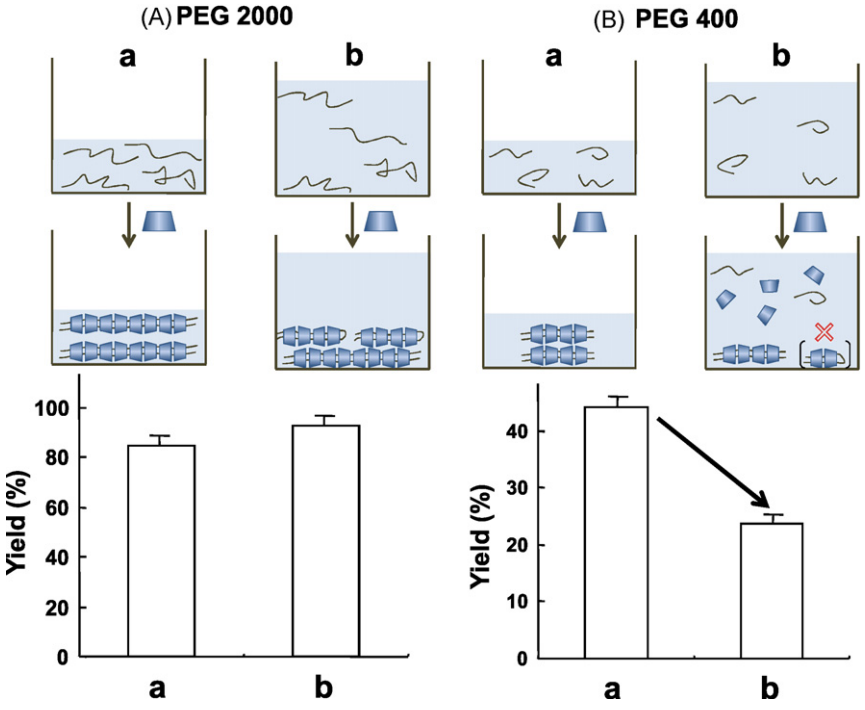

Fig. 4. Yields of PEG polypseudorotaxanes in different volumes of CyD solutions. 0.7 mL (a) or 7.0 mL (b) of γ-CyD solution (232 mg/mL) was added to 20 mg of PEG. Each point represents the mean ± S.E. of three experiments.

