Polypseudorotaxane Formation of Randomly-Pegylated Insulin with Cyclodextrins: Slow Release and Resistance to Enzymatic Degradation

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Pegylation technology has been widely used to improve therapeutic efficacies of protein drugs and a number of selective- or randomly-substituted pegylated proteins are on the market. In this study, we prepared a insulin derivative substituted randomly with poly(ethylene glycol) (PEG, MW about 2200) and its polypseudorotaxanes with cyclodextrins (CyDs). The pegylated insulin formed polypseudorotaxanes with α- and γ-CyDs, by inserting one PEG chain in the α-CyD cavity and two PEG chains in the γ-CyD cavity. The pegylated insulin/CyD polypseudorotaxanes were less soluble in water. The release rate of the pegylated protein from its polypseudorotaxanes decreased in the order of drug alone>the γ-CyD polypseudorotaxane>the α-CyD polypseudorotaxane. The pegylated insulin/γ-CyD polypseudorotaxane displayed a significantly higher resistance to proteolysis. The results indicated that the CyD polypseudorotaxanes could be formed with randomly-pegylated insulin and work not only as a sustained release system, but also as a stabilizing agent to enzymatic degradations of pegylated insulin.

Key words cyclodextrin; randomly-pegylated insulin; polypseudorotaxane; sustained release; stabilization; pegylated insulin

Pegylation technology is one of the useful methods for sustained release systems, because when poly(ethylene glycol) (PEG) is covalently attached to a protein, it transfers many of the polymer's favorable characteristics to the resulting conjugate, *i.e.* a number of benefits such as increased circulating half-life, enhanced proteolytic resistance, reduced antigenicity and immunogenicity, reduced aggregation, and improved bioavailability, *etc.* There are many examples of protein conjugates that are mono- or randomly-substituted with PEG, such as adenosine deamidase, insulin, interferon-α2, β-lactoglobulin, α-chymotrypsin, lipase, bovine liver catalase, asparaginase, and superoxide dismutase, *etc.* of which the first three conjugates are on the market.[1—3] Most of commercially available pegylated drugs are randomly-substituted derivatives, because of synthetic difficulty of the mono-pegylation.

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, *etc.* Cyclodextrins (CyDs) are cyclic oligosaccharides composed of 6 (α-CyD), 7 (β-CyD), and 8 (γ-CyD) glucopyranose units and can form inclusion complexes with various organic and inorganic compounds.[4] Harada *et al.* first reported the supramolecular assemblies of PEG and α-CyD, in which a number of the cyclic molecule are spontaneously threaded onto the polymer chain.[5,6] These complexes are called polypseudorotaxanes, because the CyD can be dethreaded from the polymer chain when dissolved in water. Recently, many studies on CyD polypseudorotaxanes with various polymers were reported, *e.g.* blanched PEG, poly(ethylene imine) and poly(lactic acid), *etc.*[7—9] In addition, a number of applications of polypseudorotaxanes as a biomaterial were reported, *e.g.* gene delivery carrier,[10—13] biodegradable hydrogel[11,14—17] and galectin binding material.[18] In previous studies,[19,20] we found that the mono-substituted pegylated insulin forms polypseudorotaxanes with α- and γ-CyDs in a similar manner as PEG does. However, there are no evidences if randomly- or multiply-pegylated proteins form polypseudorotaxanes with CyDs. In this study, therefore, we prepared randomly-substituted pegylated insulin and its CyD polypseudorotaxanes and evaluated it as a sustained release system. Further, proteolytic behavior of insulin in the polypseudorotaxane was investigated.

Experimental

Materials Bovine Zn-insulin (27.5 IU/mg, approximately 0.5% Zn) was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). α-Succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (MW about 2300) was obtained from NOF 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.

