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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].

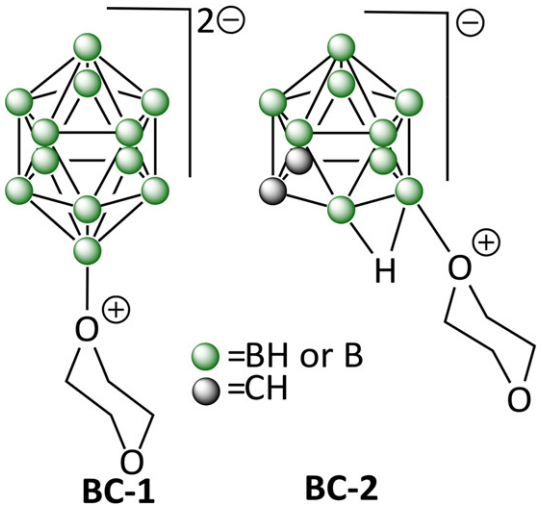

Fig. 1. Boron cluster donors used for lysozyme modification. **BC-1** — oxonium adduct of dioxane and closo-dodecaborate anion $[B_{12}H_{11}O(CH_2CH_2)_2O]^-$, **BC-2** — oxonium adduct of dioxane and 7,8-dicarba-nido-undecaborate anion $[10\text{-}O(CH_2CH_2)_2O\text{-}7,8\text{-}C_2B_9H_{11}]$.

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

5 to 70% of solution B at 20 min, with a flow rate of 0.2 mL min^{-1} and an injection volume of 5 μL, detection via MS. Average molecular weight and standard deviation were calculated from multiple charge states signals observed in the protein envelope. Analysis of the conjugation reaction progress was performed by quantitative MS analysis based on the integrated area of the extracted ion chromatogram (EIC) of +10 and +9 charged ions for each protein/conjugate.

2.6. Proteolysis of lysozyme and *L-(BC)* conjugates

Lysozyme and **L-(BC)** conjugates (0.1 mM) were first reduced by TCEP (50 mM) in phosphate buffer (32 mM containing 5% w/w glycerol, pH 4.5). Completeness of disulfide bridges reduction was checked by mass spectrometry. After 2 min of incubation at 90 °C, samples were diluted in 100 mM HCl (final protein concentration of 2 μM) and finally pepsin in 100 mM HCl was added to the mixture reaching a final concentration of 0.02 μM. Hydrolysis was achieved in 2 h at room temperature.

2.7. Identification of the boronated residues

Separation of the hydrolytic peptides was performed using a BetaBasic-4 column (C4, 300 Å, 3 μm, 1 × 50 mm, LOT no.: 9371; Thermo Scientific, Waltham, MA) and the following conditions: linear gradient of solutions A and B (eluents were the same as above) from 5 to 70% of solution B at 20 min, with a flow rate of 0.2 mL min^{-1} and an injection volume of 5 μL. Eluting peaks were subjected to collision induced dissociation (CID) MS/MS measurements. The precursor ions were selected individually in the quadrupole collision cell with the collision energy between 10 and 90 eV. The obtained fragment ions were directed to the TOF mass analyzer and registered as an MS/MS spectrum. In this type of experiment, only ions resulting from the fragmentation of the selected parent ion were observed. Boron cluster modified peptide identification and sequencing were performed on the basis of MS and MS/MS spectra using the Swiss-Prot database, FindPept program (http://web.expasy.org/findpept/), and PeptideMass program (http://web.expasy.org/peptide_mass/). The mass list generated by the DataAnalysis program (Bruker Daltonics, Bremen, Germany) was analyzed using an Excel spreadsheet to find the ions with specific mass difference (the accepted error of mass difference was below 0.02 Da.). We calculated the theoretical masses of the peptides obtained from in silico pepsin digestion (including b and y fragment ions) and assigned this data to peaks from the scans of the LC–MS/MS data set generated lists of potential boronated peptides (specific mass shifts characteristic for boronated products). The peaks corresponding to the boronated peptides were also identified by their specific isotopic distribution. The analyses were performed separately for ions of different charges.

