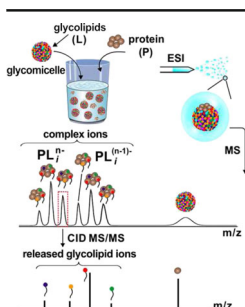


RESEARCH ARTICLE

Detecting Protein–Glycolipid Interactions Using Glycomicelles and CaR-ESI-MS

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Abstract. This study reports on the use of the catch-and-release electrospray ionization mass spectrometry (CaR-ESI-MS) assay, combined with glycomicelles, as a method for detecting specific interactions between water-soluble proteins and glycolipids (GLs) in aqueous solution. The B subunit homopentamers of cholera toxin (CTB₅) and Shiga toxin type 1 B (Stx1B₅) and the gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2 served as model systems for this study. The CTB₅ exhibits broad specificity for gangliosides and binds to GM1, GM2, GM3, GD1a, GD1b, and GT1b; Stx1B₅ does not recognize gangliosides. The CaR-ESI-MS assay was used to analyze solutions of CTB₅ or Stx1B₅ and individual gangliosides (GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2) or mixtures thereof. The high affinity

interaction of CTB₅ with GM1 was successfully detected. However, the apparent affinity, as determined from the mass spectra, is significantly lower than that of the corresponding pentasaccharide or when GM1 is presented in model membranes such as nanodiscs. Interactions between CTB₅ and the low affinity gangliosides GD1a, GD1b, and GT1b, as well as GD2, which served as a negative control, were detected; no binding of CTB₅ to GM2 or GM3 was observed. The CaR-ESI-MS results obtained for Stx1B₅ reveal that nonspecific protein-ganglioside binding can occur during the ESI process, although the extent of binding varies between gangliosides. Consequently, interactions detected for CTB₅ with GD1a, GD1b, and GT1b are likely nonspecific in origin. Taken together, these results reveal that the CaR-ESI-MS/glycomicelle approach for detecting protein–GL interactions is prone to false positives and false negatives and must be used with caution.

Keywords: Protein, Glycolipid, Interactions, Affinity, Micelles, Mass spectrometry, Electrospray ionization

Received: 6 April 2016/Revised: 16 June 2016/Accepted: 24 June 2016/Published Online: 22 August 2016

Introduction

Cell-surface glycolipids (GLs) are involved in a number of critical cellular processes, including recognition and adhesion, pathogen infection, signal transduction, trafficking, and immune response [1–3]. Glycolipids are amphipathic molecules consisting of a hydrophobic lipid moiety, which inserts into the cell membrane, and a hydrophilic mono-, oligo-, or polysaccharide head group that is exposed to the aqueous environment. Glycolipids are readily immobilized on hydrophobic surfaces and, thus, their interactions with water-soluble proteins can be studied using enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) spectroscopy,

and thin layer chromatography (TLC) [4–6]. In addition, microarrays prepared using naturally-occurring GLs or synthetic GLs (neoGLs), which enable GL-based glycan screening, have been successfully used for the discovery of protein–GL interactions [7–9]. However, a shortcoming of these methods is the non-native environment of the GLs, which could influence the nature of protein–GL interactions. An alternative approach is to incorporate the GLs in a lipid monolayer or bilayer, such that the protein–GL interactions can be studied in a more native-like environment [10]. For such studies, a variety of different model membranes have been used to solubilize the GLs, including supported lipid bilayers, liposomes, micelles, bicelles, nanodiscs, and picodiscs [11–14], and the protein–GL interactions probed using diverse analytical techniques (e.g., fluorescence, nuclear magnetic resonance (NMR), and SPR spectroscopy) [15–18].

Recently, electrospray ionization mass spectrometry (ESI-MS) has emerged as a promising method for the studying of protein–GL interactions in aqueous solution. Interactions

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1461-6) contains supplementary material, which is available to authorized users.

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between water-soluble lectins and GLs, solubilized using nanodiscs (NDs), have been detected using the catch-and-release (CaR)-ESI-MS assay [19,20]. Nanodiscs are discoidal phospholipid bilayers surrounded by two copies of an amphipathic membrane scaffold protein [12,13]. It was also shown that NDs can serve as GL arrays and be combined with the CaR-ESI-MS assay to rapidly screen mixtures of GLs (defined or natural libraries) against target proteins [21]. The successful detection of both high and low affinity protein–GL interactions using this approach has been reported [21]. The CaR-ESI-MS assay has also been combined with picodiscs (PDs) [14], which are smaller lipid-transporting macromolecular complexes composed of the human sphingolipid activator protein, saposin A (SapA), and phospholipids, for the detection of protein–GL complexes [22,23]. More recently, Zamfir and coworkers reported on the detection of interactions between proteins and gangliosides (which are glycosphingolipids that contain sialic acid) in aqueous solution using direct ESI-MS analysis [24]. Using this approach, in which the gangliosides presumably form GL micelles (glycomicelles) in solution, the authors identified the interactions between B subunits of cholera toxin (CTB) and GM1, GD1, GT1, GQ1, GP1, as well as the fucosylated GD1, GT1, and GQ1 [24]. However, it is notable that these measurements were carried out at acidic pH (5.8), conditions under which the native homopentameric structure of CTB (i.e., CTB₅) disassembles into individual subunits. Given that the ganglioside binding pocket, as identified from the X-ray crystal structure [25], is comprised of residues from adjacent B subunits, the nature of the interactions involving a single subunit is unclear.

