Biomaterials 29 (2008) 3866–3871

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Design and evaluation of polypseudorotaxanes of pegylated insulin with cyclodextrins as sustained release system

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ARTICLE INFO

Article history:
Received 22 April 2008
Accepted 17 June 2008
Available online 14 July 2008

Keywords:
Cyclodextrin
Pegylated insulin
Polypseudorotaxane
Sustained release
Drug delivery system

ABSTRACT

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. In this study, we prepared the monosubstituted insulin with poly(ethylene glycol) (PEG, MW about 2200) and its cyclodextrin (CyD) polypseudorotaxanes. 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/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 plasma levels of the pegylated insulin after subcutaneous administration of the γ-CyD polypseudorotaxane to rats were significantly prolonged, accompanying an increase in the area under plasma concentration-time curve, which was clearly reflected in the prolonged hypoglycemic effect. The results indicated that the pegylated insulin/CyD polypseudorotaxanes can work as a sustained drug release system, and the polypseudorotaxane formation with CyDs may be useful as a sustained drug delivery technique for other pegylated proteins and peptides.

1. Introduction

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, i.e. a number of benefits such as increased circulating half-life, 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 [1–3].

Recently, supramolecular assemblies have attracted a great attention, due to its intriguing topologies and its 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 [4,5]. CyDs are cyclic oligosaccharides composed of six (α-CyD), seven (β-CyD), and eight (γ-CyD) glucopyranose units that can form inclusion complexes with various organic and inorganic compounds [6]. 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 [7,8]. 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, i.e. 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 [9,10]. In spite of many studies on the formation of polypseudorotaxanes and polyrotaxanes reported so far, little is know about the combination of pegylated drugs and CyDs and their application to drug release controls. In a preliminary study [11], we found that the pegylated insulin forms polypseudorotaxanes with α- and γ-CyDs in a similar manner as PEG does, and the resulting polypseudorotaxanes may be useful as one of sustained drug delivery techniques of pegylated insulin. In this

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doi:10.1016/j.biomaterials.2008.06.019

paper, we report in detail the design and *in vitro* and *in vivo* evaluations of polypseudorotaxanes of pegylated insulin with CyDs as a sustained release system.

2. Experimental methods

2.1. Materials

Bovine Zn-insulin (27.5 IU/mg, approximately 0.5% Zn) was obtained from Sigma Chemicals (St Louis, MO). α-Succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (M.W. 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.

2.2. Preparation of monosubstituted pegylated insulin

Pegylated insulin was synthesized according to the method of Lee et al. [12]. Briefly, insulin (molecular weight 5734, 20 mg) was incubated with α-succinimidyl-oxysuccinyl-ω-methoxy-polyoxyethylene (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 additions 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 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 analysis.

2.3. Preparation of pegylated insulin/CyD polypseudorotaxanes

Pegylated insulin/CyD polypseudorotaxanes were prepared by adding 0.5 mL of 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 filtrated and dried under reduced pressure.

2.4. Structures of pegylated insulin/CyD polypseudorotaxanes

Powder X-ray diffraction pattern of CyD polypseudorotaxanes were measured using a powder X-ray diffractometer (Rigaku RINT 2500) 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 operating at 500 MHz, using a 5-mm sample tube.

2.5. In vitro *release of pegylated insulin from CyD polypseudorotaxanes*

The *in vitro* release rate was measured by the modified dispersed-amount method, i.e. various volumes (1, 0.85 or 0.45 mL) of pH 7.4 phosphate buffer or 1 mL phosphate buffer containing various CyD concentrations (α-CyD system: 0, 4.5, 9, 72.5, 145 mg/mL and γ-CyD system: 0, 14.5, 29, 116 or 232 mg/mL) 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 10,000 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), 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.

2.6. Subcutaneous administration of pegylated insulin/γ-CyD polypseudorotaxane to rat

Plasma insulin and glucose levels of rats were measured as follows: the suspension (0.459 mL/kg) of the γ-CyD polypseudorotaxane (equivalent to 0.38 mg/mL pegylated insulin) in pH 7.4 isotonic phosphate buffer in the absence or presence of γ-CyD (116 mg 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 insulin and glucose were determined by enzyme immunoassay using Glyzyme Insulin-EIA Test Wako (Wako Pure Chemicals Ind., Osaka, Japan) and the mutarotase-glucose oxidase method using Glucose-CII-Test Wako

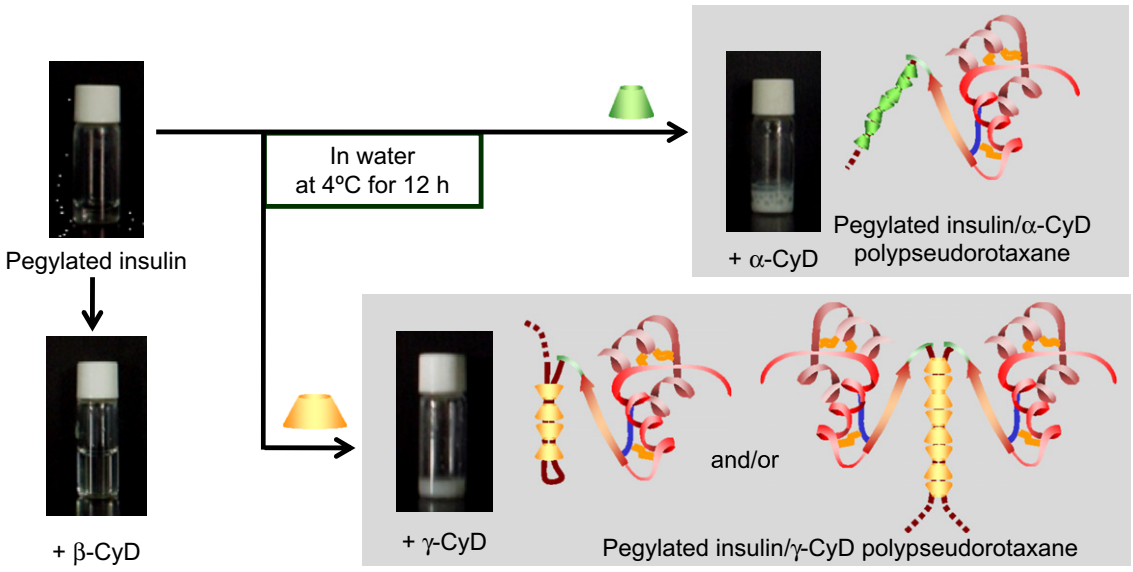

Fig. 1. Macroscopic photographs of precipitates of pegylated insulin/α- and γ-CyD polypseudorotaxanes and their interaction modes.