Preparation of Randomly-Pegylated Insulin Randomly-pegylated insulin was synthesized according to the modified method of Hinds *et al.*[3,21] Briefly, insulin (MW 5734, 14 mg) was incubated with α-succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (12 mg) in DMSO (1.4 ml) at room temperature for 5 h. The reaction was stopped by addition of 4.0 ml water and the reaction solution was dialyzed using a membrane filter (Spectra/Por® membrane MWCO: 3500) and lyophilized. The random substitution of the PEG chain on insulin molecule was confirmed by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass-spectrometry, and no contamination of free PEG in the pegylated insulin was confirmed by TLC and FAB mass-spectrometry. In the following part of this paper, we call the resulting randomly-pegylated insulin simply as pegylated insulin.

Preparation of Polypseudorotaxanes of Pegylated Insulin with CyDs Pegylated insulin/CyD polypseudorotaxanes were prepared by adding 0.5 ml of aqueous pegylated insulin solution (10 μmol of PEG chain, 54.6 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 them for 12 h at 4 °C. The resulting precipitates of the polypseudorotaxanes were filtered and dried under reduced pressure.

***In Vitro* Release of Pegylated Insulin from CyD Polypseudorotaxanes** The *in vitro* release rate of pegylated insulin was measured by the modified dispersed-amount method,[20] *i.e.* various volumes (1, 0.85 or 0.45 ml) of pH 7.4 phosphate buffer were added in the pegylated insulin/CyD 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 10000 rpm for 5 min, and analyzed for the pegylated insulin 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)

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and acetonitrile/water/trifluoroacetic acid (95 : 4.9 : 0.1) and a gradient flow increasing the ratio of the latter solution (0—100%/60 min), a flow rate of 1.0 ml/min, and a detection at 280 nm).

Proteolytic Studies Native insulin (5 mg) or equimolar amounts of pegylated insulin or its γ-CyD polypseudorotaxane were added in 5 ml of phosphate buffer (pH 7.4) or 232 mg/ml γ-CyD solution, to which the trypsin solution (0.05 ml of 1.0 mg/ml dissolved in the same buffer) was added. The resulting solutions were incubated at 25 °C, 50 rpm. At appropriate intervals (0, 1, 3, 5, 8, 24 h), 0.2 ml of samples were withdrawn and added to 0.8 ml of 0.1% trifluoroacetic acid/H_2O solution to stop the enzyme reaction. The solution was analyzed for the intact native and pegylated insulin by HPLC.

Apparatuses 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$K\alpha$ 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.

Results and Discussion

Chemistry Insulin has three primary amino groups, *i.e.* A-chain 1 Gly (pK_a≈8.0), B-chain 1 Phe (pK_a<7.0) and B-chain 29 Lys (pK_a≈10.5) which are possible sites of pegylation. Hinds *et al.* reported the selective introduction of PEG to amino groups of insulin by using blocking agents.[3,21] In this study, the random modification of insulin by PEG was carried out according to the modified method of Hinds, using α-succinimidyloxysuccinyl-ω-methoxy-polyoxyethylene in DMSO without blocking agents. Native insulin and its mono-, di- and tri-substituted pegylated insulin gave single peaks at the retention times of 12.4, 15.8, 18.4 and 20.5 min, respectively, in HPLC chromatogram under the conditions described in the experimental section. By comparing the peaks areas, the contents of native insulin and its conjugates were estimated to be 7% insulin, 35% mono-substituted, 49% di-substituted and 9% tri-substituted pegylated insulins, *i.e.* degree of average substitution of PEG=1.6. In MALDI-TOF mass spectra, insulin gave a peak at 5734.6 (calculated MW 5734.3) and its mono-, di- and tri-substituted conjugates gave dispersed peaks between 7500—8500, 9000—11100 and 11500—13200, respectively, due to the dispersed molecular weights of PEG chains.