Given the versatility and ease of implementation, the ESI-MS approach, performed directly on aqueous solutions of protein and GL, for detecting protein–GL interactions is very attractive. The goal of the present study was to more thoroughly investigate the reliability of using ESI-MS (and CaR-ESI-MS) and glycomicelles to detect protein–GL interactions. The B subunit homopentamers of cholera toxin (CTB₅) and Shiga toxin type 1 (Stx1B₅) and seven gangliosides (GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2) served as model systems for this study. CTB₅ exhibits broad specificity for gangliosides; Stx1B₅ is known to bind globosides (neutral glycosphingolipids) such as Gb3 and Gb4, but does not recognize gangliosides [26,27]. The CaR-ESI-MS assay, which is outlined in Figure 1, was used to analyze aqueous solutions of CTB₅ or Stx1B₅ and individual gangliosides (GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2) or mixtures thereof. With the exception of GM3, which does not form micelles [28,29], the concentrations of gangliosides were above the critical micelle concentration [29,30]. The ganglioside interactions identified for CTB₅ and Stx1B₅ by CaR-ESI-MS, together with binding data acquired for CTB₅ and gangliosides solubilized in NDs or PDs, as well as affinity data measured for CTB₅ and the ganglioside oligosaccharides, were used to assess the reliability of using ESI-MS (and CaR-ESI-MS) and glycomicelles for detecting protein–GL interactions in aqueous solutions.

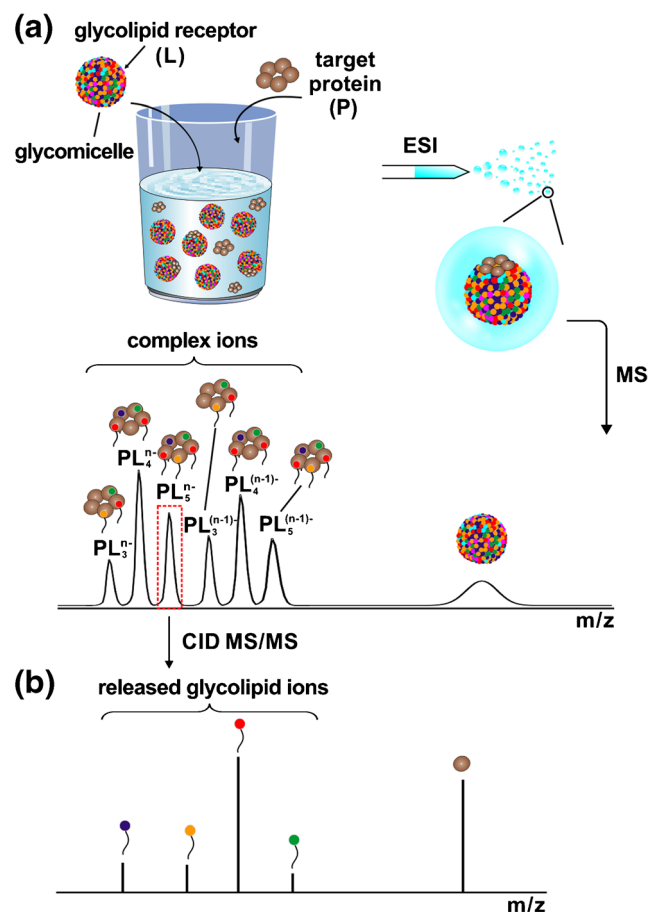


Figure 1. Schematic representation of the catch-and-release (CaR)-ESI-MS assay for detecting protein-glycolipid interactions using glycomicelles. **(a)** The soluble carbohydrate-binding protein (P, shown as a homopentameric species) is incubated in aqueous solution with glycomicelles consisting of one or more glycolipid species (L) and analyzed by ESI-MS in negative ion mode. **(b)** Identification of glycolipid ligands is achieved by subjecting the gaseous protein-glycolipid (PL_n) complexes ions to CID and measuring the MWs of released ligand ions

Experimental

Materials and Methods

Proteins Cholera toxin B subunit homopentamer (CTB₅, MW 58,040 Da) was purchased from Sigma-Aldrich Canada (Oakville, Canada). Shiga toxin type 1 B subunit homopentamer (Stx1B₅, MW 38,455 Da) was a gift from Professor G. Armstrong (University of Calgary). A single chain variable fragment (scFv, MW 26,539 Da) of the monoclonal antibody Se155-4, which served as a reference protein (P_{ref}) [31,32] for direct ESI-MS binding measurements, was produced using recombinant technology as described elsewhere [33]. To prepare stock solutions of CTB₅ and Stx1B₅, each protein was dialyzed against 200 mM ammonium acetate (pH 6.8) using 0.5 mL Amicon microconcentrators (EMD Millipore, Billerica, MA, USA) with a 30 kDa MW cutoff. A similar procedure, using microconcentrators with a 10 kDa MW cutoff, was applied to scFv. The concentration of CTB₅

stock solution was determined using a Pierce BCA assay kit (Thermo Scientific, Ottawa, Canada) following the manufacturer's instruction; the concentrations of the Stx1B₅ and scFv solutions were estimated by UV absorption (280 nm). All protein stock solutions were kept at 4 °C until used.

