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ORIGINAL ARTICLE

# β-Lactoglobulin detected in human milk forms noncovalent complexes with maltooligosaccharides as revealed by chip-nanoelectrospray high-resolution tandem mass spectrometry

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**Abstract** Cow's milk protein allergy in exclusively breast-fed infants, the main cause of food intolerance during the first 6 months of life, is triggered by the mother's diet. β-Lactoglobulin (BLG) present in cow's milk is one of the most potent allergens for newborns. Since no prophylactic treatment is available, finding ligands capable of binding BLG and reducing its allergenicity is currently the focus of research. In this work, an innovative methodology encompassing microfluidics based on fully automated chip-nanoelectrospray ionization (nanoESI), coupled with high-resolution mass spectrometry (MS) on a quadrupole time-of-flight (QTOF MS) instrument was developed. This platform was employed for the assessment of the noncovalent interactions between maltohexaose ($Glc_6$) and β-lactoglobulin extracted from human milk upon deliberate intake of cow's milk. The experiments were carried out in (+) ESI mode, using ammonium acetate (pH 6.0) as the buffer and also in pure water. In both cases, the MS analysis revealed the formation of BLG–$Glc_6$ complex, which was characterized by top-down fragmentation in tandem MS (MS/MS) using collision-induced dissociation (CID). Our findings have a significant biomedical impact, indicating that $Glc_6$ binds BLG under conditions mimicking the in vivo environment and therefore might represent a ligand, able to reduce its allergenicity.

**Keywords** β-Lactoglobulin · Human milk · Noncovalent interaction · Maltooligosaccharides · Mass spectrometry

**Abbreviations**

| | |
|---|---|
| BLG | β-Lactoglobulin |
| CID | Collision-induced dissociation |
| ESI | Electrospray ionization |
| $Glc_6$ | Maltohexaose |
| HPLC | High-performance liquid chromatography |
| MS | Mass spectrometry/spectrometer |
| MS/MS | Tandem mass spectrometry |
| Mr | Molecular mass |
| QTOF MS | Quadrupole time-of-flight mass spectrometry/spectrometer |

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## Introduction

Human milk, the first choice in infant nutrition, is a complex liquid ideally designed to fulfill the newborn's dietary needs. It contains macronutrients, immunoglobulins and antibodies, and has a major role in the healthy development of the newborn (Walker 2010). During the last few years, the study of breast milk has focused on the analysis of micromolecular content, mainly on the immunological and inflammatory components (Gregory and Walker 2013), bioactive proteins (Lönnerdal 2013), glycoconjugates (Giuffrida et al. 2014) and oligosaccharides (Underwood et al. 2014). The mother's diet during lactation essentially influences the quality of the breast milk (Marincola et al. 2015). For instance, cow's milk protein allergy in infants was shown to occur even during exclusive breast-feeding, if mother consumed cow's milk and/or derived products. β-Lactoglobulin (BLG) present in cow's milk is one of the most aggressive allergens for the newborn, triggering a complex clinical picture. Earlier research conducted in the field (Bu et al. 2010; Yoshida et al. 2005) has suggested that some classes of oligosaccharides bind β-lactoglobulin and reduce its allergenicity this way.

Nowadays, mass spectrometry (MS) with either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) is considered to be one of the most efficient methods in the analysis of biomolecules and molecular aggregates, and the key technology in the emerging fields of proteomics, glycomics and genomics. The success of modern MS is driven by the highly innovative instrumentation designs and the large scale of strategies, which use this technique to detect, de novo identify and structurally characterize in detail molecular species, originating from various biological matrices (Sarbu et al. 2014; Ziegler and Abel 2014; Zamfir et al. 2013; Sisu et al. 2011). Moreover, the development of the integrated micro- and nanofluidics systems for MS emerged in the past decade as a hot research topic, primarily due to numerous advantages of chip-based analyses. The superior sensitivity, the possibility to screen and sequence all classes of biomolecules in a high-throughput mode and the remarkable performances in terms of data quality and reproducibility are among the benefits of this method (Flangea et al. 2013; Schiopu et al. 2012).

In this context, in the present study, we have conceived an innovative microfluidics-MS-based assay for the characterization of the noncovalent interaction between maltooligosaccharides and β-lactoglobulin extracted from human milk, following a deliberate intake of cow's milk. The protein was incubated with $Glc_6$ and the reaction products were subjected to detailed compositional and structural analysis by fully automated chip-nanoESI QTOF MS. The MS survey of the reaction products revealed the formation of a BLG–$Glc_6$ noncovalent complex, which was structurally characterized in a top-down fragmentation experiment based on collision-induced dissociation (CID).

## Materials and methods

### Reagents and materials

Ammonium acetate, acetic acid and formic acid of analytical grade were purchased from Merck, Darmstadt, Germany. Distilled and deionized water from Milli-Q water systems, Millipore, Bedford, MA, USA, was utilized for the preparation of the solutions. SpeedVac concentrator SPD 111 V-230 for drying the samples was purchased from Thermo Electron, Asheville, NC, USA. The SpeedVac was coupled to a vacuum pump PC 2002 Vario with CVC 2000 controller from Vaccubrand, Wertheim, Germany. The dialysis was performed using Micro DispoDialyzer systems from Harvard Apparatus, Holliston, MA, USA. The thermomixer employed for stirring was an Eppendorf Thermomixer from Eppendorf, Hamburg, Germany, $Glc_6$ (Mr 990.86) and standard BLG (90 %) was purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA) and used without further purification. The pH was measured using a pH meter from Mettler-Toledo, AG, Schwerzenbach, Switzerland.

