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Polypseudorotaxanes of pegylated insulin with cyclodextrins: Application to sustained release system

Taishi Higashi,[a] Fumitoshi Hirayama,[b] Hidetoshi Arima[a] and Kaneto Uekama[b,*]

[a]*Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan*
[b]*Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan*

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Abstract—The monosubstituted insulin with poly(ethylene glycol) (PEG, MW about 2200) formed polypseudorotaxanes with α- and γ-cyclodextrins (CyDs), by inserting one PEG chain of the pegylated insulin in the α-CyD cavity and two PEG chains in the γ-CyD cavity. The pegylated insulin/α- and γ-CyD polypseudorotaxanes were less soluble in water and the release rate of the drug decreased in the order of drug alone > the γ-CyD polypseudorotaxane > the α-CyD polypseudorotaxane. The subcutaneous administration of the pegylated insulin/γ-CyD polypseudorotaxane in rats significantly sustained plasma glucose levels with an enhanced hypoglycemic effect. The results indicated that the pegylated insulin/CyD polypseudorotaxanes can work as a sustained drug release system and the polypseudorotaxane formation may be useful as a sustained drug delivery technique for pegylated proteins and peptides.

Pegylation technology has been widely used to improve therapeutic efficacies of a range of molecules, from proteins both small and large, through liposomes and viruses.[1] For example, when poly(ethylene glycol) (PEG) is covalently attached to a protein, it transfers many of the polymer's favorable characteristics to the resulting conjugate, that is, 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 pegylation of proteins 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.[1,2]

Recently, supramolecular assemblies have attracted a great attention due to their intriguing topologies and their application in various fields such as nanodevices, sensors, molecular switches, and drug delivery systems etc. 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.[3] CyDs are cyclic oligosaccharides composed of 6 (α-CyD), 7 (β-CyD), and 8 (γ-CyD) glucopyranose units that 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 cyclic molecules are spontaneously threaded onto the polymer chain.[5] These assemblies are formed by mixing both components in water, giving precipitates of the supramolecular complexes. These complexes are called polypseudorotaxane, because the CyDs can be dethreaded of the polymer chain when dissolved in water. This complexation shows the size-dependency, that is, the small cavity of α-CyD forms the polypseudorotaxane with PEG, while the large cavity of β-CyD with poly(propylene glycol). When both ends of the polymer chains in polypseudorotaxanes are covalently capped with bulky molecules, CyDs are trapped in and cannot be dethreaded from the assembly, giving so-called polyrotaxane. Yui et al. prepared PEG/α-CyD polyrotaxanes capped with amino acids, oligopeptides, and polypeptides, which work as biodegradable drug carriers or stimuli-responsive hydrogels.[6] In spite of many studies on the formation of polypseudorotaxanes and polyrotaxanes reported so far, little is known about the combination of pegylated drugs and CyDs and their application to drug release controls. In this paper, we report the formation of polypseudorotaxanes of pegylated insulin with CyDs and their application to sustained release system.

Keywords: Cyclodextrin; Pegylated insulin; Polypseudorotaxane; Sustained release system.
* Corresponding author. Tel./fax: +81 96 326 4096; e-mail: uekama@ph.sojo-u.ac.jp

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Pegylated insulin was synthesized according to the method of Lee et al.[7] Briefly, insulin (molecular weight 5734, 20 mg) was incubated with α-succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (molecular weight about 2300, 12 mg) in DMF/water (3:2 v/v, 1.4 mL) solution (pH 10 adjusted by 1.0 M NaOH) at room temperature for 15 min. After the reaction was stopped by addition of 3.6 mL water and the pH of the solution was adjusted to 2 with 1.0 M HCl, the reaction solution was dialyzed using a membrane filter (Spectra/Por® membrane MWCO: 3500), lyophilized, and purified for the monosubstituted insulin by HPLC (yield 45%) described later. The monosubstitution of the PEG chain on the insulin molecule was confirmed by MALDI-TOF mass spectrometry, and no contamination of free PEG and insulin in the pegylated insulin by TLC, FAB-mass spectrometry, and HPLC analyses. Pegylated insulin/CyD polypseudorotaxanes were prepared by adding 0.5 mL of the aqueous pegylated insulin solution (10 μmol, 79.2 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.

The in vitro release rate was measured by the modified dispersed-amount method, that is, 1 mL of pH 7.4 phosphate buffer was 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 10,000 rpm for 5 min, and analyzed for the pegylated insulin by HPLC (YMC Pack C18 AP-type column) (4.6 mm id × 150 mm), 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 (0–100%/60 min), a flow rate of 1.0 mL/min, and a detection at 280 nm. Blood glucose levels of rats were measured as follows: the suspension (0.459 mL/kg) of the γ-CyD polypseudorotaxanes (equivalent to 0.38 mg/mL pegylated insulin) in pH 7.4 isotonic phosphate buffer in the absence or presence of γ-CyD (116 and 232 mg/mL) was subcutaneously injected in male Wistar rats (200–250 g), and at appropriate intervals blood samples were taken from the jugular veins. Plasma glucose was determined by the mutarotase-glucose oxidase method using Glucose-CII-Test Wako. Blood glucose levels after the administration of pegylated insulin and its γ-CyD polypseudorotaxane were expressed as a percentage of the initial glucose level.