Gangliosides The gangliosides β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)- β -D-Glc-ceramide (GM1, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 1545.88 Da, 1573.91 Da), β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)- β -D-Glc-ceramide (GM2, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 1383.82 Da, 1411.86 Da) and α -D-Neu5Ac-(2,3)- β -D-Gal-(1,4)- β -D-Glc-ceramide (GM3, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 1180.74 Da, 1208.78 Da) were purchased from Cedarlane Labs (Burlington, Canada); α -D-Neu5Ac-(2,3)- β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)- β -D-Glc-ceramide (GD1a, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 1836.97 Da, 1865.00 Da), β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,8)- α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)- β -D-Glc-ceramide (GD1b, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 1836.97 Da, 1865.00 Da) and α -Neu5Ac-(2,3)- β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -Neu5Ac-(2,8)- α -Neu5Ac-(2,3)]- β -D-Gal-(1,4)- β -D-Glc-ceramide (GT1b, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 2128.07 Da, 2156.10 Da) were purchased from Sigma-Aldrich Canada (Oakville, Canada); and β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,8)- α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)- β -D-Glc-ceramide (GD2, major isoforms *d*18:1–18:0 and *d*20:1–18:0 have MWs 1674.92 Da, 1702.95 Da) was purchased from MyBioSource Inc. (San Diego, CA, USA). The structures of the gangliosides are given in Figure S1 (Supporting Information). Stock solutions (2 mM) of each ganglioside in HPLC grade methanol/chloroform (1:1, v/v, Thermo Fisher, Ottawa, Canada) were prepared and stored at –20 °C until needed.

Oligosaccharides

The ganglioside oligosaccharides β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)-D-Glc (GM1_{os}, MW 998.34 Da); β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)-D-Glc (GM2_{os}, MW 836.29 Da); α -D-Neu5Ac-(2,3)- β -D-Gal-(1,4)-D-Glc (GM3_{os}, MW 633.21 Da); α -D-Neu5Ac-(2,3)- β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)-D-Glc (GD1a_{os}, MW 1289.44 Da); β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,8)- α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)-D-Glc (GD1b_{os}, MW 1289.44 Da); β -D-GalNAc-(1,4)-[α -D-Neu5Ac-(2,8)- α -D-Neu5Ac-(2,3)]- β -D-Gal-(1,4)-D-Glc (GD2_{os}, MW 1127.39 Da); and α -Neu5Ac-(2,3)- β -D-Gal-(1,3)- β -D-GalNAc-(1,4)-[α -Neu5Ac-(2,8)- α -Neu5Ac-(2,3)]- β -D-Gal-(1,4)-D-Glc (GT1b_{os}, MW 1580.53 Da) were purchased from Elicityl SA (Crolles, France). The structures of the oligosaccharides are shown in Figure S2 (Supporting Information). Stock solutions [1 mM in Milli-Q

water (Millipore, MA, USA)] of each of the oligosaccharides were stored at –20 °C until needed.

Preparation of Glycolipid Micelles

To prepare micellar solutions, the ganglioside (or ganglioside mixture) was diluted in 1:1 methanol/chloroform and dried under gentle stream of nitrogen to form a lipid film. The dried lipid film was stored at room temperature overnight. Subsequently, the lipid film was resuspended in 200 mM aqueous ammonium acetate solution (pH 6.8, 25 °C) by vortexing for 5 min, followed by 30 min of sonication [18]. The resulting solution was stored at room temperature until used.

Mass Spectrometry

ESI-MS measurements were carried out using a Waters Synapt G2S quadrupole-ion mobility separation-time of flight (Q-IMS-TOF) mass spectrometer (Manchester, UK) equipped with a nanoflow ESI (nanoESI) source. Sample solutions were prepared in 200 mM aqueous ammonium acetate buffer (pH 6.8, 25 °C) and each solution was loaded into a nanoESI tip, which was produced by pulling the borosilicate capillaries (1.0 mm o.d., 0.68 mm i.d.) to ~5 μ m using a P-1000 micropipette puller (Sutter Instruments, Novato, CA, USA). To perform nanoESI, a voltage of –0.8 kV (negative ion mode) or 1.0 kV (positive ion mode) was applied to a platinum wire inserted into the nanoESI tip. For the ESI-MS measurements, the source temperature was 60 °C, the cone voltage was 50 V (negative ion mode) or 35 V (positive ion mode), and the Trap and Transfer voltages were 5 and 2 V, respectively. For the CaR-ESI-MS measurements, the quadrupole mass filter was set to pass a window of ions corresponding to the complexes of interest (using quadrupole parameters of LM = 8, HM = 15, window width ~50 *m/z* units; LM = 4, HM = 15, window width ~100 *m/z* units; or LM = 2, HM = 15, window width ~200 *m/z* units). Collision-induced dissociation (CID) was performed in the Trap using argon (2.15 \times 10^{–2} mbar) and 100 V collision energy. All data were processed using MassLynx software (ver. 4.1).

The abundances of free and GL-bound protein ions were calculated from the ESI mass spectra using peak heights (intensities), with no background subtraction performed. Details of the procedure used to calculate the normalized distributions of free and GL-bound proteins have been reported previously [20]. The quantitative binding measurements performed on Stx1B₅ and the ganglioside oligosaccharides were carried out using the direct ESI-MS assay. A detailed description of the method can be found elsewhere [31,32]. For these measurements, a P_{ref} was added to the solutions in order to quantitatively correct the mass spectra for the occurrence of nonspecific protein-carbohydrate binding during the ESI process. A description of the correction method and its implementation is reported elsewhere [31,32].

Results and Discussion

In the present study, the interactions of CTB₅ and Stx1B₅ with seven different gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD2, and GT1b), either alone or present as an equimolar mixture in aqueous solution, were studied using CaR-ESI-MS. With the exception of GM3 (which forms large vesicles at concentrations >3 nM) [28,29], each of the gangliosides is expected to form micelles in aqueous solution at the concentrations used. The critical micelle concentrations (CMC) of GM1, GM2, GD1a, GD1b, and GT1b are reported as: 20 nM, 11 nM, 2 μM, 1 μM, and 10 μM, respectively [29,30]. To our knowledge, the CMC for GD2 has not been reported. However, given the structural similarity between GD2 and GD1a/b, the CMC of GD2 is likely 1–2 μM.

