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Nanodiscs and Electrospray Ionization Mass Spectrometry: A Tool for Screening Glycolipids Against Proteins

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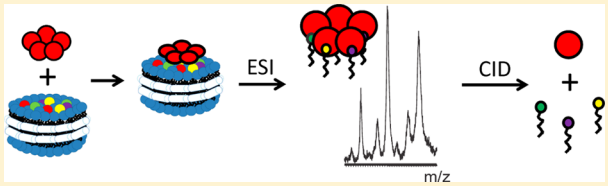
Supporting Information

ABSTRACT: Electrospray ionization-mass spectrometry (ESI-MS) is extensively employed to detect and quantify protein−carbohydrate interactions *in vitro* and is increasingly used to screen carbohydrate libraries against target proteins. However, current ESI-MS methods are limited to carbohydrate ligands that are relatively soluble in water and are, therefore, not generally suitable for studying protein interactions with glycolipids, an important class of cellular receptors. Here, we describe a catch-and-release (CaR)-ESI-MS assay, which exploits nanodiscs (NDs) to solubilize glycolipids and mimic their natural cellular environment, suitable for screening libraries of glycosphingolipids (GSL) against proteins to identify specific interactions and to rank their relative affinities. Using the B subunit homopentamers of cholera toxin and heat labile toxin as model GSL-binding proteins, the CaR-ESI-MS was applied to NDs containing mixtures of gangliosides. The results demonstrate that the CaR-ESI-MS assay can simultaneously detect both high and low affinity GSL ligands using either a library of NDs that each contains one GSL or incorporating a mixture of GSLs into a single ND. Moreover, the relative abundances of the released ligands appear to reflect their relative affinities in solution. Application of the CaR-ESI-MS assay using NDs containing gangliosides extracted from porcine brain led to the discovery of a neolacto GSL as a cholera toxin ligand, highlighting the power of the assay for identifying specific protein−glycolipid interactions from biologically relevant mixtures.



The binding of pathogen-generated proteins, e.g., toxins and surface lectins, with glycans found in glycosphingolipids (GSL) within cell membranes are critical events in many infectious diseases.[1,2] Consequently, there is interest in the development of glycan receptor analogues as competitive binders to disrupt the binding of microbial lectins to host cell receptors and prevent infection of the host by these organisms.[3] To be an effective strategy, the natural glycan receptors must first be identified and their interactions with pathogen-generated proteins characterized. Because of the poor solubility of cellular glycolipids and the low affinities that are typical for protein−carbohydrate interactions, the detection and quantification of these interactions is often impossible with standard binding assays, such as isothermal titration calorimetry (ITC),[4,5] surface plasmon resonance (SPR) spectroscopy,[6,7] and electrospray ionization mass spectrometry (ESI-MS).[8] Consequently, while human glycolipid receptors have been established for some bacterial[9−12] and viral proteins,[13,14] their identification remains a significant experimental challenge, and the development of new analytical methods represents an important and active area of research.

Currently, glycan microarrays are the dominant technology for screening GSLs against water-soluble proteins.[15] Such assays typically involve the immobilization of glycolipids, extracted from cells, onto a glass slide, followed by interrogation of the microarray by the target protein. Although this technique has been used to characterize and identify multiple protein−glycolipid interactions, the assay suffers from the important limitation that the structural and functional properties of glycolipids may differ when attached to a surface compared with glycolipids present in their native cell membrane environment.[16,17] More recently, assays employing liposomes, to capture glycolipids in their native environment, have been developed for protein−glycolipid interaction screening.[18,19] However, because of their dynamic behavior, liposomes have limited stability in aqueous solution and are typically heterogeneous in nature. Nanodiscs (NDs), which consist of a lipid bilayer surrounded by a membrane scaffold protein (MSP),[20] are an attractive alternative to liposomes. Because of the fixed size of the MSP surrounding the lipid bilayer, NDs are relatively homogeneous in size and can remain stable for several weeks in aqueous solution.[21] A number of different detection strategies, based around NDs, have been applied to the detection of protein−GSL interactions, including SPR spectroscopy,[22] silicon photonic sensor arrays,[23] and catch-and-release (CaR) ESI-MS.[24,25]