The introduction of PEG moiety at the lysine residue (Lys^{B29}) of insulin B-chain was carried out according to the method of Lee,[7] using α-succinimidyloxysuccinyl-ω-methoxy-polyoxyethylene in a basic (pH 10) 60% DMF solution where the Lys^{B29} of insulin is most reactive. Insulin and the monosubstituted pegylated insulin gave a single peak at the retention times of 12.4 and 15.4 min, respectively, in HPLC chromatogram under the conditions described above. The conjugate was obtained with above 99% purity and about 45% product yield. In MALDI-TOF mass spectra, insulin gave a peak at 5734.6 (calculated molecular weight 5734.3) and the monosubstituted conjugate gave dispersed peaks between 7430 and 8400 with a center at 7915 due to the dispersed molecular weights of PEG chain. Polypseudorotaxanes of the pegylated insulin with CyDs were prepared by mixing aqueous solutions of both components. Figure 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 insulin with α- and γ-CyDs, the phenomenon same as those observed for PEG/CyD systems reported by Harada et al.[5,8] 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

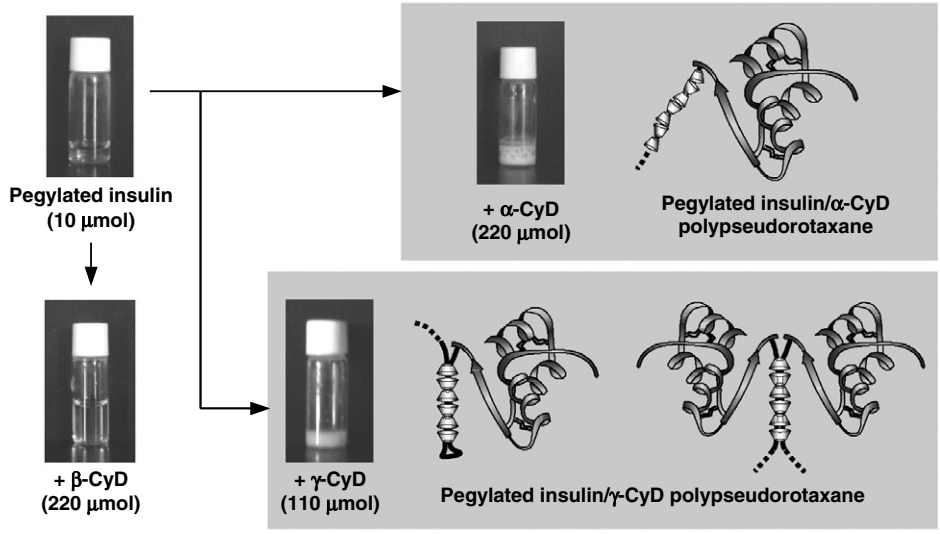

Figure 1. Macroscopic photographs of precipitates of pegylated insulin/CyD polypseudorotaxanes and their interaction modes.

that 20 and 11 moles of α- and γ-CyDs, respectively, are involved in the polypseudorotaxane formation with one PEG chain in the pegylated insulin, that is, the coverage of the PEG chain by α- or γ-CyD is 89% or 49% when assumed that two (ethylene glycol) repeat units are included in one CyD cavity.[8] Figure 2 shows powder X-ray diffraction patterns of the α- and γ-CyD polypseudorotaxanes with the pegylated insulin, in comparison with those of PEG. The diffraction pattern of the pegylated insulin/CyD polypseudorotaxanes was different from those of physical mixtures, but same as those of PEG/CyD polypseudorotaxanes.[9] These results indicate that the PEG moiety of the pegylated insulin forms the polypseudorotaxane with α-CyD, where the long PEG chain is embedded in the stacked host channel, as shown in Figure 1. In the case of the γ-CyD polypseudorotaxane, two PEG chains of a single or separate pegylated insulin molecules are in the host channel.

Figure 3 shows the release behavior of the pegylated insulin from its α- and γ-CyD polypseudorotaxanes in phosphate buffer (pH 7.4) at 37 °C. The release rate decreased in the order of pegylated insulin alone > γ-CyD polypseudorotaxane > α-CyD polypseudorotaxane, suggesting that the polypseudorotaxane can work as a sustained release system for insulin. The release rate of the pegylated insulin from its polypseudorotaxanes was dependent on the amounts of the dissolution medium and on the presence of CyDs in the medium, that is, the rate decreased with decrease in the amount of dissolution medium and it was decreased by the addition of CyDs in the medium (data not shown) because the threading and dethreading of polypseudorotaxanes are in equilibrium with free host and guest molecules.[9] Further, the ternary structure of the pegylated insulin was negligibly changed before and after the release from the polypseudorotaxanes, because of no changes in circular dichroism spectra of the drug.