Shown in Figure S3 (Supporting Information) are representative ESI mass spectra acquired in negative ion mode for aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of 400 μM of GM1, GM2, GD1a, GD1b, GT1b, or GD2. In each case, a broad feature, centered at m/z 7000–10,000, is evident. This feature, which is qualitatively similar to the results obtained by ESI-MS analysis on the detergent micelles, is attributed to the ganglioside micelle ions [34–36]. The ESI mass spectrum acquired for GM3 also exhibits a broad feature, centered at m/z 13,000–16,000, although with lower abundance (Figure S3m, Supporting Information). This finding is consistent with the fact that GM3 tends to form large vesicles [28,29]. To confirm the presence of each ganglioside, CID was performed on ions with a range of m/z values (window width ~200 m/z units) centered at an m/z corresponding to the most abundant micellar ions. In each case, signal corresponding to the deprotonated ions of each of the gangliosides was detected (Figure S3b, d, f, h, j, l, and n, Supporting Information).

The high affinity interactions between CTB₅ and GM1 served as a starting point for testing the reliability of the CaR-ESI-MS assay, implemented with glycomicelles, for detecting protein interactions with GLs in vitro. Measurements were performed on aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of CTB₅ (3 μM) and GM1, at concentrations ranging from 20 to 360 μM. Shown in Figure 2 are representative ESI mass spectra measured for three GM1 concentrations, 20, 80, and 360 μM. At lowest concentration investigated (20 μM), only signal corresponding to free CTB₅ ions (i.e., CTB₅^{*n*-} at $n = 13–16$) was evident in the mass spectrum (Figure 2a). Moreover, there was no obvious signal that could be attributed to GM1 micelle ions. CID performed using a ~50 m/z window centered at m/z 4587, which corresponds to the –13 charge state of the putative (CTB₅ + GM1) complex, resulted in the appearance of B subunit monomer ions (at charge states –5 and –6) and GM1 ions, albeit at very low abundance (Figure 2b). Analogous CID measurements performed using identical experimental conditions, but in the absence of CTB₅, failed to produce any detectable signal corresponding to GM1 (Figure S4, Supporting Information). Taken together, these results indicate that CTB₅–GM1 interactions exist in solution, but are presumably at very low

concentration. At higher concentrations of GM1 (Figure 2c and e), signal consistent with complexes of CTB₅ bound to one or more GM1 molecules was detected, i.e., (CTB₅ + q GM1)^{*n*-} with $q = 2–5$ and $n = 13–16$ (Table S1, Supporting Information), and the number of bound GM1 increased with GM1 concentration. The presence of bound GM1 was also confirmed by CID, performed using conditions identical to those described above (Figure 2d and f). It can also be seen that signal corresponding to GM1 micelle ions becomes more abundant with increasing ganglioside concentration (Figure 2e).

The normalized distributions of (CTB₅ + q GM1) species determined from the mass spectra and the corresponding distributions expected for the GM1 pentasaccharide (GM1_{os}), which were calculated based on the reported apparent affinities for the stepwise binding of GM1_{os} to CTB₅, at the same concentration as GM1 are shown in Figure 2a, c, and e [37,38]. Notably, at 20 μM GM1_{os}, CTB₅ is expected to be almost fully bound, whereas CTB₅ appears to exist almost exclusively in the unbound form at 20 μM GM1 micelle (Figure 2a). At GM1_{os} concentrations >30 μM, CTB₅ is essentially fully bound; in contrast, a significant fraction of the binding sites remain unoccupied even at GM1 concentrations as high as >80 μM (Figure 2c). The effect of concentration is, perhaps, more clearly seen in Figure 2g, where the fraction (f) of occupied CTB₅ binding sites is plotted versus GM1 concentration. Also shown is the fraction of occupied binding sites expected for GM1_{os} at concentrations between 0 and 370 μM and the fraction of bound CTB₅ measured experimentally using NDs to solubilize GM1 [20]. It can be seen that f appears to reach a limiting value of ~0.85 at GM1 concentrations >200 μM, which contrasts with an f of 0.99 at 30 μM GM1_{os}. It is also important to note that the significantly higher concentrations of GM1 are required to achieve near saturation (of the binding sites) compared with the case when GM1-containing NDs are used, wherein an f of ~0.9 is reached at GM1 concentrations of ~30 μM [20].

To confirm that nonspecific binding between CTB₅ and GM1 during the ESI process (due to concentration effects in the droplets) does not contribute appreciably to the measured distributions of (CTB₅ + q GM1) species, analogous CaR-ESI-MS measurements were performed using Stx1B₅, which, to the best of our knowledge, does not bind to gangliosides. To further support the use of Stx1B₅ as a negative control, binding measurements were carried on the GM1 pentasaccharide (GM1_{os}), as well as the oligosaccharides of the six other gangliosides using the direct ESI-MS assay in positive ion mode (Figure S5, Supporting Information). Notably, no interactions between Stx1B₅ and GM1_{os} or to the other six oligosaccharides were detected. Application of CaR-ESI-MS to an aqueous ammonium acetate solution (200 mM, pH 6.8, 25 °C) of Stx1B₅ (3 μM) with GM1 (320 μM) produced no evidence of the presence of (Stx1B₅ + q GM1) complexes, either directly (Figure S6a, Supporting Information) or by release of bound ganglioside by CID (Figure S6b, Supporting Information). These results suggest that nonspecific binding of CTB₅ and