### Sampling of human milk

Human milk samples, of 3 mL volumes each, were collected from five mothers at 4 h after the intake of 300 mL cow's milk. These particular subjects were chosen for the research, since their babies were diagnosed with cow's milk protein allergy, although they were exclusively breast-fed. The patients freely agreed to participate in this study and signed a written informed consent.

### Extraction and purification of the human milk BLG

Human milk BLG was extracted following the protocol described by Cohen and Chait (1997) and Schneider et al. (2014). Briefly, 3 mL of raw human milk from each subject was submitted to centrifugation, using an Eppendorf 5417R centrifuge at 13.000 rpm and 4 °C for 30 min. The lipid layer accumulated on the top was removed. Whey proteins were separated by adjusting the pH to 4.8, using acetic acid and subsequent centrifugation. The collected whey was further dialyzed against 20 mM phosphate buffer and submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). As the standard for comparison of the relative mobility, commercially available BLG was used. The final purification step was performed by

cation-exchange chromatography, following the protocol described by Neyestani et al. (2003).

### Chip-based nanoESI MS and CID MS/MS

Mass spectrometry was conducted in positive ion mode on a hybrid quadrupole time-of-flight mass spectrometer (QTOF MS, Waters, Manchester, UK), controlled by MassLynx v. 4.1 software, in-laboratory coupled to a NanoMate robot (Advion BioSciences, Norfolk, UK) via a specially designed mounting bracket. The MS scans were acquired with a resolution of 5000 (for *m/z* 500). The incorporated nanoESI chip exhibited 400 nozzles of 2.5 μm inner diameter. Using the ChipSoft software, the robot was programmed to aspirate the whole volume of sample, followed by 2 μL of air to prevent sample dripping during manipulation. In the case of BLG–$Glc_6$ interaction in ammonium acetate/formic acid buffer, chip-based nanoESI was initiated at 1.8 kV on the pipette tip, 40 V cone voltage, 0.40 psi nitrogen back pressure, and 60 psi pressure of the nitrogen nebulizer. For the detection of the BLG–$Glc_6$ complex formed in water, 1.9 kV on the pipette tip and 60 V cone voltage values were applied. For optimal desolvation of the sample droplets, the source block was maintained at 80 °C. The top-down fragmentation of the formed complex was carried out by CID at variable energy, using argon as the collision gas. The nomenclature of the oligosaccharide fragment ions followed the recommendations of Domon and Costello (1988). In all figures, the monoisotopic masses are provided. The in-run and run-to-run reproducibility of the experiments was situated between 98 and 100 %, while the sample-to-sample reproducibility was almost 95 %.

### BLG–$Glc_6$ interaction assays

The solvent employed for studying the noncovalent interactions was 10 mM aqueous ammonium acetate, adjusted with 98 % formic acid to pH 6.0. Although a higher ionic strength positively influenced the extent of interaction, 10 mM ammonium acetate concentration at pH 6.0 was found as a fair compromise between the complex formation and the solvent system requirements for electrospray ionization and MS detection. The interaction assay was carried out in the Eppendorf thermomixer, by incubating at 37 °C 30 μL BLG solution in buffer at a concentration of 5 pmol $\mu L^{-1}$ with 6 μL $Glc_6$ solution in buffer, at a concentration of 10 pmol $\mu L^{-1}$. 5 μL aliquots were collected after 1, 5, 10, 15, 20, 25 and 30 min of incubation and submitted to MS analysis. Following a series of optimization procedures, the stoichiometry of 1:2.5 $Glc_6$:BLG was found to be the best suited for both complex formation and its detection by MS. To minimize the interference in the reaction process during the collection procedure, the vial was not removed from the Thermomixer.

For the interaction assay carried out in pure water, the best results were obtained by incubating for 50 min at 37 °C 30 μL of BLG solution at a concentration of 5 pmol $\mu L^{-1}$ with 6 μL $Glc_6$ solution at a concentration of 10 pmol $\mu L^{-1}$.

## Results

### BLG–$Glc_6$ interaction in ammonium acetate/formic acid

BLG extracted and purified from human milk following cow's milk intake was dried and subsequently dissolved in 10 mM aqueous ammonium acetate, adjusted with 98 % formic acid (pH 6.0) to a final concentration of 5 pmol $\mu L^{-1}$. 5 μL BLG/buffer solution was loaded into the well of the microtiter plate of the NanoMate robot. The sample was automatically infused in the hybrid QTOF MS by positive ion mode chip-nanoESI. The spectrum, as a sum of scans acquired for only 2 min, is presented in Fig. 1. A detailed evaluation of the spectrum reveals that, obviously, ammonium acetate/formic acid buffer at pH 6.0 induced a fair ionization of BLG. Five signals of high intensity, forming the protein-specific envelope at *m/z* 2026.63, 2279.86, 2605.38, 3039.41 and 3647.00 were detected. Using the deconvolution program of MassLynx software v. 4.1, the signals were assigned to the series of protonated BLG molecules, from $[BLG + 5H^+]^{5+}$ to $[BLG + 9H^+]^{9+}$. According to calculation, the experimentally determined monoisotopic molecular mass (Mr) of BLG eliminated in human milk is (18,230.50 ± 0.2) Da.