2.8. Dynamic light scattering (DLS) analysis

Lysozyme and conjugates with boron clusters were characterized by dynamic light scattering in order to obtain hydrodynamic parameters and polydispersity characteristics. The sample solutions were illuminated with a laser at λ = 633 nm, then the intensity of light scattered at an angle of 173° was measured. All samples were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) analyzer in a low volume quartz cuvette (12 μL) and data were analyzed using DTS 6.10 software (Malvern Instruments, Worcestershire, UK). Particle-size distributions were obtained by using the General Purpose algorithm included in the DTS software. Solutions of **L-(BC)** conjugates were prepared in phosphate buffer (64 mM, containing 10% w/w glycerol, pH 7.2), and the final concentration of the conjugates was ca. 0.2 mM. The following parameters were used: protein refractive index (1.450), solvent viscosity (1.212×10^{-4} Pa × s), and temperature (25 °C). At least six consecutive measurements of each sample were carried out.

In addition, the effect of temperature on the hydrodynamic parameters of **L-(BC)** conjugates was estimated by DLS at 1 °C intervals from 25 to 80 °C and a 3 min equilibrium time at each measurement temperature.

2.9. Circular dichroism (CD) analysis

CD spectra were recorded at 25 °C on a Jasco 815 (Jasco, Tokyo, Japan) spectropolarimeter equipped with a Peltier temperature controller cell holder. Three spectra (recorded with a data pitch of 0.1 nm, band width of 1 nm, with a detector response time of 4 s at 50 nm min^{-1}) were averaged for each sample. Quartz cells with 1 mm light path length were used for the measurements. Samples were prepared in a phosphate buffer (64 mM, containing 10% w/w glycerol, pH 7.2). Lysozyme and **L-(BC)** conjugate concentrations were in the range 6–8 μM for far UV measurements and 60–80 μM for near UV measurements. The mean residue ellipticity ($[\theta]_{MRW}$) was calculated using the formula: $[\theta]_{MRW} = (\theta_{obsd}MRW) / (10lc)$ where θ_{obsd} is the observed ellipticity in degrees, MRW stands for mean residue molecular weight (111.8 for lysozyme), l is the path length in centimeters, and c is the protein concentration in g mL^{-1}.

2.10. Enzymatic activity assay

The enzymatic activity of lysozyme and **L-(BC)** conjugates was determined using *Micrococcus lysodeikticus* (Sigma, LOT no.: 111M8601V; ATCC no. 4698) according to the standard procedure [23]. Lytic activity was measured at λ = 450 nm for 5 min (25 °C) in a total volume of 2.6 mL of phosphate buffer (66 mM, pH 6.20) containing 0.2 mg mL^{-1} of suspended bacteria.

3. Results

The physicochemical properties (molecular weight, hydrodynamic parameters and conformational changes) and identification of the amino acids involved in thermal reaction were studied using a combination of mass spectrometry (MS), dynamic light scattering (DLS) and circular dichroism (CD) techniques with the aid of chromatographic techniques (HPLC).

Control lysozyme samples treated the same way as during preparation of the conjugates (dissolving in water/DMSO mixture, freeze drying, and heating at 80 °C for 10 min), but without the addition of the boron cluster donor (t-lysozyme), show no differences in physicochemical parameters or biological activity (variation in measurement error ± 1.8%) as compared to untreated lysozyme (Supplementary data).

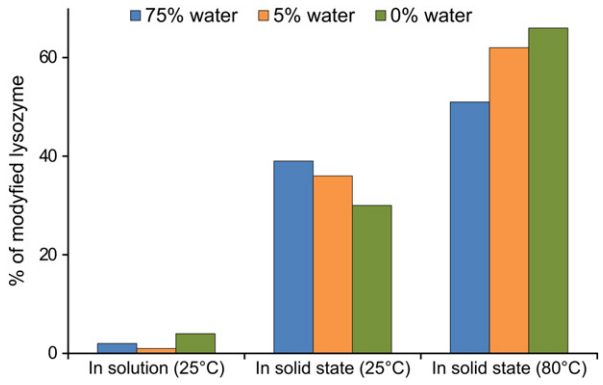

Fig. 2. Comparison of the conjugation reaction between lysozyme and **BC-1** yield carried out in solution (water/DMSO) at 25 °C, after freeze-drying, in solid state, at 25 °C and after freeze-drying, in solid state, at 80 °C. The progress of the reaction was monitored by LC–MS. For details of the reaction conditions, see Section 2.2 of the Materials and methods section.