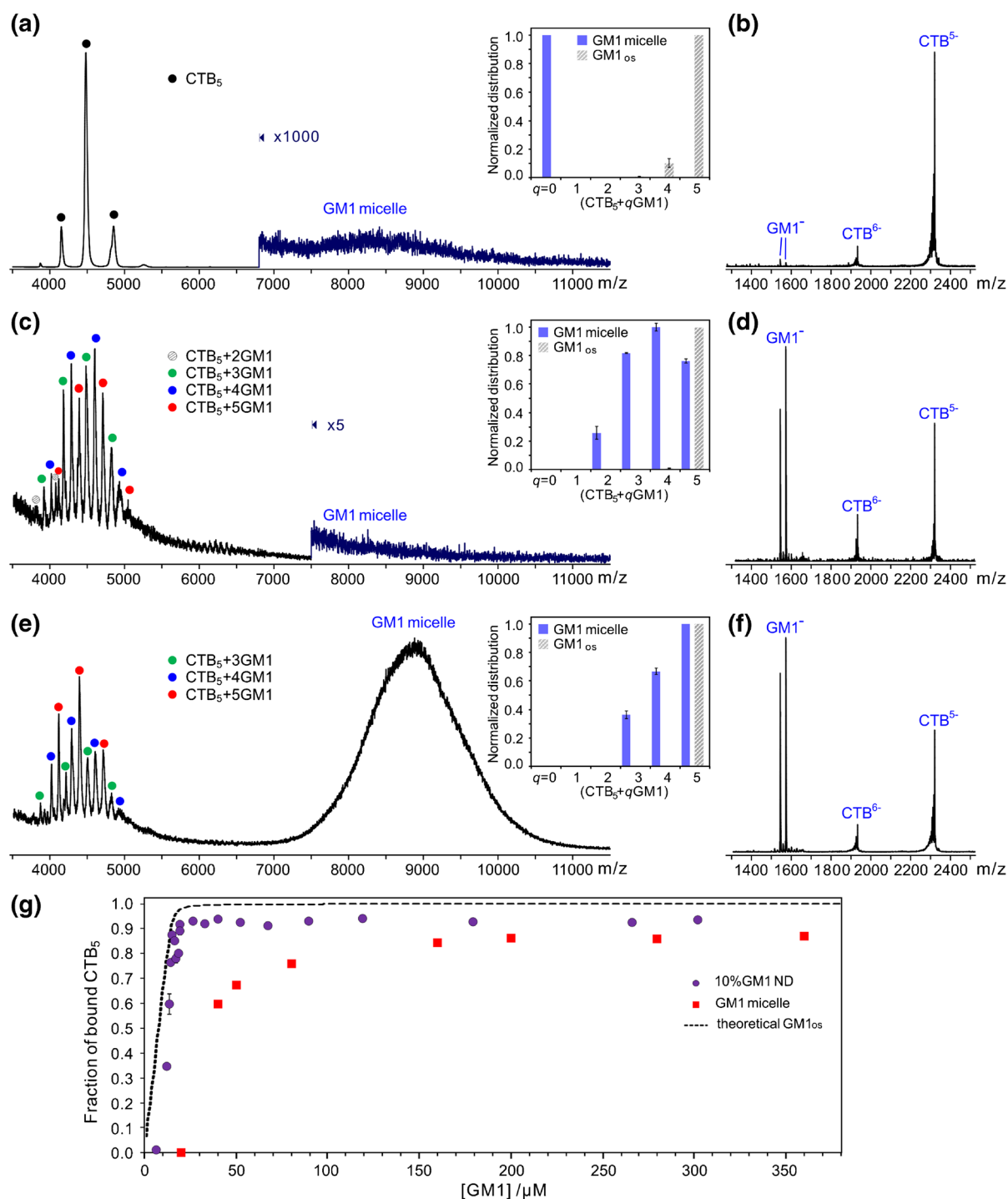


Figure 2. ESI mass spectra acquired in negative ion mode for aqueous ammonium acetate solutions (200 mM, 25 °C and pH 6.8) of CTB₅ (3 μM) with GM1 at concentrations of (a) 20 μM, (c) 80 μM, and (e) 360 μM. Insets show the normalized distributions of free and GM1-bound CTB₅; the errors correspond to one standard deviation. Also shown are the distributions of bound GM1_{os} expected based on the association constants reported in reference [37]; the errors were calculated from propagation of uncertainties in the reported association constants. (b), (d), and (f) CID mass spectra measured for ions produced by ESI for the solutions described in (a), (c), and (e), respectively. For (b), ions within a window of m/z values (~50 m/z units wide) centered at m/z 4587 (which corresponds to the -13 charge state of CTB₅ bound to one ganglioside) were isolated; for (d) and (f) ions centered at m/z 4395 (which corresponds to the -15 charge state of CTB₅ bound to five gangliosides) were isolated; CID was performed in the Trap using a collision energy of 100 V. (g) Plot of fraction of occupied CTB₅ binding sites versus GM1 concentration, as measured by ESI-MS. The 10% GM1 ND binding data were adapted from reference [20]. The dashed line represents the theoretical plot calculated using affinities for the stepwise binding of GM1_{os} to CTB₅ reported in reference [37]

GM1 during the ESI process does not contribute in a meaningful way to the mass spectrum. Based on these findings, it is concluded that the (CTB₅ + *q*GM1) complexes detected by ESI-MS are the result of specific interactions between CTB₅ and GM1 micelles in solution. Moreover, the measured distributions of (CTB₅ + *q*GM1) species suggest that the affinity of GM1, when present as a glycomicelle, for CTB₅ is significantly lower than when GM1 is presented in a ND [20]. Although the origin of the reduced affinity cannot be elucidated based on the present experimental data, it is reasonable to conclude that it arises from differences in the oligosaccharide environment when presented in micelles and NDs.