Following the incubation of BLG and $Glc_6$, aliquots of the reaction products were collected at 1, 5 and 10 min after mixing and incubation.

After 1 min, 5 μL was collected and loaded directly into the 96-well plate of the NanoMate robot. MS screening, as a sum of scans acquired for 2 min in positive ion mode, is depicted in Fig. 2. Besides the characteristic BLG envelope with 5+ to 9+ charge states of the molecular ions, chip-nanoESI mass spectrum depicted in Fig. 2 presents a signal at *m/z* 2750.10. This *m/z* corresponds to an Mr of 19,242.51 Da at a 7+ charge state. Based on calculation, this value is consistent with the molecular mass of the noncovalent complex of BLG with the sodiated form of $Glc_6$; in the MS screening of $Glc_6$ (data not shown), the sodiated form of $Glc_6$ was found as the most abundant ion at *m/z* 1013.85.

The spectrum of the reaction products, collected after 5 min of reaction is presented in Fig. 3. As compared to the spectrum in Fig. 2, only one dissimilarity is noticed. The

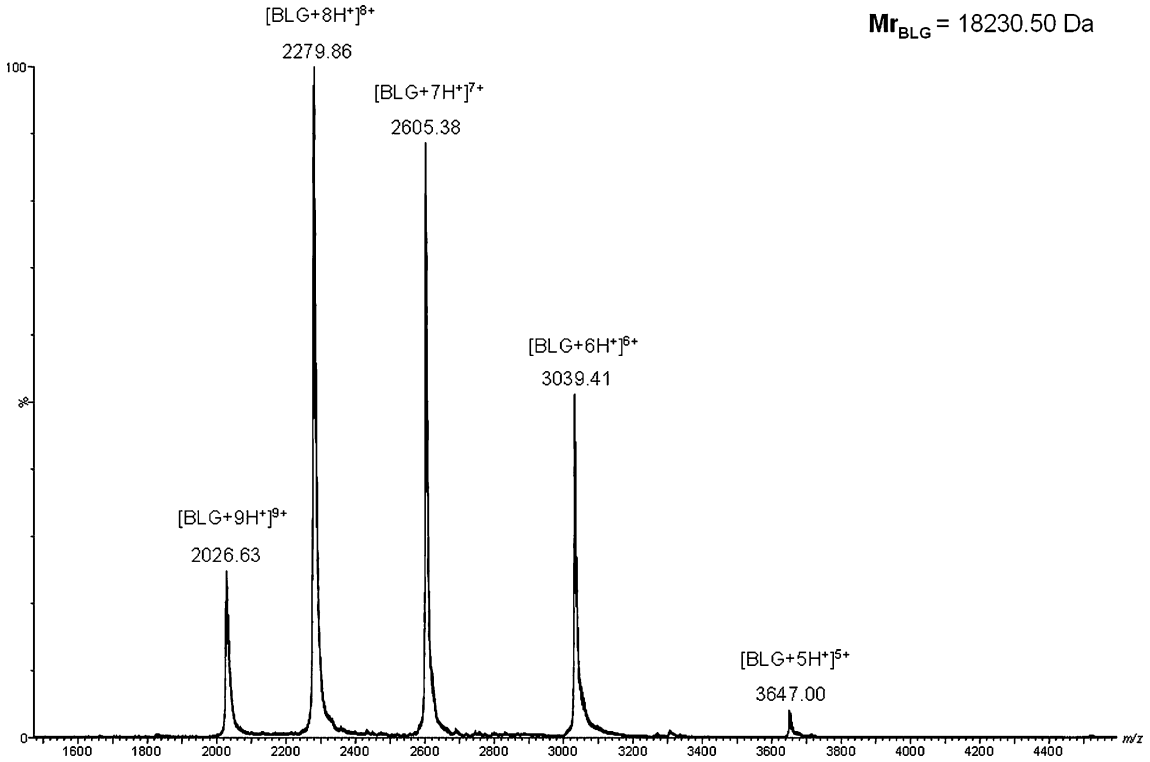


**Fig. 1** (+) Chip-nanoESI QTOF MS of BLG extracted from human milk upon intake of 300 mL cow's milk. Concentration, 5 pmol $\mu L^{-1}$ in 10 mM ammonium acetate/formic acid, pH 6.0. NanoESI voltage, 1.8 kV; cone voltage, 40 V; nitrogen back pressure, 0.40 psi; nitrogen nebulizer pressure, 60 psi; acquisition time, 2 min

intensity of the ionic signal, corresponding to BLG complex with $Glc_6$, is higher than that of the homolog ions in Fig. 2. This feature suggests that, between min 1 and min 5, the reaction was still ongoing. Based on these observations, the process was further monitored.

Hence, at min 10 after the incubation, 5 µL was collected and submitted to positive chip-based nanoESI QTOF MS under identical conditions. The recorded spectrum is depicted in Fig. 4. In comparison with the other two experiments, the spectrum in Fig. 4 shows a much higher intensity of the signal, corresponding to the noncovalent complex at *m/z* 2750.09. The spectra of the aliquots collected further, up to 30 min with a 5 min time interval, did not exhibit any significant changes.