Table 1
Molecular mass differences between lysozyme and **L-(BC)** conjugates. For details see Section 2.5 of the Materials and methods section.

Protein/conjugate	$M_{protein/conjugate}$ (Da) calcd/found	$\Delta M_{protein\ vs.\ conjugate}$ (Da) calcd/found	$\Delta M_{native\ vs.\ reduced}$ (Da) calcd/found
Lysozyme[a]	14,304.98/14,304.64	–	–
Lysozyme reduced[b]	14,313.05/14,312.56	–	8.07/7.92
L-(BC-1)[a]	14,533.91/14,534.90	229.93/230.26	–
L-(BC-1) reduced[b]	14,541.98/14,542.60	229.93/230.04	8.07/7.70
L-(BC-2)[a]	14,524.49/14,525.24	220.51/220.60	–
L-(BC-2) reduced[b]	14,532.56/14,533.30	220.51/220.74	8. 07/8.06

[a] Samples were analyzed without disulfide bridges reduction.
[b] Samples were analyzed after disulfide bridges reduction by tris-(2-carboxyethyl) phosphine (TCEP).

3.1. Thermal reaction in solid state

Optimization of the conjugation reaction in solid state was carried out. The effect of the temperature on the reaction yield as well as the water/DMSO ratio in lysozyme and **BC-1** solutions before freeze-drying was studied (Fig. 2). It is of interest that the reaction between lysozyme and boron cluster donors in solution, i.e. in water/DMSO mixtures (25–100% DMSO content), does not occur to any practical degree, though after freeze-drying of the reaction mixture, it proceeds efficiently in the solid state providing suitable lysozyme–boron cluster conjugates. The solubility of the conjugates in water was good and comparable with unmodified lysozyme.

3.2. Characterization of intact *L-(BC)* conjugates by LC–MS

Incubation of a solid lysozyme sample at a high temperature (80 °C) without boron clusters donor has no effect on the protein as demonstrated with the ESI-MS analysis (Supplementary data, Fig. S1). This suggests that there are no significant changes in the chemical structure or significant alterations in the stereochemistry of the protein during a short heating period (10 min). The molecular weight of the lysozyme and **L-(BC)** conjugates was assigned (Table 1). Deconvolution of m/z mass spectra estimated the average molecular mass differences between lysozyme and **L-(BC-1)** and **L-(BC-2)** conjugates to be 230.04 and 220.74 Da, respectively, which is equivalent to the mass of a covalently bound **BC-1** or **BC-2** boron cluster moiety. MS data also show an 8 Da increase in mass for lysozyme and **L-(BC)** conjugates after reduction by TCEP, demonstrating that all four of the disulfide bonds were reduced and the cysteine residues did not participate in the reaction with the oxonium adduct of dioxane and boron cluster. According to the spectra presented in Fig. 3, charge-state envelopes are the same for the unmodified lysozyme and for boronated conjugate forms. Slight differences concern only the **L-(BC-2)** conjugate in native state.

3.2.1. Identification of the boronated residues

Two methods for hydrolysis of the conjugates were used: 1) 2% formic acid hydrolysis [24] and 2) pepsin. The highest number of boronated peptides and most appropriate signal intensity was identified after pepsin digestion. Therefore, pepsin enzymatic digestion was chosen for the collision induced dissociation (CID) experiment. To identify the boronated residues, lysozyme and **L-(BC)** conjugates were reduced by TCEP, digested with pepsin, and then the digests were analyzed using LC–MS/MS (Table 2, Table 3, Fig. 4). In the **L-(BC)** conjugate digests, two common modified sites were identified: R128 [123–129] and T51 [39–56]. Additionally, R5 modification [1–8] was localized in the **L-(BC-2)** conjugate. However, it is noteworthy that during the CID experiment the ions representing the **BC-2** boronated peptides first eliminate the cluster (133 Da) and then subsequent b and y linker containing ions (Fig. 4B).