Formation of Polypseudorotaxanes of Pegylated Insulin with CyDs Polypseudorotaxanes of the pegylated insulin with CyDs were prepared by mixing aqueous solutions of both components. Figure 1 shows appearances of the solutions after mixing the conjugate and α-, β- and γ-CyDs 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 insulin with α- and γ-CyDs, the phenomena same as those observed for PEG/CyD systems reported by Harada *et al.*[5,22] The stoichiometry of the polypseudorotaxanes was determined by measuring peak areas of the anomeric proton of CyDs and the ethylene protons of the pegylated insulin in ^{1}H-NMR spectra after dissolving the soild polypseudorotaxanes in DMSO. The results indicated that 21 and 11 mol of α- and γ-CyDs, respectively, are involved in the polypseudorotaxane formation with one PEG chain in the pegylated insulin, *i.e.* the coverage of the PEG chain by α- or γ-CyD is 92 or 48% when assumed that 2 (ethylene glycol) repeat units are included in one CyD cavity (Table 1).[5,22] These results suggest that α-CyD forms

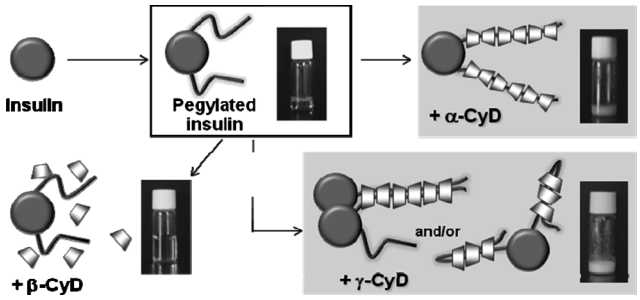

Fig. 1. Macroscopic Photographs of Precipitates of Pegylated Insulin/α-CyD and γ-CyD Polypseudorotaxanes and Their Interaction Modes

Table 1. Yields and Compositions of Pegylated Insulin/CyD Polypseudorotaxanes

Protein	CyDs	Yield (%)	$CyDs_{number}$ [a)]	Coverage [b)] (%)
Insulin	α-CyD	80.9	20.6	91.6
	γ-CyD	89.4	10.7	47.6

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

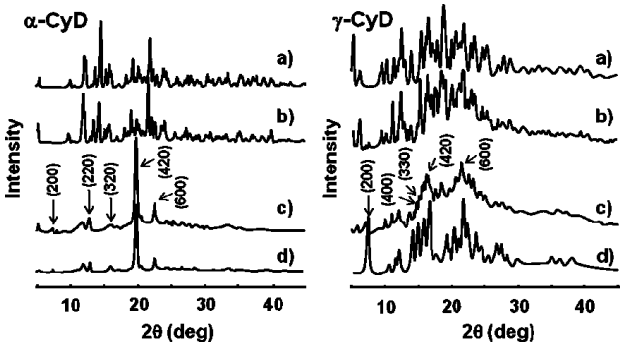

Fig. 2. Powder X-Ray Diffraction Patterns of Pegylated Insulin/α-CyD (Left Figure) and γ-CyD (Right Figure) Polypseudorotaxanes

a, CyD alone; b, physical mixtures of pegylated insulin and CyDs; c, polypseudorotaxanes of pegylated insulin/CyDs; d, PEG/CyD polypseudorotaxanes.

polypseudorotaxane with one PEG chain in the pegylated insulin, while in the case of the γ-CyD polypseudorotaxane, two PEG chains of single or separate pegylated insulin molecules are in the host channel, as shown in Fig. 1.