To study in detail the formed complex of BLG with $Glc_6$, the ion detected at the nominal *m/z* 2750.09 in the MS of the pools collected at min 1, 5 and 10 was selected within an isolation window of 2u and submitted to top-down fragmentation by collision-induced dissociation, at variable ion acceleration energies.

In Fig. 5, the top-down analysis carried out using as the precursor the ion corresponding to the complex detected in the sample collected after 10 min is presented. Fragmentation under identical conditions of the same precursor ion in the pools after 1 and 5 min of reaction yielded almost identical results. Inspection of the spectrum in Fig. 5 reveals a number of fragment ions diagnostic for the BLG–$Glc_6$(Na) complex. Moreover, the fragmentation pattern documents that two CID events occurred in one and the same top-down experiment: (1) cleavage of the noncovalent BLG–$Glc_6$ bond, supported by $[Glc_6 + Na^+]^+$ at *m/z* 1013.86, corresponding to $Glc_6$, and the series of ions at *m/z* 2026.65; 2279.86; 2605.42; 3039.46 and 3647.06, related to the different charge states of the protein, ranging from 5+ to 9+; (2) sequencing of $Glc_6$ with the formation of the product ions in the low *m/z* region, characteristic for the sodiated form of $Glc_6$ (inset Fig. 5).

For comparison, in Fig. 6, CID MS/MS of $[Glc_6 + Na^+]^+$ detected at *m/z* 1013.85 by MS screening of the $Glc_6$ alone dissolved in buffer is provided together with the fragmentation scheme. Obviously, the fragmentation pathways experienced by $[Glc_6 + Na^+]^+$ in the two experiments are similar, which substantiates the formation of the noncovalent complex of BLG with $Glc_6$.

## BLG–$Glc_6$ interaction in water

5 µL aliquots of the BLG–$Glc_6$ reaction products were collected at min 1, 5, 10 and 30 after incubation in water and loaded into the well of the NanoMate microtiter plate. In all

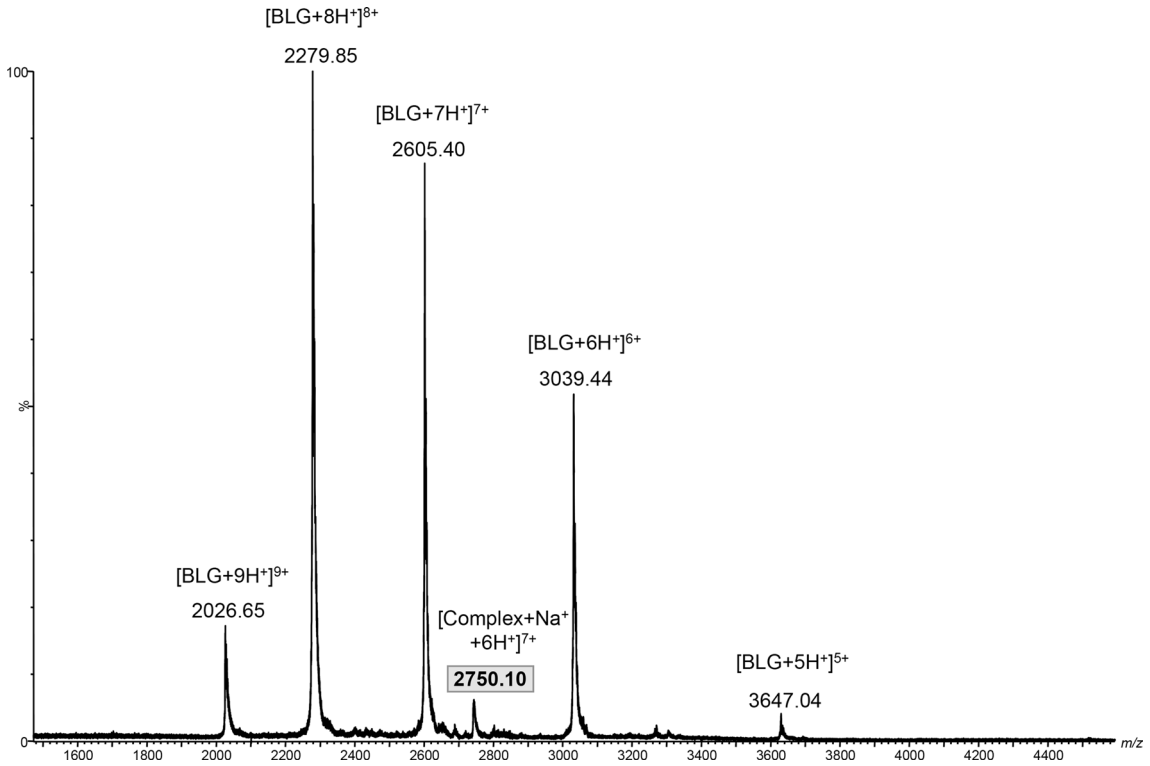


**Fig. 2** (+) Chip-nanoESI QTOF MS of the BLG–$Glc_6$ reaction products after 1 min incubation in buffer at 37 °C. Other MS conditions are as in Fig. 1