3.3. Hydrodynamic parameters of lysozyme and *L-(BC)* conjugates

The in-solution behavior of conjugates **L-(BC-1)** and **L-(BC-2)** was studied using the DLS technique. The conjugates, directly after synthesis and purification, at room temperature and in aqueous solution (phosphate buffer, 64 mM, containing 10% w/w glycerol, pH 7.2) are fairly monodisperse, although the hydrodynamic diameter and polydispersity of conjugates are slightly higher than for unmodified protein (Table 4, Fig. 5A). The temperature exerted a significant effect on the hydrodynamic size for both lysozyme and the **L-(BC)** conjugates, as can be seen in Fig. 5B. The position and width of the peaks obtained from the DLS autocorrelation function vary with temperature. For temperatures higher than 70 °C, the hydrodynamic diameter of lysozyme increased significantly, suggesting the appearance of large aggregates. The temperature at which aggregation occurs is referred to as the protein thermal aggregation point (TAP). TAP can be determined from the curves as a point when the hydrodynamic diameter starts to increase exponentially with temperature. TAP for the conjugates has significantly lower values than TAP for the lysozyme. **L-(BC-1)** conjugate exhibits higher thermal stability than **L-(BC-2)** (TAP = 51 °C), although its aggregation process is multi-stage, and at least two TAPs can be distinguished — 1st TAP = 48 °C and 2nd TAP = 63 °C (Fig. 5B).

3.4. Spatial structure analysis of *L-(BC)* conjugates

The effect of lysozyme modification with **BC-1** or **BC-2** and conjugate synthesis conditions (80 °C for 10 min) on the conformational properties of lysozyme, as shown by a comparison of CD spectra (Fig. 6), seems to be negligible. The two minima at 208 and 222 nm are characteristic of polypeptides with high helical content. Tertiary structure was investigated via CD in the near UV. As shown in the inset of Fig. 6, no essential conformational changes were observed for **L-(BC)** conjugates, indicating that the presence of the boron cluster (as well as nido- and closo-forms) did not significantly alter the tertiary structure of lysozyme.

3.5. Catalytic activity of lysozyme and *L-(BC)* conjugates

Enzymatic activity analysis of boron cluster conjugates shows a decreased ability to hydrolyze bacterial cell walls: 44.5% and 43.0% for **L-(BC-1)** and **L-(BC-2)**, respectively, as compared to the control (unmodified) lysozyme samples that were given the same treatments (Table 4).

4. Discussion

Knowledge of the relationship between protein functions and structure not only is important for a better understanding of biological processes at the molecular level, but also has a fundamental importance for drug development. Lysozyme, a small globular protein from hen egg white (14,305.04 Da), has 129 amino acids in the primary sequence and 4 intrachain disulfide bridges between sulfhydryl groups containing amino acids [26]. Its biological substrate represents glycosidic