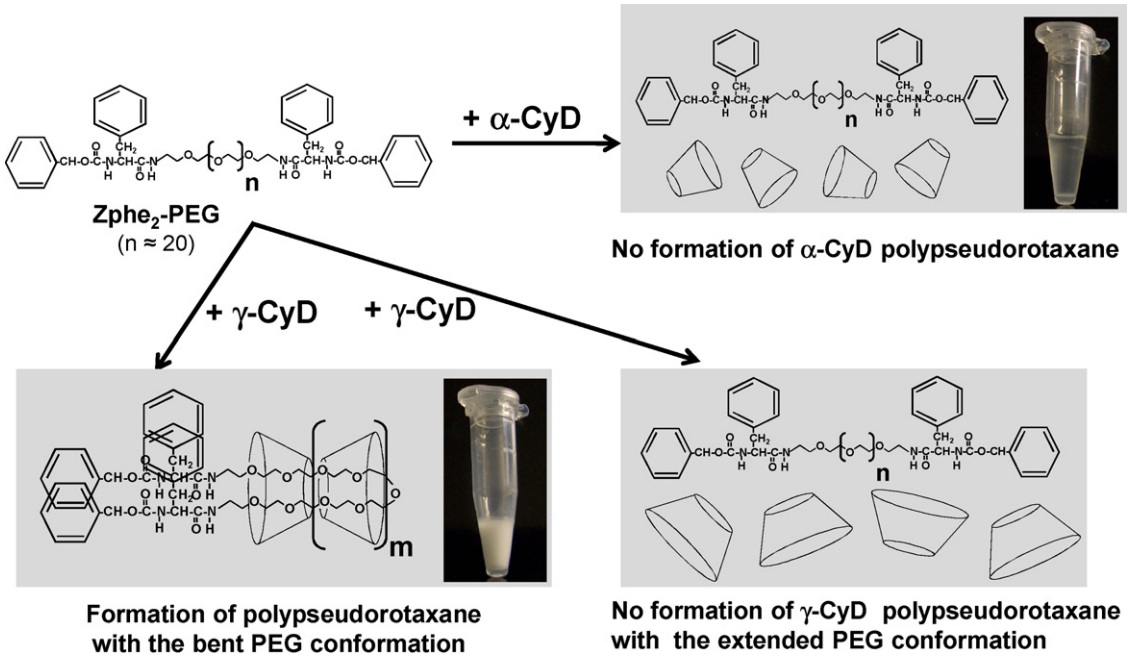

Fig. 5. Macroscopic photographs of precipitates of $Zphe_2$-PEG/CyD polypseudorotaxanes and their interaction modes.

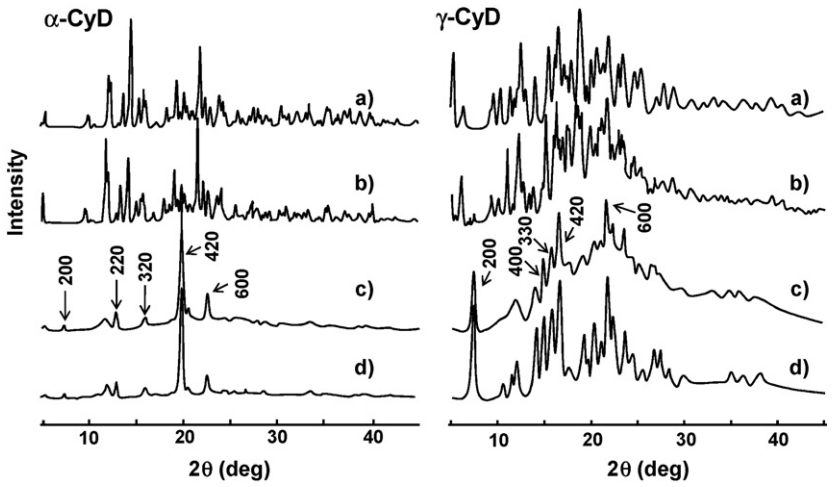

Fig. 6. Powder X-ray diffraction patterns of pegylated lysozyme/α-CyD and γ-CyD polypseudorotaxanes. (a) CyD alone; (b) physical mixture of pegylated lysozyme and CyDs; (c) polypseudorotaxanes of pegylated lysozyme/CyDs; (d) PEG/CyD polypseudorotaxane.

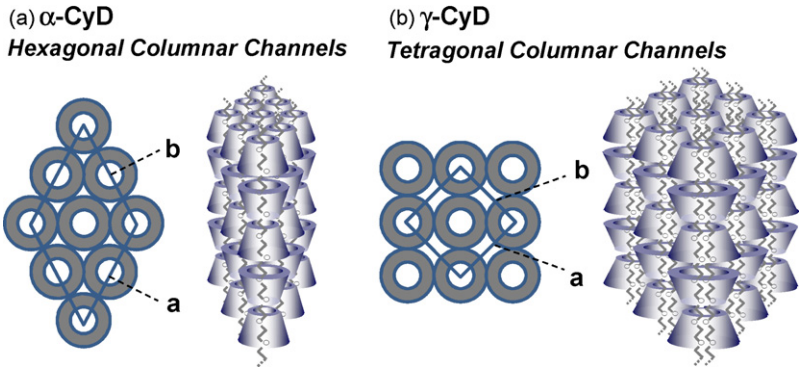

Fig. 7. Schematic representations of crystal structures of pegylated lysozyme/CyD polypseudorotaxanes. (a) α-CyD; (b) γ-CyD.

