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ORIGINAL ARTICLE

β-Cyclodextrin/protein conjugates as a innovative drug systems: synthesis and MS investigation

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Abstract The design of proteins whose structure and function can be manipulated by binding with specific ligands such as cyclodextrins, has been of great interest in the field of protein engineering and also could be used as drug delivery systems in targeted cancer therapy (Loftsson and Duchêne, Int. J. Pharm. 329:1–11, 1; Loftsson et al., Expert. Opin. Drug Deliv. 2:335–351, 2). CD/proteins conjugates are synthesized using original high temperature method in which mono-6-*O*-formyl-β-CD reacts with two proteins: basic pancreatic trypsin inhibitor and lysozyme. The proposed synthesis method has a high reproducibility which makes it useful for pharmaceutical purposes. That method allows to obtain the conjugate without losing protein's biological and enzymatic activity which will used in the reaction, and without violating the chemical structure of cyclodextrin molecules.

Keywords Cyclodextrin · Protein · Conjugate · Drug carrier · MALDI-TOF MS

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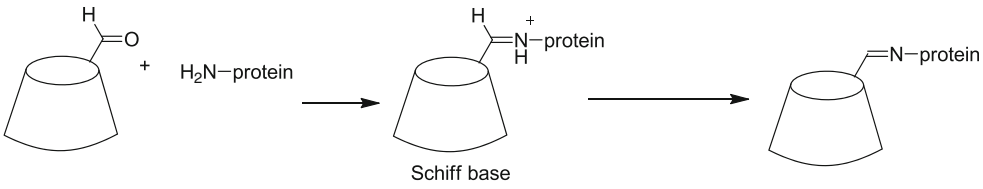
Introduction

Cyclodextrins (CDs) are truncated cone-shaped molecules with hydrophilic rims and hydrophobic cavities [3–7]. The most common CDs are α-, β-, and γ-CD each composed of six, seven, and eight α-1,4 linked D-glucopyranose units arranged in a circular fashion, respectively. CDs have been reported to interact with hydrophobic residues at the surface of proteins, leading to an increase in solubility and suppression of protein aggregation [8–10]. CD aldehyde, is perhaps the most appropriate reactive CD candidate for attachment to biomolecules. Unlike CD tosylates and halides, CD aldehyde is highly water soluble and can be efficiently reacted with proteins or other water soluble polymers under the facile conditions of Schiff base or hydrazone formation and subsequent reduction [11]. Recently one of us proposed a novel approach to the synthesis of glycoconjugates [12, 13]. Our method enables a direct attachment of carbohydrate moieties (CD monoaldehyde) to proteins without any prior modification or chemical activation of the reagents. β-CD can be converted to the monotosylate by using various procedures. The monotosylate would afford selectively the monoaldehyde via oxidation with DMSO in the presence of week base. The main chemical reaction would be the reaction between the terminal amino acid containing a free amino group and mono-6-*O*-formyl-β-CD. In the reaction of aldehyde groups with amino groups, unstable Schiff base are formed, which at high temperature is rearranged to a stable product [11]. Summarizing, we found that it suffices to mix the CD monoaldehyde with a protein, lyophilize them together to dryness and then briefly heat to 120 °C. In that temperature the process of modified glycation can occur (Scheme 1).

Scheme 1 Synthesis of β-CD/protein conjugate



Experimental

Reagents and solvents

Crystalline β-CD, imidasole, *p*-toluenesulfonyl chloride, DMSO, collidine (2,4,6-trimethylpyridine), 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid), acetonitrile (ACN), trifluoroacetic acid (TFA) were purchased from Aldrich (Germany). Dichloromethane and other solvents were purchased from POCh Gliwice (Poland).

Dimethylsulfoxide (DMSO) and collidine were distilled under vaccum and store under molecular sieve 4 Å. Other solvents were used without initial purification.

Synthesis of mono-6-*O*-formyl-β-CD

Mono 6-*O*-formyl-β-CD was obtained by two steps procedure. At the first step a 1-(*p*-toluenesulfonyl)imidazole as a tosylation agent was used [14]. At the next step, the homogeneous sample of the monotosylate would afford selectively the monoaldehyde via oxidation with DMSO in the presence of week base such as collidine [15]. The obtained mono-6-*O*-formyl-β-CD was fully characterized by ^{1}H and ^{13}C NMR. Proton NMR spectra shows relevant peaks at 9.7 ppm (CHO), 4,2 ppm (CHCHO) and typical peaks corresponding to β-CD. At carbon NMR spectra we can also observed an aldehyde peak at 198 ppm.

