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Solid state, thermal synthesis of site-specific protein–boron cluster conjugates and their physicochemical and biochemical properties

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ABSTRACT

Background: Boron clusters represent a vast family of boron-rich compounds with extraordinary properties that provide the opportunity of exploitation in different areas of chemistry and biology. In addition, boron clusters are clinically used in boron neutron capture therapy (BNCT) of tumors. In this paper, a novel, in solid state (solvent free), thermal method for protein modification with boron clusters has been proposed.
Methods: The method is based on a cyclic ether ring opening in oxonium adduct of cyclic ether and a boron cluster with nucleophilic centers of the protein. Lysozyme was used as the model protein, and the physicochemical and biological properties of the obtained conjugates were characterized.
Results: The main residues of modification were identified as arginine-128 and threonine-51. No significant changes in the secondary or tertiary structures of the protein after tethering of the boron cluster were found using mass spectrometry and circular dichroism measurements. However, some changes in the intermolecular interactions and hydrodynamic and catalytic properties were observed.
Conclusions: To the best of our knowledge, we have described the first example of an application of cyclic ether ring opening in the oxonium adducts of a boron cluster for protein modification. In addition, a distinctive feature of the proposed approach is performing the reaction in solid state and at elevated temperature.
General significance: The proposed methodology provides a new route to protein modification with boron clusters and extends the range of innovative molecules available for biological and medical testing.

1. Introduction

Of the various icosahedral boron clusters closo-dodecaborate anion $(B_{12}H_{12})^{2-}$, electrically neutral dicarba-closo-dodecacarborane ($C_2B_{10}H_{12}$) and its charged derivative 7,8-dicarba-nido-undecaborate anion $(7,8\text{-}C_2B_9H_{12})^-$ are frequently used for modification of biological molecules [1]. Their remarkable properties, such as near spherical geometry, chemical, biological and thermal stability, low toxicity, high (depending upon structure) hydrophilicity, hydrophobicity or amphiphilicity and susceptibility to derivatization [2], promise to use them in designing innovative, biologically active molecules. Since the skeletal electrons are delocalized within the cluster, these boron clusters are considered to be three-dimensional aromatic systems [3]. Their size of approximately 5.5 Å (diameter just a little larger than a rotating phenyl group — ca. 4.7 Å) facilitates replacement of organic aromatics in biomolecules with an abiotic boron cluster [4]. Hydrogen atoms of some B–H groups in boron clusters have a partial negative charge, which prevents them from forming classical hydrogen bonds resulting in the highly hydrophobic character of the clusters. Simultaneously, the electronegativity of these hydrogens enables boron clusters to form dihydrogen bonds (proton–hydride bonds) such as NH···HB, CH···HB and SH···HB [5]. Hydrophilicity, hydrophobicity or amphiphilicity together with the ability to form dihydrogen bonds is involved in the interactions of boron clusters with biomolecules, in particular proteins. These features provide the opportunity to exploit boron clusters in different areas of medicinal chemistry, such as the modification of the activity of biologically important molecules [2,6]. The application of boron clusters as modifying entities for biomolecules has been explored for several decades. However, this research has been driven mainly by the quest for better boron carriers for boron neutron capture therapy (BNCT) [7–9]. These days, there is a growing interest in less explored advantages of boron clusters. For example, their use as pharmacophores and modulators in drug design, and hydrophilic or lipophilic components of biomolecules to tune their interactions with other biomolecules captures scientists' attention. The recent appeal of boron clusters to the pharmaceutical industry lies also in the fact that these clusters are abiotic in nature and therefore resistant to catabolism, a property desirable for biological applications. In addition, boron clusters may be used to target receptors that appear to be unaffected by non-boron-containing organic molecules, by interacting with these receptors through diverse mechanisms [10,11].

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The ring-opening reaction of oxonium derivatives of cyclic ethers and polyhedral boron hydrides with nitrogen and oxygen nucleophiles is well recognized and widely used in the bioorganic boron chemistry of low-molecular weight compounds [12,13]. More recently, sulfur nucleophiles such as thiols have been also successfully used [14,15].

In the present study, we have focused on the development of a new method for the coupling of two types of boron clusters with protein in thermal, solid state (solvent free) conditions. The thermal, solid state approach for synthesis of protein conjugates was first proposed by Boratynski and Roy [16] as a method for protein glycation [16,17]. Further experiments on albumin, fibrinogen–methotrexate and lysozyme glycation have shown that careful choice of temperature and reaction time assures the retaining of the biological activity of the proteins without significant changes in their molecular structure [18–20].

In this study, cyclic oxonium derivatives of two different boron clusters i) closo-dodecaborate anion and 7,8-dicarba-nido-undecaborate anion, **(BC-1)** and **(BC-2)**, respectively, as boron cluster donors were used (Fig. 1). As a model protein, lysozyme from egg white, an easily available molecule with a well-defined structure and biological functions, was chosen.

Herein, we have described the first example of an application of cyclic ether ring opening in the oxonium adducts of a boron cluster for protein modification. In addition, a distinctive feature of the proposed approach is performing the reaction in solid state and at elevated temperature. A further aim of our study was the systematic testing of the effect of boron cluster conjugation on protein physicochemical and biological properties.

2. Materials and methods

2.1. Materials

Inorganic salts were kindly provided by POCH, Gliwice, Poland. Pepsin from porcine gastric (Sigma, >2500 units/mg, LOT no.: 101M7001V), tris(2-carboxyethyl)phosphine, TCEP (Aldrich, LOT no.: 040M1232) and all other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, and used without further purification. High purity water was generated by a Direct-Q apparatus, Millipore, Billerica, MA. Two different boron cluster donors were used, an oxonium adduct of dioxane and a closo-dodecaborate anion — **(BC-1)** and an adduct of dioxane and a 7,8-dicarba-nido-undecaborate anion — **(BC-2)**. The structures of these compounds are shown in Fig. 1. Both boron cluster donors have been obtained according to the previously described methods [12,21].