Table 2
Crystallographic characteristics of pegylated lysozyme/CyD polypseudorotaxanes.

α-CyD polypseudorotaxane			
2θ (°)	*h k l*	d_{obs} (Å)	d_{calc} (Å)[a]
7.40	200	11.95	11.95
12.90	220	6.86	6.90
15.92	320	5.57	5.48
19.84	420	4.48	4.52
22.56	600	3.94	3.98
γ-CyD polypseudorotaxane			
2θ (°)	*h k l*	d_{obs} (Å)	d_{calc} (Å)[b]
7.42	200	11.91	11.91
14.80	400	5.99	5.96
15.76	330	5.62	5.62
16.56	420	5.35	5.33
21.64	600	4.11	3.97

[a] Calculated assuming a hexagonal unit cell with $a = b = 27.59$ Å, packing diameter 11.95 Å.
[b] Calculated assuming a tetragonal unit cell with $a = b = 23.83$ Å, packing diameter 11.91 Å.

of the polymer to the cavity. On the other hand, the γ-CyD solution gave white precipitates immediately after the mixing. These results indicate that the $Zphe_2$-PEG conjugate forms polypseudorotaxanes with γ-CyD, in spite of the end-capping with the bulky group, suggesting γ-CyD includes the two PEG chains of the bent conformation, as shown in Fig. 5. Therefore, we concluded that both the intermolecular and intramolecular complexes of the pegylated lysozyme with γ-CyD coexist in the polypseudorotaxane, as shown in Fig. 1.

Fig. 6 shows powder X-ray diffraction (XRD) patterns of the α- and γ-CyD polypseudorotaxanes with the pegylated lysozyme, in comparison with those of PEG. The diffraction patterns of the pegylated lysozyme/CyD polypseudorotaxanes were different from those of physical mixtures, but same as those of PEG/CyD polypseudorotaxanes (Harada, 1996; Choi et al., 2004). The XRD patterns of the α-CyD and γ-CyD polypseudorotaxanes gave diffraction peaks at $2\theta = 7.40$, 12.9, 15.9, 19.8, and 22.6° and 7.42, 14.8, 15.8, 16.6 and 21.6°, respectively, and resembled the diffraction patterns of the hexagonal and tetragonal columnar channels of the linearly aligned α-CyD and γ-CyD cavities in the crystalline phase (Takeo and Kuge, 1969, 1970; Topchieva et al., 2004; Toropainen et al., 2007). Therefore, the diffraction patterns of the α- and γ-CyD polypseudorotaxanes with the pegylated lysozyme were indexed on the basis of the two-dimensional hexagonal and tetragonal unit cells with dimensions $a = b = 27.59$ Å and $a = b = 23.83$ Å, respectively, as shown in Table 2. The *d*-spacings of the *h k l* (2 0 0) reflection were used to calculate the unit cell dimensions (indicated in Fig. 6). The calculated *d*-spacings (d_{calc}) were in excellent agreement with those observed (d_{obs}), confirming that α- and γ-CyD polypseudorotaxanes of pegylated lysozyme formed the hexagonal or tetragonal structure, respectively. Therefore, we concluded that the one linearly extended PEG chain of the pegylated lysozyme is included in the hexagonal columnar channels formed by the stacking of α-CyD molecules in the crystalline part of the solid polypseudorotaxane, as shown in Fig. 7A. On the other hand, two linearly extended PEG chains of two pegylated lysozyme molecules or two PEG chain of the bent conformation are included in the γ-CyD cavity, as shown in Fig. 7B.

3.3. Release of pegylated lysozyme from CyD polypseudorotaxanes

Fig. 8 shows the release profiles of the pegylated lysozyme from its α- and γ-CyD polypseudorotaxanes in phosphate buffer (1.0 mL, pH 7.4) at 37 °C. The dissolution rate of the pegylated lysozyme was very rapid and dissolved completely within about 5 min. On the other hand, the release rate from the polypseudorotaxanes was significantly retarded and decreased in the order of pegylated lysozyme alone > γ-CyD polypseudorotaxane > α-CyD polypseudorotaxane. These results indicate that the CyD polypseudorotaxanes, particularly the α-CyD assembly, can slow down the release rate of the pegylated lysozyme, and thus serve as a slow release system for pegylated proteins and peptides.