The results obtained for CTB₅ and GM1 demonstrate that high affinity protein–GL interactions can be detected directly by ESI-MS performed on solutions containing lectin and GL. However, significantly higher GL concentrations are required in order to achieve the same extent of binding compared with the corresponding GL oligosaccharide or when using NDs (or PDs) to solubilize the GLs [20,22,37]. The next step was to establish whether low affinity protein–GL interactions could be detected. To answer this question, the CaR-ESI-MS assay was applied to aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of CTB₅ (3 μM) and GM2, GM3, GD1a, GD1b, or GT1b. The affinities measured for the oligosaccharides GM2_{os}, GM3_{os}, GD1a_{os}, GD1b_{os}, and GT1b_{os} are approximately three orders of magnitude lower than for the GM1 pentasaccharide [23]. Shown in Figures S7 and S8 (Supporting Information) are representative

mass spectra measured for solutions containing CTB₅ with GM2 and GM3, respectively. Surprisingly, no interaction between CTB₅ and either of these gangliosides was detected, even at concentrations as high as 360 μM ganglioside. In contrast, binding of CTB₅ to GD1a, GD1b, and to GT1b was readily detected by CaR-ESI-MS and the measured distributions of free and bound CTB₅ are similar to those predicted, based on the reported affinities, for the corresponding oligosaccharides (Figures S9–S11, Supporting Information) [23].

Measurements were also performed on solutions containing CTB₅ with GD2, which served as a negative control. Direct ESI-MS binding measurements carried out on the GD2 pentasaccharide (GD2_{os}) and CTB₅ revealed no evidence of binding [23]. The results of an SPR spectroscopy binding study also suggest that CTB₅ does not bind to GD2 liposomes [39]. Moreover, application of the CaR-ESI-MS assay to solutions of CTB₅ and GD2, incorporated into NDs or PDs, failed to identify any binding [21,23]. Shown in Figure S12 (Supporting Information) are representative CaR-ESI-MS data acquired for aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of CTB₅ (3 μM) and GD2 at 80 and 200 μM. Unexpectedly, signal corresponding to the (CTB₅ + GD2) complex is clearly evident in both ESI mass spectra (Figures S12a and S12c, Supporting Information). Collision-induced dissociation performed on the ions of the (CTB₅ + GD2) complex conclusively established the presence of bound GD2 (Figures S12b and S12d, Supporting Information).

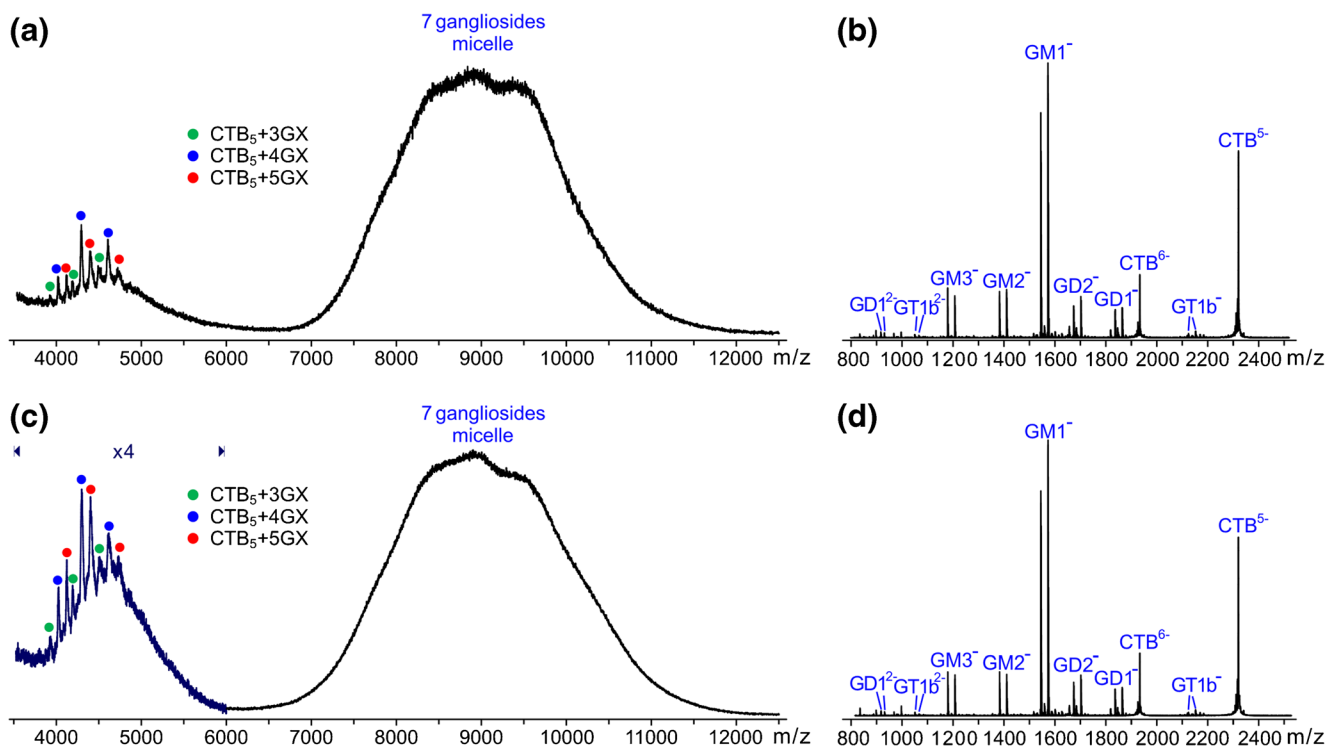


Figure 3. (a) and (c) ESI mass spectra acquired for aqueous ammonium acetate solutions (200 mM, 25 °C and pH 6.8) of CTB₅ (3 μM) and a mixture of GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2, each a concentration of (a) 80 μM and (c) 150 μM. GX represents any of the seven gangliosides. (b) and (d) CID mass spectra measured for ions produced by ESI for the solutions described in (a) and (c), respectively. Ions within a window of *m/z* values (~100 *m/z* units wide) centered at 4400 (which corresponds to the -15 charge state of CTB₅ bound to five gangliosides) were isolated and subjected to CID in the Trap using a collision energy of 100 V