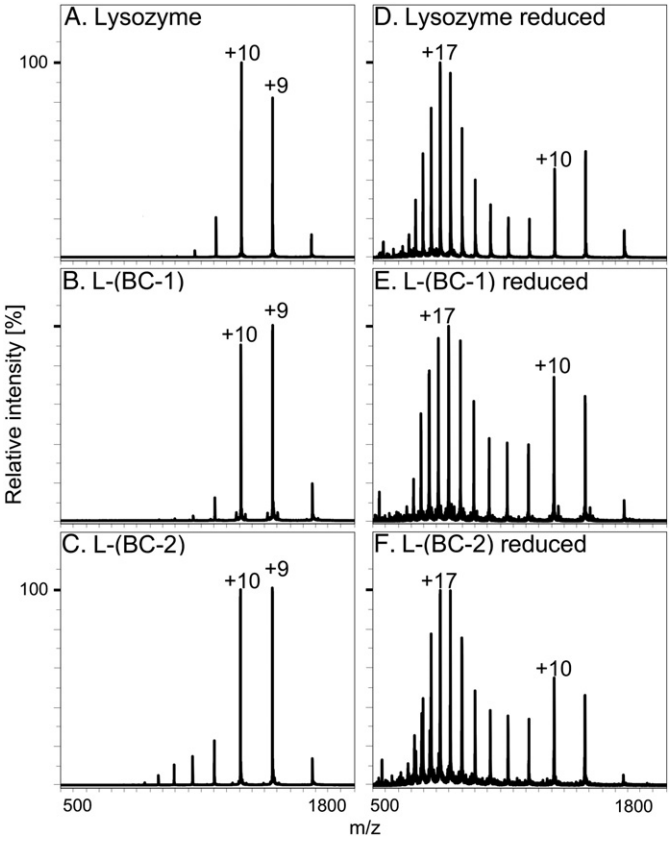

Fig. 3. Comparison of the protein charge envelopes of the lysozyme (A, D), **L-(BC-1)** conjugate (B, E) and **L-(BC-2)** conjugate (C, F). Samples were analyzed by mass spectrometry without disulfide bridges reduction (left panel) or after S–S bridges reduction by tris-(2-carboxyethyl) phosphine (TCEP) (right panel).

linkage between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan polymer of the bacterial cell wall, especially of Gram-positive bacteria [27,28]. Due to the wealth of data regarding its three-dimensional structure, folding, and stability [29,30], lysozyme is a useful model protein for investigation of the effect of chemical modifications on protein physicochemical properties and biological activity. Furthermore, relatively simple structure of lysozyme and its low-molecular mass simplifies characterization of the conjugates.

Herein, we demonstrated that lysozyme–boron cluster conjugates can be obtained using an innovative method based on the thermal treatment (80 °C for 10 min) of the protein with a suitable boron cluster donor in the solid state. We have also proved that such a treatment does not cause any significant secondary or tertiary structure changes. The influence of the reaction conditions (freeze-drying, temperature) on the protein properties is negligible. However, exposure of the lysozyme for an extended time (up to 7 days) to the temperature of 60–80 °C in solid state causes significant loss of enzymatic activity and decreased stability and side covalent modifications of the protein [31]. Our method minimizes a reaction time to 10 min and runs under the conditions in the absence of oxygen. Shortening of the thermal reaction time together with running the reaction under an inert atmosphere proved to be advantageous in comparison with other methods used for the preparation of lysozyme conjugates with different low-molecular weight compounds and had a crucial role in preserving lysozyme activity during the reactions [31,32]. Our results present that the oxonium derivatives of polyhedral boron hydrides are useful starting material not only for low-molecular weight biomolecule mod-

Table 2
Boronated peptides identified via LC–MS/MS analyses of fractionated products of pepsic hydrolysis of **L-(BC-1)** conjugate.

Modified peptide	m/z (calcd/found)	MS/MS analysis m/z (calcd/found)[a]	
[123–129] WIRGCRL	567.377/567.378	455.265/455.272(b3); 512.286/512.307(b4); 131.095/131.079(y1);	831.555/831.563(y5); 1130.718/1130.721(y7)
[39–56] NTQATNRNT DGSTDYGIL	724.722/724.719	414.187/414.190(b4); 515.235/515.231(b5); 785.379/785.374(b7); 1115.496/1115.496(b10); 1172.518/1172.527(b11); 1259.550/1259.528(b12); 1703.851/1703.845(b14); 1866.915/1866.935(b15);	1923.936/1923.939(b16); 2037.020/2037.045(b17); 2150.104/2150.095(b18); 244.179/244.177(y2); 301.201/301.201(y3); 464.264/464.264(y4); 2054.072/2054.053(y17); 2168.115/2168.127(y18)

[a]Nomenclature for peptide fragment ions according to Roepstorff and Fohlman [25].