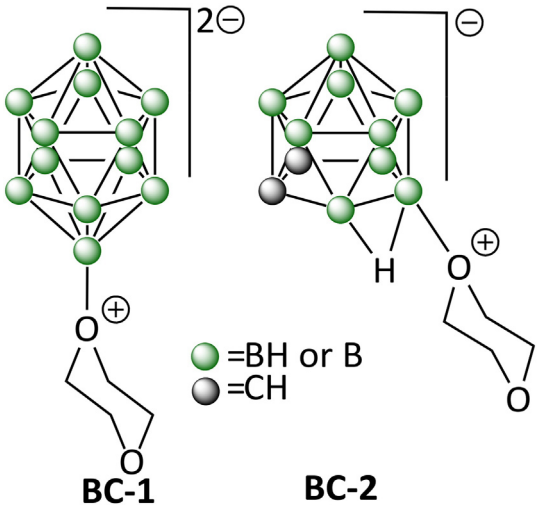

Fig. 1. Boron cluster donors used for lysozyme modification. **BC-1** — oxonium adduct of dioxane and closo-dodecaborate anion $[B_{12}H_{11}O(CH_2CH_2)_2O]^-$, **BC-2** — oxonium adduct of dioxane and 7,8-dicarba-nido-undecaborate anion [10-$O(CH_2CH_2)_2$O-7,8-$C_2B_9H_{11}$].

2.2. Synthesis of lysozyme–boron cluster conjugates via ring-opening reaction in oxonium adducts of cyclic ethers and boron clusters (thermal treatment in the solid state)

Lysozyme from chicken egg white (Fluka, LOT no.: BCBD8746V, 20 mg, 1.39 μmol) was dissolved in dry dimethylsulfoxide (DMSO) (Sigma, LOT no.: SHBB1129V, 3.85 mL). The precise lysozyme concentration was determined by the measurement of the absorbance at $\lambda = 280$ nm, using $\varepsilon = 37{,}750\ cm^{-1}\ M^{-1}$ [22]. After mixing with a solution of the appropriate boron cluster donor, **BC-1** or **BC-2** (5.3 μmol dissolved in 0.1 mL DMSO), 0.05 mL water was finally added to the reaction mixture reaching a final concentration of 1% (v/v). The final protein concentration was in the range 0.25–0.30 mM. Subsequently, the whole mixture was stirred, frozen in liquid nitrogen and freeze-dried (Christ, Alpha 2-4 LSC, Osterode am Harz, Germany) at 0.1 mBar and room temperature. After lyophilization, lysozyme samples used as references (t-lysozyme) and the reaction mixtures, in powdered form, were placed in hermetically capped glass tubes, under argon atmosphere. The samples were heated for 10 min in an oven with forced air circulation (Elkon KC-100/200, Lodz, Poland), equilibrated at 80 ± 0.5 °C, then cooled to room temperature and dissolved in acetate buffer (4 mL, 100 mM, containing 400 mM sodium chloride, pH 4.0). Precipitates of unreacted **BC-1** and **BC-2** were separated from the resultant solution by centrifugation (20,000 × *g*, 5 min; Eppendorf 5424 centrifuge).

2.3. High performance liquid chromatography (HPLC) and mass spectrometry analysis (MS)

HPLC analyses were carried using the Ultimate 3000 RS HPLC system (Dionex, Sunnyvale, CA) equipped with a DAD detector. MS analyses were carried out on a MicrOTOF-Q II hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. All ESI-MS experiments were performed in the positive ion mode and calibrated with a sodium formate (10 mM) in water/isopropanol mixture (50/50 v/v) in the quadratic + HPC regression mode. The mass accuracy was within the range of 5 ppm. The potential between the spray needle and the orifice was set at 4 kV. The capillary temperature was 180 °C, and N_2 was used as a nebulizing gas. Data were acquired with micrOTOF control 3.0 and processed for calibration and charge deconvolution of spectra with DataAnalysis 4.0 software (Bruker Daltonics GmbH, Bremen, Germany).

2.4. Purification of the obtained *L-(BC)* conjugates

Separation in acetate buffer (100 mM, containing 400 mM sodium chloride, pH 4.0) was carried out on a BioSuite phenyl column (10 μm, 7.5 × 75 mm; Waters, Milford, MA). All runs were performed at room temperature using conditions as follows: isocratic elution of A from 0 to 2 min and then, linear gradient from 0% to 45% eluent B from 2 to 15 min, buffer A — acetate buffer (100 mM, containing 400 mM sodium chloride, pH 4.0); B — CH_3CN, with a flow rate of 1.0 mL min^{-1} and an injection volume of 250 μL. The fractions containing lysozyme–boron cluster conjugates were collected together, then concentrated to ca. 0.2 mM using Nanosep™ Omega 3 kDa centrifuge filters (Pall corp., Port Washington, NY) with simultaneous exchange to phosphate buffer (64 mM, containing 10% w/w glycerol, pH 7.2).

2.5. Mass spectrometry of the obtained *L-(BC)* conjugates

When the entire mass of protein/conjugate was investigated (without S–S bridges reduction or after S–S bridges reduction by TCEP), experiments were performed using a BioBasic column (C8, 300 Å, 5 μm, 2.1 × 50 mm, LOT no.: 9420; Thermo Scientific, Waltham, MA) and the following conditions: linear gradient of solutions A (0.1% aqueous formic acid (FA)) and B (CH_3CN with 0.1% FA) from