The results of the CaR-ESI-MS measurements performed on solutions of CTB₅ and seven gangliosides are intriguing and, seemingly, contradictory. The absence of detectable binding to the low affinity ligands, GM2 and GM3, could be explained, in principle, by a reduced affinity resulting from the micellar presentation of these gangliosides. This explanation finds support in the results obtained for GM1, *vide supra*. However, the finding that GD1a, GD1b, and GT1b exhibit affinities that are apparently similar to those of the corresponding oligosaccharides is at odds with this general explanation. Moreover, the detection of CTB₅ binding to GD2, which is a negative control, adds further confusion to the situation.

In an effort to make sense of these observations, CaR-ESI-MS measurements were performed on aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of Stx1B₅ (3 μM) and each of the six gangliosides—GM2, GM3, GD1a, GD1b, GT1b, and GD2 (at 320 μM). As expected, no binding of Stx1B₅ to GM2 (Figure S13, Supporting Information) or GM3 (Figure S14, Supporting Information) was observed. However, binding was detected for GD1a, GD1b, GT1b, and GD2 (Figures S15–S18, Supporting Information). To facilitate comparison of these results with those obtained for CTB₅, the normalized distributions of ligand (ganglioside or ganglioside oligosaccharide)-bound CTB₅ and Stx1B₅ measured for each ganglioside or ganglioside oligosaccharide are given in Figure S19 (Supporting Information).

As discussed above, Stx1B₅ exhibits no measurable affinity for the oligosaccharides of these gangliosides. Consequently, the identified interactions are likely formed during the ESI

process as a result of nonspecific interactions. The absence of nonspecific binding observed for GM2 and GM3 (as well as GM1, *vide supra*) is likely due to the very low CMC for these gangliosides [29], which presumably translates to a very low concentration of free ganglioside in solution. It is also possible that in-source (*i.e.*, gas-phase) dissociation may be responsible for the absence of observed binding for these protein–ganglioside complexes. The CaR-ESI-MS results obtained for Stx1B₅ and GD2 also provide a possible explanation for the observation of CTB₅ binding to GD2. The occurrence of nonspecific binding could also explain the unexpected distributions of bound GD1a, GD1b, and GT1b observed for CTB₅.

The aforementioned results suggest that the environment of ganglioside oligosaccharides in glycomicelles influences lectin binding in solution. To further probe this phenomenon, the CaR-ESI-MS assay was applied to solutions containing CTB₅ and all seven of the gangliosides. Shown in Figure 3 are ESI mass spectra acquired for aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of CTB₅ (3 μM) with GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2, each at 80 μM (Figure 3a) or 150 μM (Figure 3c) concentrations. The broad feature centered at ~9000 *m/z* is attributed to ganglioside micelle ions (Figure S20a, Supporting Information); CID of these ions produced signal corresponding to the anions of GM1, GM2, GM3, GT1b, and GD2, as well as GD1 (Figure S20b, Supporting Information). Because GD1a and GD1b are structural isomers, the presence of both gangliosides could not be established simply from the CID mass spectrum. Also identified in the mass spectrum are ions corresponding to (CTB₅ + *q*GM1)^{*n*-} with *q* = 3–5 and *n* = 14–16