Table 3
Modified peptides identified via LC–MS/MS analyses of fractionated products of pepsic hydrolysis of **L-(BC-2)** conjugate.

Modified peptide	m/z (calcd/found)	MS/MS analysis m/z (calcd/found)a	
[123–129] WIRGCRL	563.367/563.364; 1125.727/1125.718	186.080/186.079(b1); 299.164/299.108(b2); 615.296/615.313(b5); 131.095/131.073(y1); 375.248/358.214(y2-NH3);	478.257/478.277(y3); 535.279/535.274(y4); 691.380/674.353(y5-NH3); 804.464/787.434(y6-NH3); 990.543/990.539(y7);
[39–56] NTQATN RNTDGS TDYGIL	721.713/721.713; 1082.065/1082.059	215.091/215.086(b2); 785.379/785363(b7); 899.421/899.416(b8); 1000.469/1000.464(b9); 1115.496/1115.494(b10); 1172.518/1172.514(b11); 1259.550/1259.540(b12);	1563.676/1563.667(b14); 1726.739/1726.735(b15); 1783.761/1783.758(b16); 1896.845/1896.843(b17); 2009.929/2009.917(b18); 131.095/131.097(y1); 1512.706/1512.736(y13)
[1-8] KVFGRC EL	587.372/587.369	128.09553/128.0937(b1); 227.1639/227.1677 (b2); 664.3213/664.3167(y5);	811.3898/811.3867(y6); 910.4581/910.4567(y7); 1038.5531/1038.5477(y8);

[a]Nomenclature for peptide fragment ions according to Roepstorff and Fohlman [25].

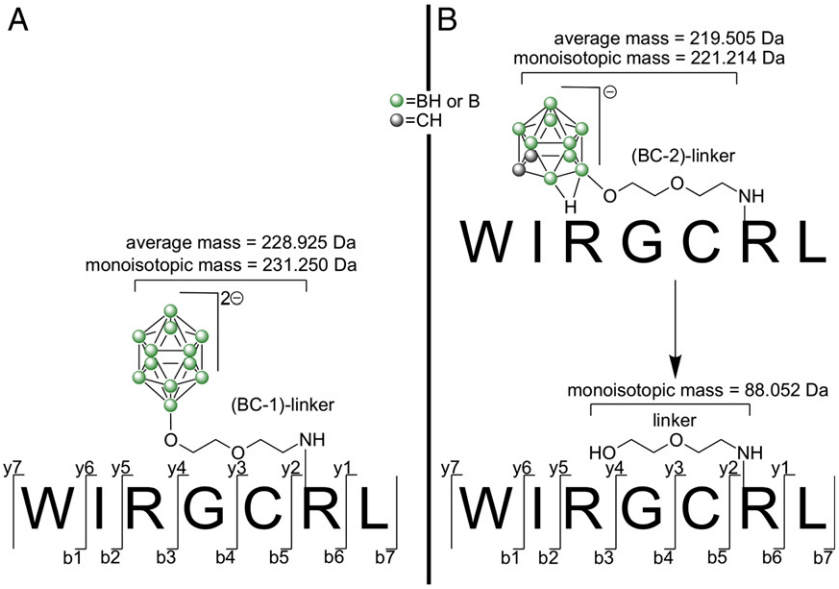

Fig. 4. Schematic representation of the boronated residue identification. Fragmentation pathway during collision induced dissociation (CID) experiment for peptide [120–129] from **L-(BC-1)** (A) and **L-(BC-2)** conjugates (B). Nomenclature for peptide fragment ions according to Roepstorff and Fohlman [25].

ification but also for the synthesis of boronated proteins.