Synthesis of β-CD-protein conjugates

Conventionally neoglycoconjugates are synthesized in the reaction of chemically modified carbohydrate moieties with macromolecules. We found that it suffices to mix the CD monoaldehyde with a protein, lyophilize them together to dryness and then briefly heat to 120 °C. In that temperature the process of glycation can occur.

The typical procedure is as follows: protein (BPTI or lysozyme) and CD derivative were dissolved in water at 1:1 weight ratio, typically 20 mg each in 1 ml solution. In some experiments aqueous solutions were supplemented with phosphate buffer. The samples were frozen in liquid N_2 and lyophilized. Dry lyophilizate was then placed in an air oven at 73, 95 and 120 °C for 20–40 min, with the tube left open. The tubes were allowed to cool down to room temperature and content of conjugates in each samples were determined by MALDI-TOF experiments.

Molecular mass measurement by the MALDI-TOF MS experiment

MALDI-TOF spectra were obtained using a Bruker Autoflex III spectrometer (Bruker Daltonics, Bremen, Germany), calibration were made on protein calibration standard I or protein standard II from Bruker company. The RP-HPLC technique can be used to "desalt" conjugates samples prior MALDI-MS measurements. RP-HPLC measurements were performed using Waters HPLC instrument equipped with diode array detector (990+) and Symmetry300™ (C4, 300 Å, 3.5 µm, 4.6 × 50 mm; Waters Corp. USA) column. Linear gradient elution was developed from 10 to 90% eluent B from 1 to 10 min. Eluent A was 0.1% TFA in water, whereas eluent B contained 0.1% TFA in ACN. Experiments were carried out at a flow rate of 0.9 ml/min at room temperature. The sample solution (20 µl) was injected. Peaks were detected at $\lambda = 220$ nm and then collected.

Samples to MALDI-MS analysis were prepared on steel plate by mixing 1 µl of conjugates samples (after RP-HPLC "desalt" procedure) with 1 µl of matrix (sinapinic acid) and was evaporated at room temperature, The matrix was dissolved at mixture ACN:water:TFA 50:50:0.1

Results and discussion

Molecular mass analysis

Our experiments we founded that a brief exposure of lyophilized powdered mixtures of BPTI or lysozyme and β-CD to temperatures up to 120 °C produced adducts which are build from protein molecule and one or two β-CD moieties.

Table 1 contains data concerning β-CD/BPTI conjugate. The reaction in water and phosphate buffer was carried out. Analysis of MALDI-TOF spectra of the conjugate obtained in water showed that at 73 °C the reaction of mono-6-*O*-formyl-β-CD with BPTI does not occur. At a temperature of 95 °C we can observe the reaction, while at 120 °C conjugates are formed with high yield. In case of application of phosphate buffer, at temperature 73 and 95 °C, conjugates containing one molecule of β-CD on the surface of the protein are formed, but only with a small yield, while at the temperature of 120 °C two molecules of β-CD are attached to the protein.