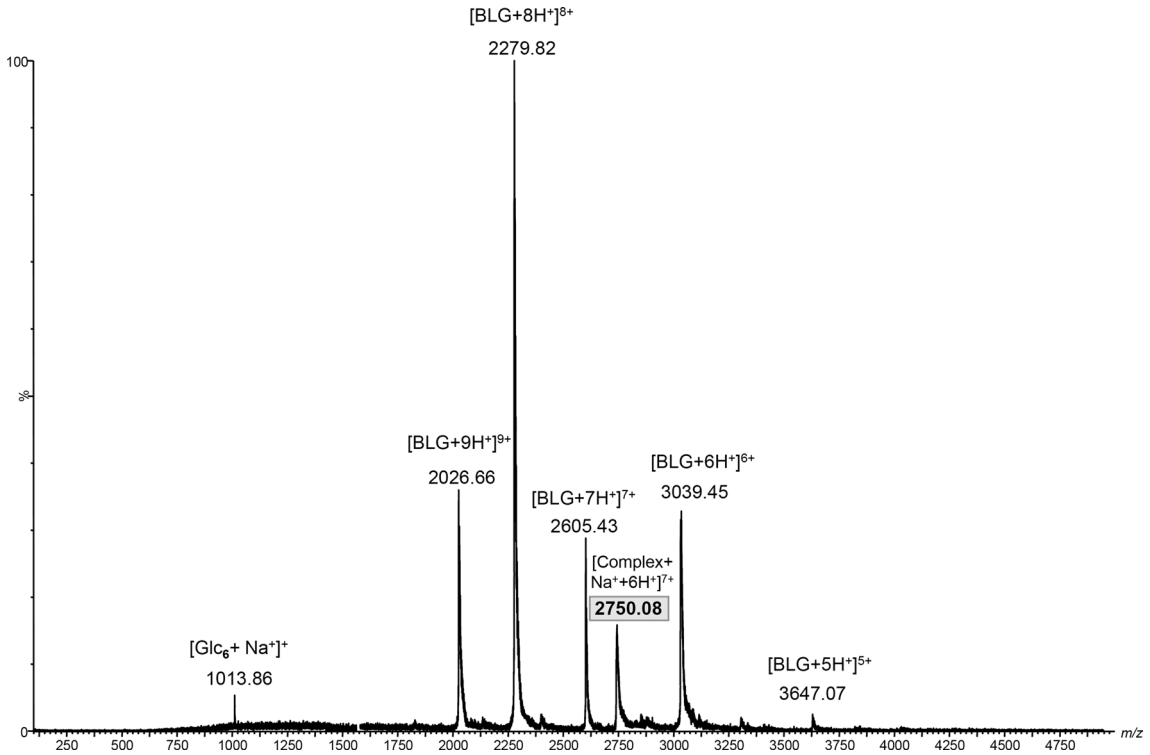


**Fig. 3** (+) Chip-based nanoESI QTOF MS of the BLG–$Glc_6$ reaction products after 5 min incubation in buffer at 37 °C. Other MS conditions are as in Fig. 1

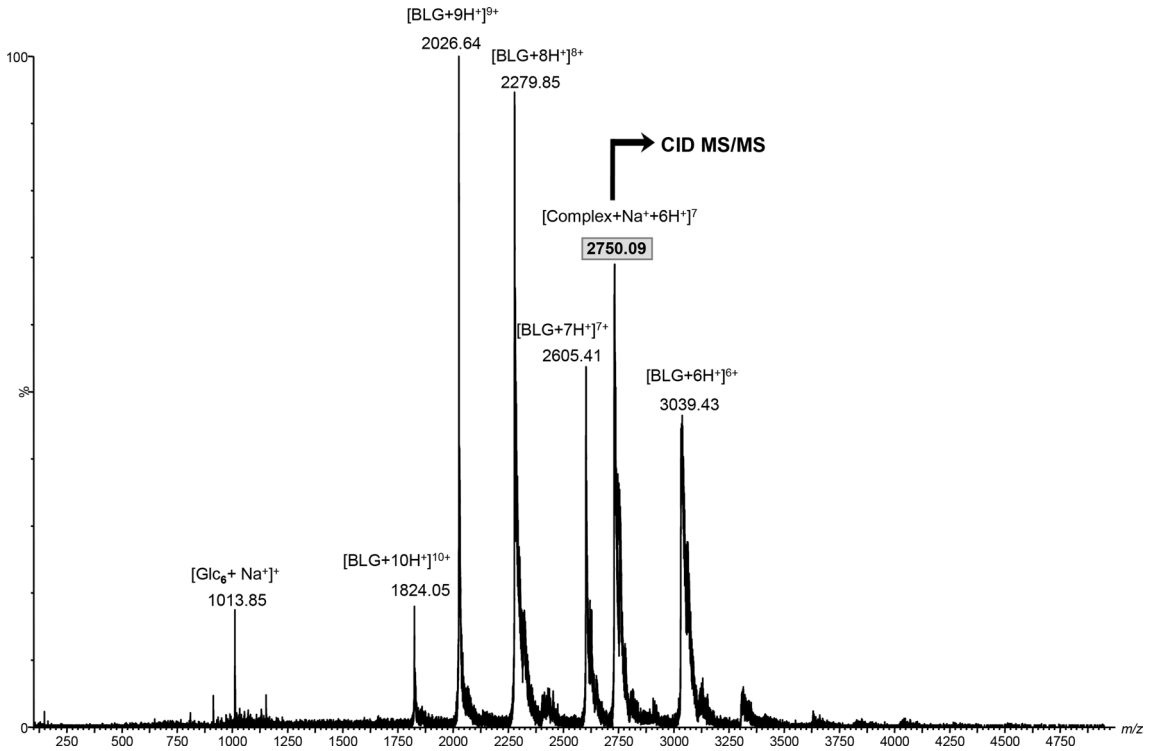


**Fig. 4** (+) Chip-based nanoESI QTOF MS of the BLG–$Glc_6$ reaction products after 10 min incubation in buffer at 37 °C. Other MS conditions are as in Fig. 1