Figure 2 shows powder X-ray diffraction (XRD) patterns of the α- and γ-CyD polypseudorotaxanes with the pegylated insulin, in comparison with those of PEG. The diffraction patterns of the pegylated insulin/CyD polypseudorotaxanes were different from those of physical mixtures, but same as those of PEG/CyD polypseudorotaxanes.[23] The XRD patterns of the α-CyD and γ-CyD polypseudorotaxanes gave diffraction peaks at 2θ=(7.44, 12.9, 15.9, 19.9, 22.6°) and (7.36, 14.8, 15.9, 16.3, 21.6°), respectively, which 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.[24—27] Therefore, the diffraction patterns of the α- and γ-CyD polypseudorotaxanes with the pegylated insulin were indexed on the basis of the two-dimensional hexagonal and tetragonal unit cells with dimensions $a=b=27.44$ Å and $a=b=24.02$ Å, respectively, as shown in Table 2. The *d*-spacings of the *hkl* (200) reflection

Table 2. Crystallographic Characteristics of Pegylated Insulin/CyD Polypseudorotaxanes

2θ (deg)	(*hkl*)	d_{obs} (Å)	d_{cal} [a)] (Å)
α-CyD polypseudorotaxane			
7.44	(200)	11.88	11.88
12.92	(220)	6.85	6.86
15.92	(320)	5.57	5.45
19.90	(420)	4.46	4.49
22.60	(600)	3.93	3.96
γ-CyD polypseudorotaxane			
2θ (deg)	(*hkl*)	d_{obs} (Å)	d_{cal} [b)] (Å)
7.36	(200)	12.01	12.01
14.80	(400)	5.99	6.01
15.86	(330)	5.59	5.66
16.34	(420)	5.43	5.37
21.64	(600)	4.11	4.00

a) Calculated assuming a hexagonal unit cell with $a=b=27.44$ Å, packing diameter 11.88 Å. *b*) Calculated assuming a tetragonal unit cell with $a=b=24.02$ Å, packing diameter 12.01 Å.

were used to calculate the unit cell dimensions (Fig. 2). The calculated *d*-spacings (d_{calc}) were in excellent agreement with those observed (d_{obs}), confirming that α- and γ-CyD polypseudorotaxanes of pegylated insulin formed the hexagonal and tetragonal structure, respectively. Therefore, we concluded that the one linearly-extended PEG chain of the pegylated insulin is included in the hexagonal columnar channels formed by the stacking of α-CyD molecules in the crystalline part of the solid polypseudorotaxane. On the other hand, two linearly extended PEG chains of two pegylated insulin molecules or two PEG chain of the bent conformation are included in the γ-CyD cavity, as reported previously.[19,20,28)]

***In Vitro* Release of Pegylated Insulin from CyD Polypseudorotaxanes** Figure 3 shows the release profiles of the pegylated insulin from its α- and γ-CyD polypseudorotaxanes in different volumes of phosphate buffer (1.0, 0.85, 0.45 ml, pH 7.4) at 37 °C. The dissolution rate of the pegylated insulin was very rapid and dissolved completely within about 5 min. On the other hand, the release rate from the polypseudorotaxane was significantly decreased in the order of pegylated insulin alone$>\gamma$-CyD polypseudorotaxane$>\alpha$-CyD polypseudorotaxane. In addition, the release rate of the pegylated insulin from its polypseudorotaxanes was dependent on amounts of the dissolution medium (the rate: 1.0 ml$>$0.85 ml$>$0.45 ml), because the threading and dethreading of polypseudorotaxanes are in equilibrium with free host and guest molecules.[23)] In our previous report, the release rate of mono-substituted pegylated insulin from CyD polypseudorotaxanes can be controlled by the addition of CyDs in the medium.[20)] These results indicate that the release rate of pegylated insulin from the polypseudorotaxanes can be controlled by adjusting volumes of dissolution medium or CyD concentrations in the medium.