New methods for the coupling of boron clusters with protein have a great interest because of their potential for biological purposes including BNCT; however, the identification of a linkage and residues involved in solid state thermal reaction remains challenging. The present study, performed in the solid state, demonstrates that under particular conditions unusual modifications of T51 were induced. Additionally, it has to be highlighted that aliphatic hydroxyl groups of serine and threonine, having about the same nucleophilicity as water, are generally unreactive in aqueous solutions [33]. Modification of T51 could also be responsible for the partial loss of the biological activity of **L-(BC)** conjugates, because the amino acid following T51 is D52 which is essential for lysozyme enzymatic activity [34–36]. As mentioned above, nitrogen, oxygen and sulfur nucleophiles bearing $-NH_2$, $-OH$ and $-SH$ groups, respectively, have been successfully used in the ring-opening reaction in oxonium derivatives of cyclic ethers and polyhedral boron hydrides in the case of low-molecular weight compounds [37]. Potential application of this type of boron cluster donors for biopolymer modification has not been described in the literature. Also, this is the first time when the usage of the solid state method for boron cluster–protein conjugate synthesis has been described. All three types of the above-listed nucleophilic groups are present in lysozyme but the reaction runs with threonine and arginine. Further studies are needed to establish the mechanism of this phenomenon.

Tethering of the boron cluster to the lysozyme does not significantly alter its secondary or tertiary structures as indicated by MS and CD measurements, but causes a nanoparticle formation. This is demonstrated by the DLS analysis of aggregation of conjugates in aqueous solution (phosphate buffer) during thermal measurements. A slight modification of the lysozyme with a boron cluster (a single **BC-1** modification consists of only 1.5% of the lysozyme molecular weight) results in the enhanced intermolecular interactions observed in DLS measurement resulting in an increased tendency to form nanoparticles. This observation indicates the great potential of boron clusters as modifying units that can be used to modulate the properties of therapeutic biomolecules including proteins. Hydrophobicity or hydrophilicity and the ability to form dihydrogen bonds play an important role in the interactions of boron clusters with biomolecules [38]. The electrostatic and van der Waals forces were found in the interaction of boron clusters with dihydrofolate reductase [39] whereas hydrophobic interactions are important for cage containing ligands with nuclear receptors, such as androgen receptor [40], estrogen receptor [41] and vitamin D receptor [42]. The nature of boron cluster-protein specific interactions has been studied and include CH···HB dihydrogen bonds [5], BH···Na^+ bridges [43] and B_2H···π and CH···π interactions [44]. On the other hand, the boron clusters presented in this paper are electrically charged and therefore can affect the electrostatic interactions of the modified biomolecule with the environment. For example, negatively charged nido cage interacts with the protein (carbonic anhydrase) mainly via electrostatic forces and forms strong dihydrogen bonds while the neutral closo-carborane cage interacts with the carbonic anhydrase mainly via dispersion interactions (dihydrogen bonds are very weak) [45]. In addition, interactions of boron clusters with selected amino acid residue can influence the regioselectivity of the cyclic dioxane ring opening reaction in the oxonium adducts of a boron cluster [5].

New methods for the coupling of boron clusters with protein can reveal possible applications of boronated proteins that range from the development of improved conjugates for BNCT to the use as bioactive molecules that change specific protein functions [46]. In addition, boron clusters have been utilized as “prosthetic groups” for radiohalogens in the design of protein-based radiotherapeutics [47]. The application of boron clusters as a pharmacophore or modifying unit for proteins creates new opportunities in drug design and protein engineering. Depending on their structure, boron clusters are characterized by different physicochemical properties. As a result, they can be used to adjust parameters critical for the biological activity of therapeutic substances and their affinity toward biological targets. In silico calculations suggest that several protein targets can interact with boron cage [48]. These factors demonstrate the potential of boron clusters for biological and medical applications. Current interest in boronated proteins for their utility as boron donors for BNCT may be just the beginning of their broader application applications in future medicinal chemistry.

Table 4
Hydrodynamic diameter (d_H), polydispersity index (PDI) — (from DLS measurement) and enzymatic activity of lysozyme and **L-(BC)** conjugates. SD: standard deviation.