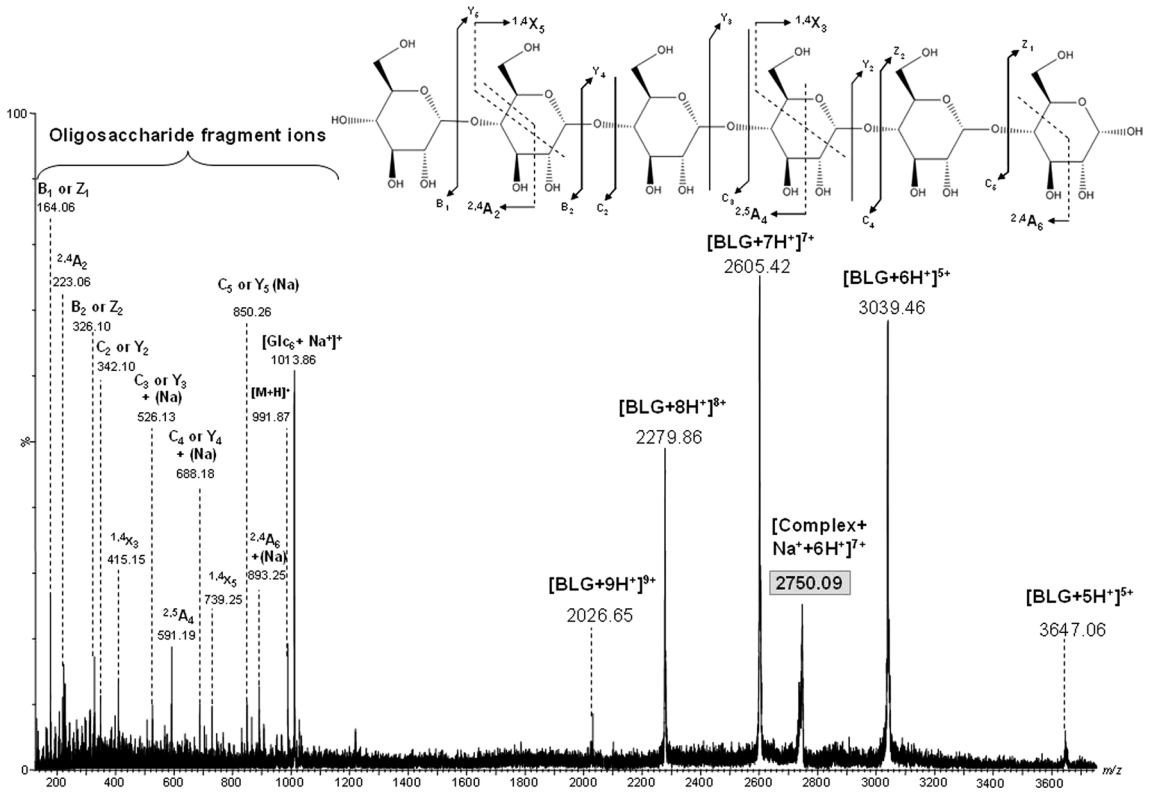


**Fig. 5** Top-down by (+) chip-nanoESI QTOF CID MS/MS of the [complex + $Na^+$+6$H^+$]$^{7+}$ detected at *m/z* 2750.09, corresponding to BLG–$Glc_6$ noncovalent complex. Variable collision energy, within (40–100) eV; acquisition time, 5 min; other MS conditions are as in Fig. 1. *Inset* ion fragmentation scheme of $Glc_6$ in top-down experiment