Table 1 MALDI TOF MS spectra of β-CD/BPTI conjugate

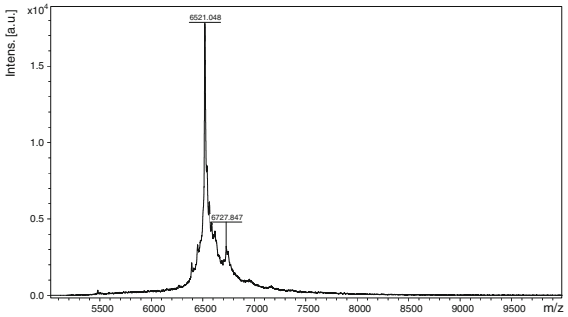
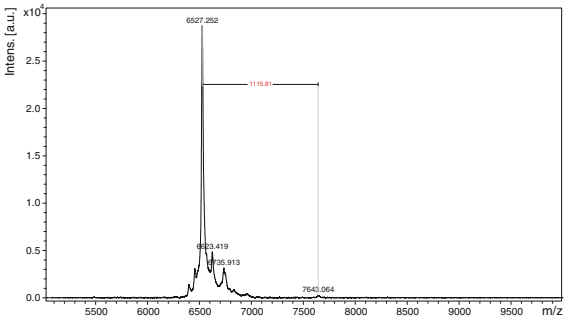
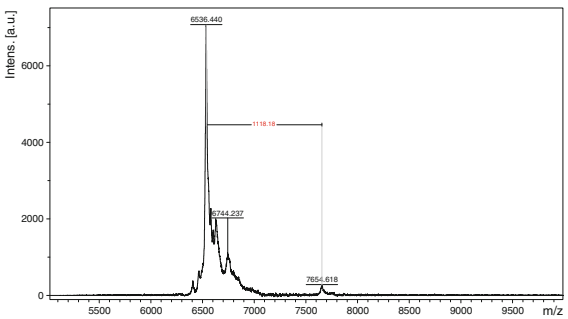
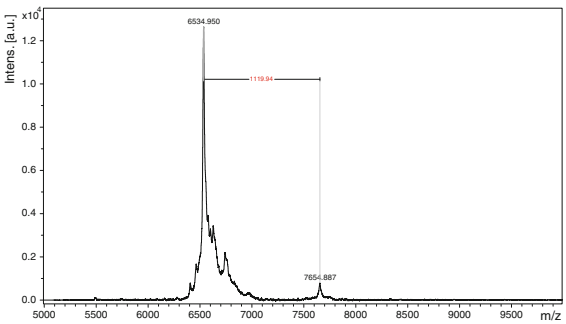
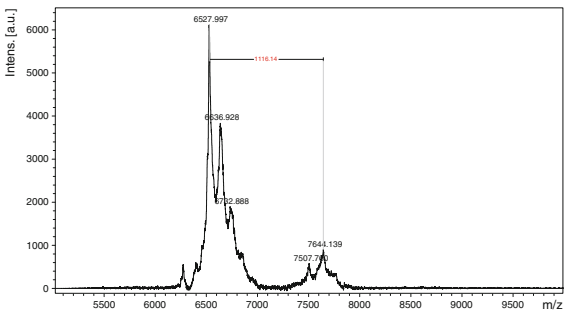
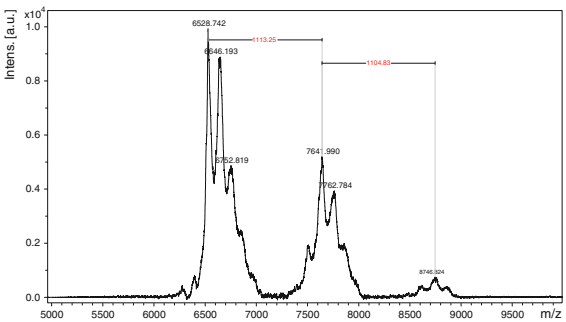
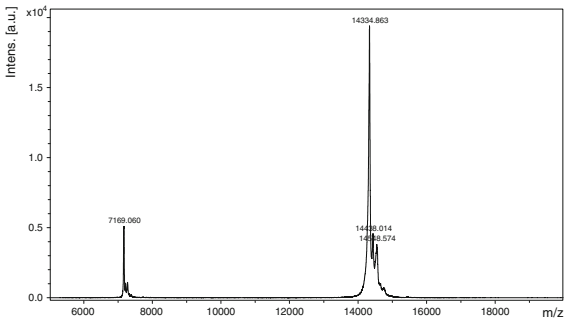
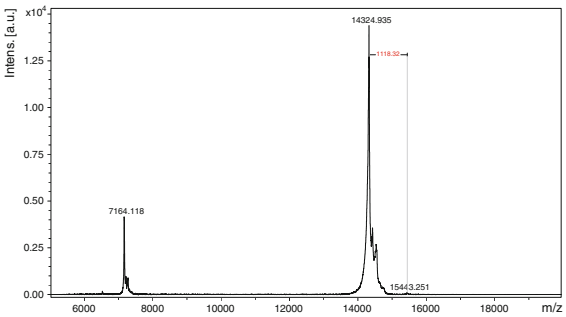
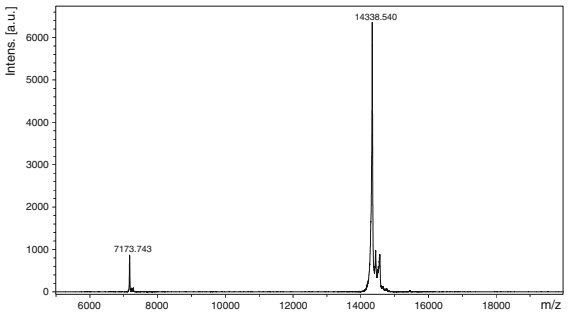
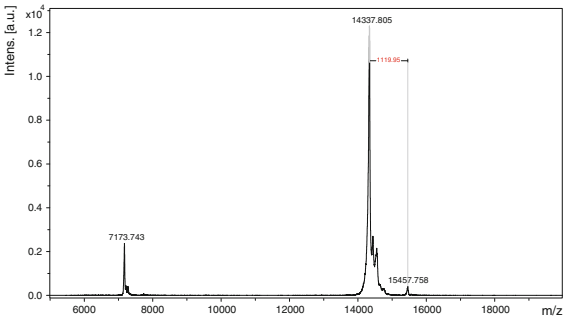
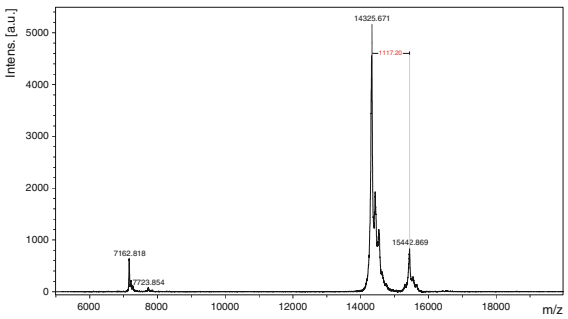
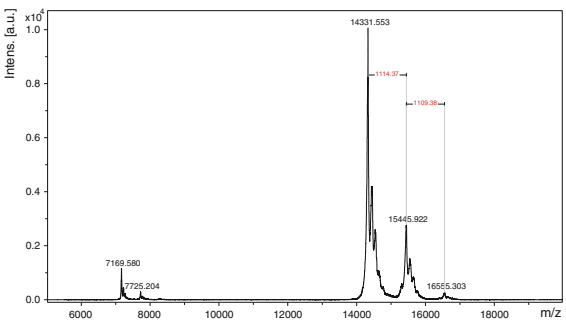
	Water	Phosphate buffer
73 °C		
95 °C		
120 °C		