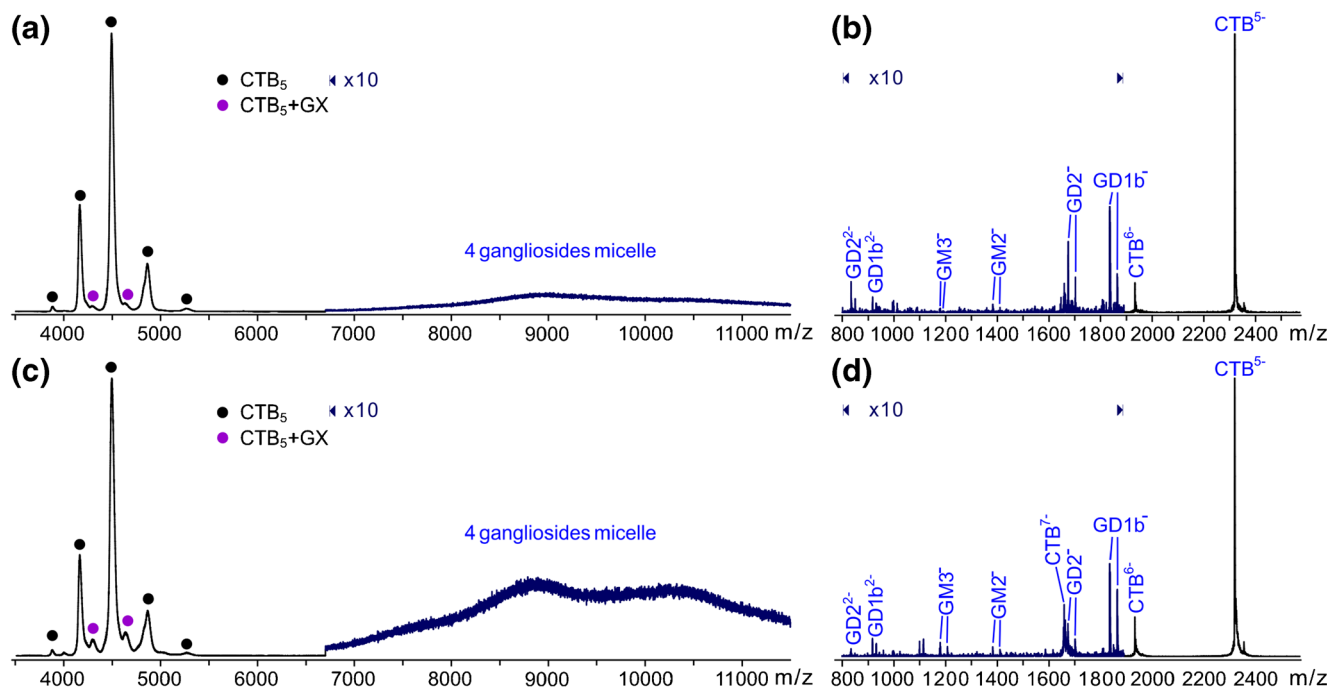


Figure 4. (a) and (c) ESI mass spectra acquired for aqueous ammonium acetate solutions (200 mM, 25 °C and pH 6.8) of CTB₅ (3 μM) and a mixture of GM2, GM3, GD1a, GD1b, GT1b, and GD2, each a concentration of (a) 160 μM and (c) 290 μM. GX represents any of the four gangliosides. (b) and (d) CID mass spectra measured for ions produced by ESI for the solutions described in (a) and (c), respectively. Ions within a window of *m/z* values (~100 *m/z* units wide) centered at 4605 (which corresponds to the –13 charge state of CTB₅ bound to a single ganglioside) were isolated and subjected to CID in the Trap using a collision energy of 100 V

(Figure 3a and c). Due to the inadequate mass resolution, it is not possible to establish directly whether other gangliosides were also bound to CTB₅. However, CID performed using a ~ 100 *m/z* window centered at *m/z* 4400, which corresponds approximately to the -15 charge state of CTB₅ bound to five gangliosides, resulted in the appearance of abundant GM1 anions, as well as the anions of GM2, GM3, GD2, GT1b, and GD1 (GD1a/GD1b), but at lower abundance (Figure 3b and d). Notably, analogous CID experiments performed on a solution containing the same mixture of gangliosides but in the absence of CTB₅ failed to produce signal corresponding to the ganglioside anions (Figure S20c, Supporting Information). Taken together, these results suggest that the ganglioside ions identified by CID were originally bound to CTB₅.

The aforementioned observation that CTB₅ binds to GM2 and GM3 is surprising, given that these gangliosides could not be detected from similar measurements performed on solutions containing the individual gangliosides, *vide supra*. One possible explanation for this finding is that CTB₅-ganglioside binding (specific or nonspecific) is enhanced by the presence of the high affinity GM1 ligand. In other words, it is possible that GM1 anchors CTB₅ to the micelle allowing the protein to interact, either specifically (in solution) or nonspecifically (during the ESI process), with other gangliosides that are present in the micelle. To test this hypothesis, the CaR-ESI-MS assay was also carried out for aqueous ammonium acetate solutions (200 mM, pH 6.8, 25 °C) of CTB₅ (3 μ M) with GM2, GM3, GD1b, and GD2, each at 160 μ M (Figure 4a) and 290 μ M (Figure 4c). It can be seen that in the absence of GM1, very little ganglioside-bound CTB₅ was detected (Figure 4a and c). CID performed using a ~ 100 *m/z* window centered at *m/z* 4605, which corresponds approximately to the -13 charge state of CTB₅ bound to a single ganglioside (Figure 4b and d), produced predominantly signal corresponding to GD1b and GD2, with GM2 and GM3 anions present at very low abundance. These results, taken together with those shown in Figure 3, suggest that the high affinity interaction between CTB₅ and GM1 promotes binding of CTB₅ to the other gangliosides. It is also possible that the presence of GM1 leads to a more favorable presentation of the other glycolipids in the micelles.

Conclusions

The present study represents the first comprehensive investigation into the detection of protein–GL interactions in aqueous solution using glycomicelles and the CaR-ESI-MS assay. The high affinity interaction between CTB₅ and GM1 was successfully detected. However, the apparent affinity, as determined from the mass spectrum, is significantly lower than that of the corresponding ganglioside pentasaccharide or when GM1 is present in model membranes, such as nanodiscs or picodiscs. Interactions between CTB₅ and the low affinity ganglioside ligands GM2, GM3, GD1a, GD1b, and GT1b could not be positively identified by CaR-ESI-MS. No interaction with GM2 or GM3 was detected. Although interactions were

identified for GD1a, GD1b, and GT1b, binding was also detected for GD2, which is not recognized by CTB₅. It is proposed that nonspecific binding during the ESI process is responsible for the interactions with GD1a, GD1b, and GT1b, as well as GD2. This conclusion is supported by the CaR-ESI-MS results obtained for Stx1B₅, which revealed the occurrence of nonspecific protein–ganglioside binding during ESI. Overall, the results of this study suggest that the CaR-ESI-MS/glycomicelle approach for detecting protein–GL interactions *in vitro* is prone to false positives and false negatives and, therefore, must be used with caution.

Acknowledgement

The authors acknowledge the Natural Sciences and Engineering Research Council of Canada and the Alberta Glycomics Center for generous funding, and Professor G. Armstrong (University of Calgary) for generously providing protein used in this study.

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