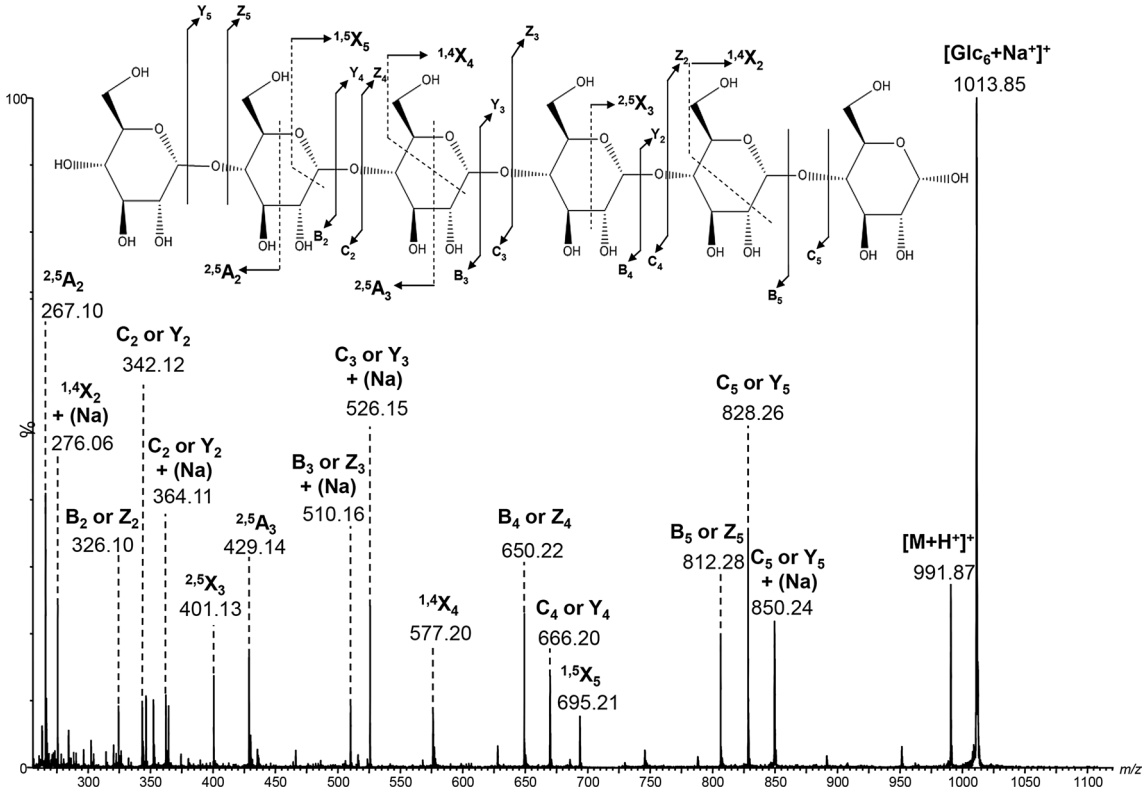


**Fig. 6** (+) Chip-nanoESI QTOF CID MS/MS of $[Glc_6 + Na^+]^+$ detected at *m/z* 1013.85 by MS screening of $Glc_6$ in buffer. Concentration, 10 pmol $\mu L^{-1}$ in 10 mM ammonium acetate/formic acid, pH 6.0. NanoESI voltage, 1.8 kV; cone voltage, 60 V; variable collision energy, within (40–60) eV; acquisition time, 2 min. *Inset* ion fragmentation scheme of $Glc_6$ in buffer

four cases, chip-nanoESI MS analysis revealed solely the characteristic BLG envelope with 5+ to 9+ charge states of the molecular ions. For this reason, the interaction assay was further monitored. In Fig. 7, the spectrum of the reaction products collected after 50 min of incubation is presented. Besides the BLG envelope, the spectrum in Fig. 7 exhibits a signal at *m/z* 2750.12 which, according to calculation, corresponds to an Mr of 19,243.84 Da. This Mr value is attributable to the noncovalent complex of BLG with the sodiated form of $Glc_6$. In view of this characteristic of the noncovalent interaction carried out in water, aliquots were further collected up to 3 h with a 30 min time interval between collections. The MS analyses of all these samples did not show any modifications.

## Discussions

Earlier reports (Ding et al. 2011; Sawyer and Kontopidis 2000; Zsila et al. 2002) have discussed the occurrence of two BLG isomers in cow's milk: isomer A of 18,363 Da and isomer B, of 18,276 Da, corresponding to 162 amino acids. These Mr values were determined by several methods such as ESI MS, capillary zone electrophoresis and high-performance liquid chromatography (HPLC)-MS. According to the calculation based on the amino acid sequence, BLG has a molecular mass of approximately 18,400 Da (Rachagani et al. 2006; Ohtomo et al. 2011). A variant having (18,230.50 ± 0.2) Da has never been reported before. Hence, we consider that the protein eliminated in human milk undergoes structural modifications. This aspect offers interesting perspectives for further research, aiming at determination of such alterations by ESI MS/MS in either bottom-up or top-down approaches.

Another interesting outcome of the interaction assays is that, according to the obtained data, the BLG–$Glc_6$ noncovalent binding in ammonium acetate/formic acid is a fast process, as compared to other protein–oligosaccharide interactions studied in the same buffer (Cederkvist et al. 2006). In ammonium acetate, BLG–$Glc_6$ noncovalent complex was formed and detected by MS already after 1 min of incubation. The mass spectra of the reaction products collected every 5 min over half an hour revealed a significant increase in the abundance of the corresponding molecular ion. Therefore, employment of fully automated chip-nanoESI, which allowed working in a high-throughput mode, was crucial for the assessment of the interaction