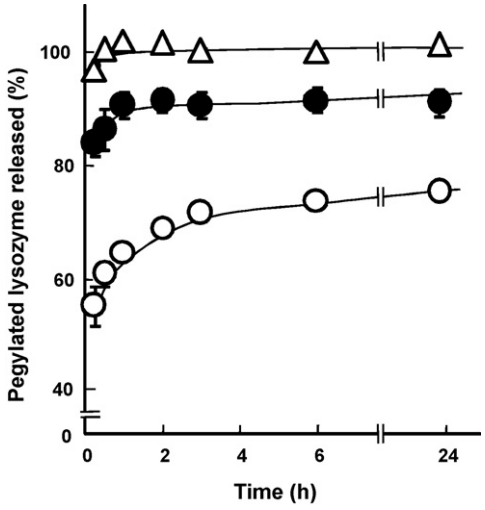

Fig. 8. Release profiles of pegylated lysozyme from its CyD polypseudorotaxanes (equivalent to 0.1 μmol of pegylated lysozyme) in phosphate buffer (pH 7.4, 1.0 mL) at 37 °C. (Δ) Pegylated lysozyme; (○) α-CyD polypseudorotaxane; (●) γ-CyD polypseudorotaxanes. Each point represents the mean ± S.E. of three experiments.

3.4. Enzymatic activity of pegylated lysozyme released from CyD polypseudorotaxanes

Fig. 9 shows the *in vitro* lytic activity of pegylated lysozyme released from CyD polypseudorotaxanes on *M. lysodeikticus* cells. In general, the pegylation is known to decrease in vitro activities of protein drugs (Harris and Chess, 2003; Fee and Van Alstine, 2006). In fact, the lytic activity of the pegylated lysozyme was 80% of native lysozyme. However, as shown in Fig. 9, the lytic activities of pegylated lysozymes released from the α- and γ-CyD polypseudorotaxanes were negligibly decreased before and after the release. These results suggest that the formation of the polypseudorotaxanes does not affect the intrinsic enzymatic activity of the pegylated lysozyme.

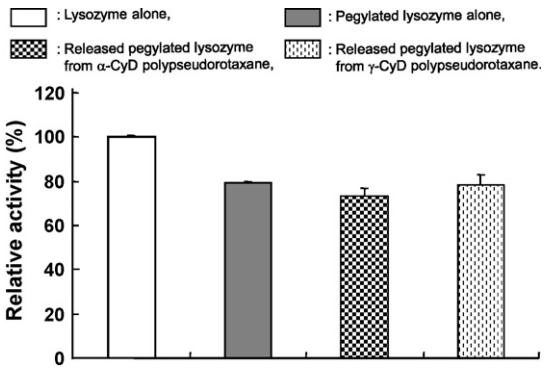

Fig. 9. Lytic activities of lysozyme or pegylated lysozyme (0.36 μM) released from CyD polypseudorotaxanes to *M. lysodeikticus* cells (0.1 mg/mL) in phosphate buffer (pH 6.5, $I = 0.03$) at 25 °C. Each value represents the mean ± S.E. of three to five experiments.

4. Conclusions

We demonstrated here that the pegylated lysozyme forms polypseudorotaxanes with α- and γ-CyD in a similar manner as PEG does. The resulting polypseudorotaxanes were less soluble in water and showed the slow release property of the pegylated protein. This technology may be applicable as a new method for preparation of slow release systems of not only other pegylated proteins and peptides, but also to pegylated low-molecular weight drugs. Furthermore, the present results suggest that the selective precipitation of polypseudorotaxanes is useful for separation of pegylated products from unpegylated and pegylated mixtures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2009.02.017.

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