The Proteolytic Degradation of Pegylated Insulin in γ-CyD Polypseudorotaxane Figure 4 shows the proteolytic profiles of insulin with or without γ-CyD, pegylated insulin, and pegylated insulin/γ-CyD polypseudorotaxane, catalyzed by trypsin. The γ-CyD polypseudorotaxane was employed in

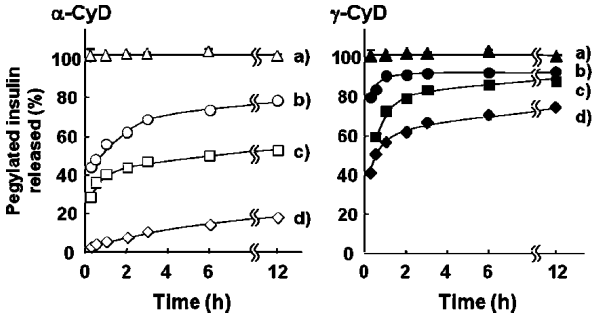

Fig. 3. Release Profiles of Pegylated Insulin from Its α-CyD (Left Figure) and γ-CyD (Right Figure) Polypseudorotaxanes (Equivalent to 0.1 μmol Pegylated Insulin) in Different Volumes of Phosphate Buffer (pH 7.4) at 37 °C

a, release of pegylated insulin alone in 1 ml of the phosphate buffer; b, release of pegylated insulin from its CyD polypseudorotaxanes in 1 ml; c, in 0.85 ml; d, in 0.45 ml, respectively. Each point represents the mean±S.E. of 3—4 experiments.

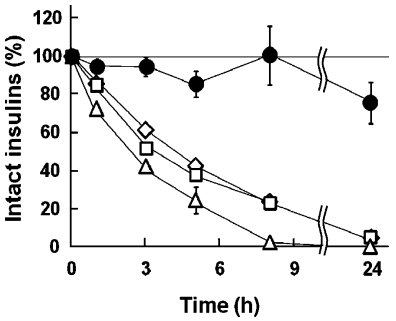

Fig. 4. Degradation Profiles of Insulin (◇), Insulin/γ-CyD[a)] (□), Pegylated Insulin (△) and Pegylated Insulin/γ-CyD Polypseudorotaxane[b)] (●) Incubated with Trypsin

Each point represents the mean±S.E. of 3 experiments. *a*) Insulin was dissolved in 232 mg/ml γ-CyD solution. *b*) γ-CyD polypseudorotaxane was diluted with 232 mg/ml γ-CyD solution.

the proteolytic degradation studies, because it exhibited the appropriate sustained-release property of mono-pegylated insulin, compared with the α-CyD polypseudorotaxane[19,20)] and the safety profiles of γ-CyD such as low hemolytic activity are higher than those of α-CyD.[29)] The native insulin with or without γ-CyD (232 mg/ml) was rapidly degraded in the presence of trypsin (100% degradation within 24 h), indicating no stabilizing effect of γ-CyD on the degradation of insulin. Moreover, the pegylation of insulin did not improve the proteolytic degradation of the drug under the experimental conditions. On the other hand, the pegylated insulin/γ-CyD polypseudorotaxane displayed the significantly higher resistance to the proteolysis (only about 25% degradation in 24 h). Actually, the area under the plasma insulin level–time curve (*AUC*) after subcutaneous administration of mono-pegylated insulin/γ-CyD polypseudorotaxane to rats markedly increased compared to that of pegylated insulin.[19,20)] This proteolytic resistance may be attributable to decrease in not only the reactivity, but also the solubility of the pegylated insulin by the polypseudorotaxane formation.

In conclusion, we demonstrated here that the randomly-pegylated insulin forms polypseudorotaxanes with α- and γ-CyD in a similar manner as PEG and the mono-substituted insulin do. The resulting polypseudorotaxanes were less soluble in water, released slowly the pegylated insulin, and were resistant to trypsin-catalyzed proteolytic degradation. The pegylation of drugs has been utilized for prolongation of sys-

temic circulation of drugs due to increase in molecular weight. This prolongation *in vivo* can be further enhanced by the polypseudorotaxane formation.[19,20] Therefore, the polypseudorotaxane technology may be applicable, as one of sustained delivery techniques for injectable and pulmonary preparations for not only mono-pegylated but also randomly-pegylated proteins and peptides.

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