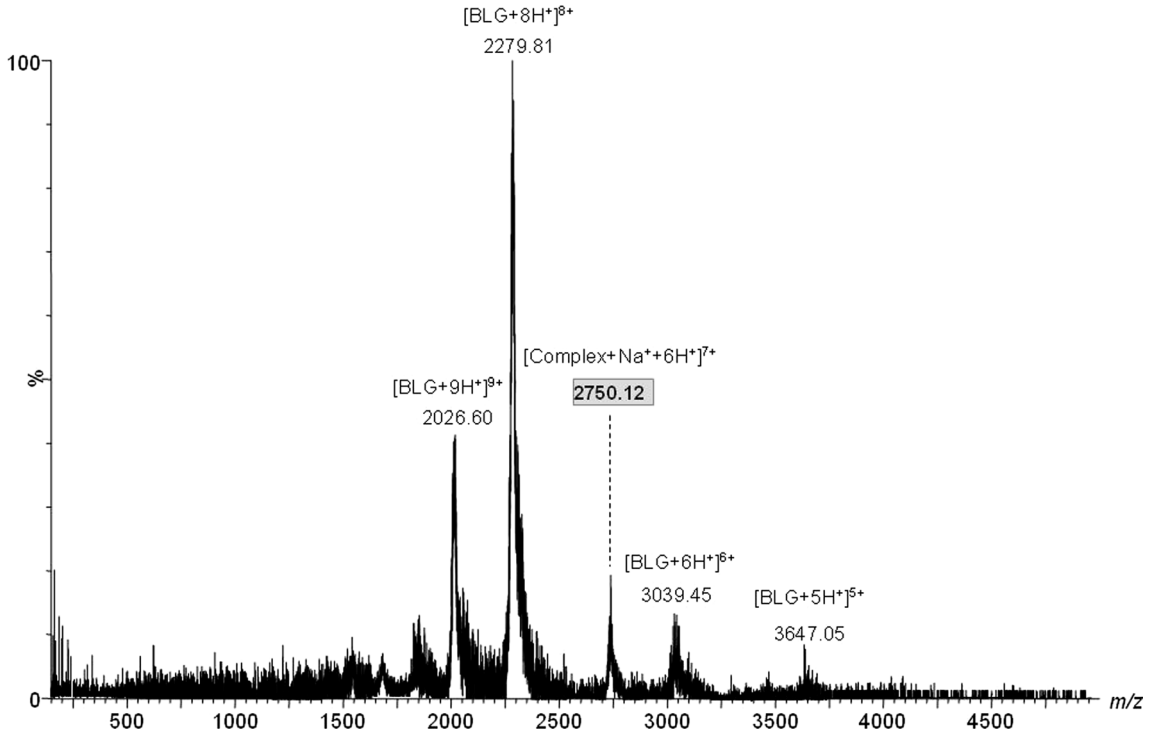


**Fig. 7** (+) Chip-nanoESI QTOF MS of BLG–$Glc_6$ reaction products after 50 min incubation in pure water at 37 °C. Concentration, 5 pmol $\mu L^{-1}$ in pure water (pH 7.0); nanoESI voltage, 1.9 kV; cone voltage, 60 V; nitrogen back pressure, 0.40 psi; nitrogen nebulizer presure, 60 psi; acquisition time, 2 min

products almost in real time, without jeopardizing the reaction conditions.

In a preceding study (Cederkvist et al. 2006) on protein–oligosaccharide interaction assay, using capillary-based nanoESI QTOF MS, 2850 scans, equivalent to almost 100 min, were accumulated for fragmentation analysis by CID MS/MS. In the present work, in view of the high sensitivity and ionization efficiency exhibited by chip-nanoESI, 2 min for a screening mass spectrum and 5 min for the top-down experiment were sufficient for a complete characterization of the noncovalent complex. Such a reduced sample consumption and high speed of analysis were critical requirements for our study, since the investigated protein is present in human milk in a very low amount.

To mimic the in vivo environment and assess whether the complex might be formed and detected by ESI MS under such conditions, in the second phase of the research, the ammonium acetate buffer was replaced by pure water. Remarkably, the formed complex could be detected by ESI MS under pH 7.0. However, since the concentration of the interacting partners and the incubation conditions were the same as in the buffer, in relation to the obtained data using pure water, we may conclude the following: (1) as expected, human milk BLG and $Glc_6$ present a lower level of ionizability. As a consequence, in the spectrum in Fig. 7, not all BLG charge states visible in buffer were formed. Additionally, $Glc_6$ ions are not even detectable; (2) a lower signal-to-noise ratio was obtained in pure water, as compared to ammonium acetate buffer; (3) the process of noncovalent complex formation is much slower in water. The complex was detected only after 50 min of reaction, but remained stable over several hours; (4) the ions corresponding to BLG–$Glc_6$ present a rather low intensity, which made the top-down experiment results less reliable. This feature could be the consequence of either the reduced ionization, the low concentration of the formed complex in water or both.

According to previous results (Boyce et al. 2010), even traces of BLG in human milk trigger allergy in some infants. It was also shown that some oligosaccharide classes (Yoshida et al. 2005) determine the reduction of BLG allergenicity. From this point of view, the outcome of the present work has an interesting biological and clinical connotation. Our data suggest that, by forming a noncovalent complex with BLG under conditions mimicking the in vivo parameters, $Glc_6$ might be among the glycans, which contribute to the reduction of BLG allergenicity. On the basis of these findings, clinical trials might be conducted for testing the allergenicity level of the BLG–$Glc_6$ complex.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflicts of interest.

**Ethical standards** This study has been approved by the Ethics Commission of Victor Babes University of Medicine and Pharmacy, Timisoara. Informed consent was obtained from all individual participants included in this research. All procedures on the human participants were in agreement with the ethical standards of Victor Babes University of Medicine and Pharmacy, Timisoara, National Authority for Scientific Research (ANCS, Romania) and with the 1964 Helsinki declaration and its later amendments.

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