Protein/conjugate	PDI	$d_H \pm$ SD (nm)	Activity ± SD (%)
Lysozyme	0.19	3.7 ± 0.05	100.0 ± 1.8
L-(BC-1)	0.20	4.3 ± 0.10	44.5 ± 1.5
L-(BC-2)	0.20	4.2 ± 0.02	43.0 ± 3.2

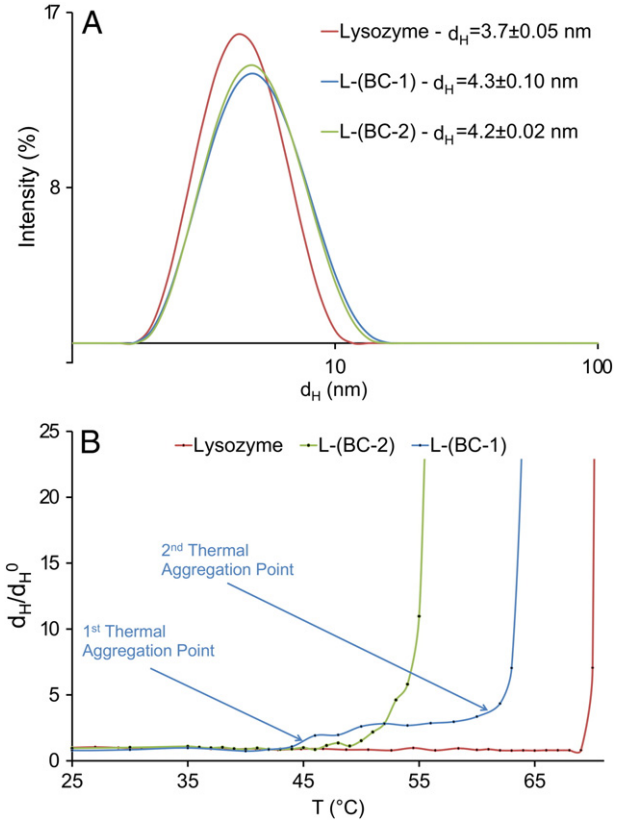

Fig. 5. Characterization of lysozyme and **L-(BC)** conjugates by DLS technique. A — hydrodynamic size distributions are shown according to intensity; d_H value calculated from the averaged scattering intensity. B — relative hydrodynamic diameter d_H/d_H^0 (where d_H^0 is the hydrodynamic diameter in T = 25 °C) of lysozyme, **L-(BC-1)** and **L-(BC-2)** conjugates were determined by the DLS method as a function of temperature. All samples (c = 0.2 mM) were prepared in phosphate buffer (64 mM, containing 10% w/w glycerol, pH 7.2).

5. Conclusion

This paper presents a novel, solid state, thermal, method for protein modification with boron clusters. Modified with boron cluster amino acids have been identified within the lysozyme structure. Additionally, the usefulness of oxonium cyclic ether boron cluster adducts as boron cluster donors not only for low-molecular weight compounds but also for biopolymers such as proteins has been demonstrated. These results open new routes for the modification of biomolecules and create new opportunities in the design of innovative bioactive molecules.

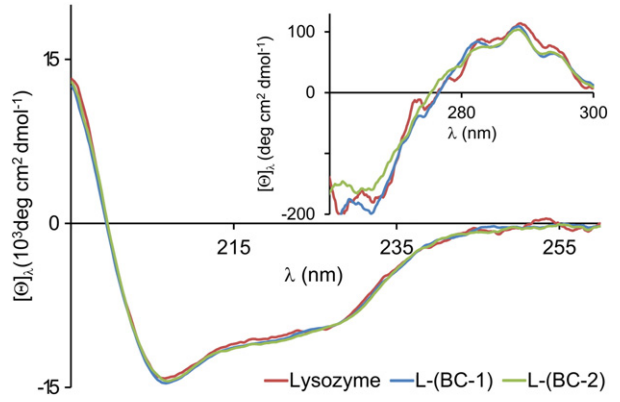

Fig. 6. Far and near (inset) UV circular dichroism spectra of lysozyme and **L-(BC)** conjugates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.11.015.

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