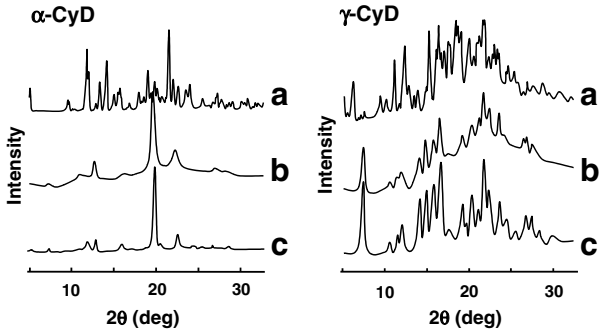

Figure 2. Powder X-ray diffraction patterns of polypseudorotaxanes of pegylated insulin with α- and γ-CyDs.

Figure 4 shows the plasma glucose level–time profiles after subcutaneous administration of the pegylated insulin in phosphate buffer (pH 7.4) or its γ-CyD polypseudorotaxane in the presence of γ-CyD. As a first trial, the γ-CyD polypseudorotaxane rather than the α-CyD polypseudorotaxane with the slower dissolution property was chosen, because of the safety profile of γ-CyD such as low hemolytic activity and superior biodegradability compared to those of α-CyD. When the pegylated insulin alone was injected, the minimal levels of glucose occurred about 2 h after injection and then the plasma glucose levels recovered within 6 h in basal levels. On the other hand, the γ-CyD polypseudorotaxane significantly sustained the hypoglycemic effect and its sustained effect became greater with increase in γ-CyD concentration added in the injection medium, for example, the glucose level recovered after more than 12 h in the presence of 232 mg/mL γ-CyD, because the dethreading rate, that is, release rate of the pegylated insulin is suppressed by the addition of excess amounts of γ-CyD. Further, the area under the plasma glucose level–time curve was significantly increased by the administration of the γ-CyD polypseudorotaxane. The time to nadir blood glucose concentration (T_{nadir}) and the cumulative percentage of changes in the plasma glucose levels up to 12 h post administration (AUC_G) values were as follows: 2 h and 265 ± 39 (% h) for the pegylated insulin alone, 2 h and 305 ± 46 (% h) for the γ-CyD polypseudorotaxane in the presence of 116 mg/

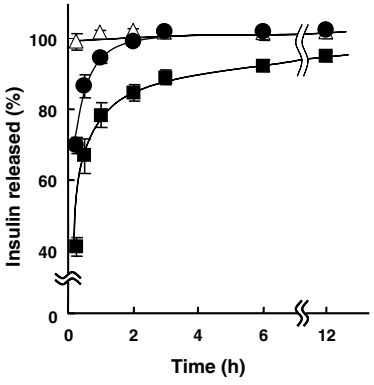

Figure 3. Dissolution behavior of pegylated insulin (△) and its α-CyD (■) and γ-CyD (●) polypseudorotaxanes (0.1 μmol) in pH 7.4 phosphate buffer (1 mL) at 37 °C. Each point represents the mean ± SE of 3 experiments.

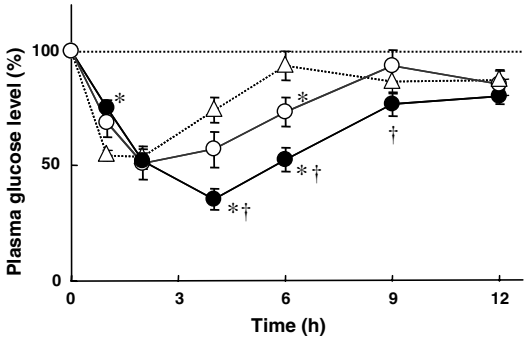

Figure 4. Plasma glucose levels after subcutaneous administration of pegylated insulin (△) and pegylated insulin/γ-CyD polypseudorotaxane in the presence of 116 mg/mL (○) or 232 mg/mL (●) γ-CyD in rats. Each point represents the mean ± SE of 6–9 experiments. *, $p < 0.05$ versus △. †, $p < 0.05$ versus ○.

mL γ-CyD, and 4 h and 443 ± 25 (% h) for that in the presence of 232 mg/mL γ-CyD.

In this study, we demonstrated that the pegylated insulin forms polypseudorotaxanes with α- and γ-CyDs in a similar manner as poly(ethylene glycol) does. The resulting polypseudorotaxanes are less soluble in water and the release rate of the pegylated drug can be controlled by regulating the threading and dethreading rates of the polypseudorotaxanes by adjustment of administration conditions such as amount of injection medium and concentration of CyDs in the medium. The pegylation of drugs has been utilized to prolong systemic circulation of drugs due to increase in molecular weight. This prolongation in vivo can be further enhanced by controlling physicochemically the release rate by the polypseudorotaxane formation, and this technology may be applicable, as one of sustained drug delivery techniques, to pegylated proteins and peptides.

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