Table 2 contains data concerning β-CD/lysozyme conjugate. The reaction in water and phosphate buffer was carried out. Analysis of MALDI-TOF spectra of the conjugates obtained in water, showed that at 73 and 95 °C the reaction of mono-6-*O*-formyl-β-CD with lysozyme does not occur. At a temperature of 120 °C we can observe the reaction. In case of application of phosphate buffer, at temperature 73 and 95 °C the reaction occurs but conjugates contain only one molecule of β-CD on the surface of the protein, while at the temperature of 120 °C we can observed, that two molecules of β-CD are attached to the protein.

Conclusions

This work shows formation of β-CD-protein conjugates. The reactions of mono-6-*O*-formyl-β-CD with two proteins: lysozyme and BPTI (basic pancreatic trypsin inhibitor) used as a model proteins were carried out. MALDI-TOF spectra clearly indicate the creation of the conjugate, in which one or two molecules of β-CD to the protein molecule were attached. The largest yields capacity for the reaction at 120 °C and in phosphate buffer solution for BPTI and lysozyme was observed, respectively. The proposed synthesis method has a high reproducibility which makes it useful for pharmaceutical purposes. BPTI and lysozyme are the good model of immunoglobulin and a protease, so it has already opened a wide range of possible applications for this method of dry thermal protein glycation to immobilize them by β-CD. Obtained conjugates will be tested as a containers in carrier-CD-drug systems, which can potentially serve as drug transfer systems in targeted cancer therapy.

Table 2 MALDI TOF MS spectra of β-CD/lisozyme conjugate

	Water	Phosphate buffer
73 °C		
95 °C		
120 °C		

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