Among these approaches, the CaR-ESI-MS assay is unique in that it allows for direct detection of the individual protein−glycolipid complexes.[24] Briefly, the assay involves transferring the protein−glycolipid−ND complexes that form in aqueous solution to the gas phase using ESI (Figure 1). Intact protein−glycolipid complexes are found to be readily released from the NDs. While the precise mechanism of release is not known, the

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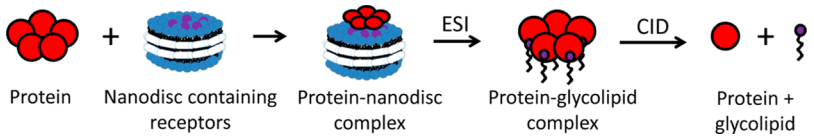

Figure 1. Cartoon representation of the CaR-ESI-MS assay for screening glycosphingolipids (GSLs) against target proteins. The protein (red) and ND (lipid bilayer (blue/black) with surrounding membrane scaffold protein (white)) containing GSLs (purple) are incubated to form a complex. Using mild in-source dissociation conditions, the protein−GSL complexes are stripped out of the ND. The GSL ligands are then released (as ions) from the protein using collision-induced dissociation (CID) and mass analyzed.

process is believed to occur in the ion source, *vide infra*, and is likely driven by mild collisional activation arising from the sampling conditions used to introduce the ND ions into the mass spectrometer and assisted by Coulombic repulsion between the multiple charges associated with the ND and the protein−glycolipid complex in the gaseous protein−glycolipid−ND complexes. The protein−glycolipid complex ions are then isolated and subjected to collision-induced dissociation (CID) to break the protein−glycolipid interactions and release the glycolipids, as ions. The identity of the glycolipid receptors is then established from accurate mass measurements of the glycolipid ions. Previously, the CaR-ESI-MS assay was used to detect protein binding to a specific GSL receptor incorporated into NDs.[24] In addition, protein binding was demonstrated using NDs made from a mixture of lipids, extracted from cell membrane, which contained the same specific receptor.[24]

Here, we extend this earlier work and demonstrate the feasibility of using the CaR-ESI-MS assay and ND-solubilized GSLs as glycan arrays to screen for specific protein−GSL interactions and determine the relative affinities of the interactions. The B subunit homopentamers of cholera toxin (CT) and heat labile toxin (HLT), i.e., CTB_5 and $HLTB_5$, respectively, served as model GSL-binding proteins for this study. Cholera toxin is a hexameric protein consisting of five B subunits in a ring structure bound to the catalytically active A subunit. During infection, the B subunits bind to cellular receptors, possibly the GM1 ganglioside (Figure S1, Supporting Information), present on the surface of the epithelial cells of the small intestine allowing the A subunit to be translocated across the membrane, whereby it causes downstream effects resulting in severe diarrhea.[26] The interactions between CT holotoxin and CTB_5 with GM1, as well as other gangliosides and their water-soluble oligosaccharides, have been extensively investigated. A recent quantitative study of the stepwise binding of GM1 pentasaccharide to CTB_5 reported apparent association constants ranging from 2×10^6 to 2×10^7 M^{-1}.[27] Binding of CTB_5 to other ganglioside oligosaccharides is found to be weaker. For example, affinities for the GM2 and GM3 oligosaccharides (Figure S1, Supporting Information) are reported to be in the $\sim 10^3$ M^{-1} range.[28] Heat labile toxin (HLT), which is the major virulent factor of enterotoxigenic *Escherichia coli*,[29] binds to the epithelial cells of the small intestine, increasing adenylate cyclase activity, resulting in severe diarrhea.[30] Like with CT, the B subunits of HLT (which also assemble into a homopentamer, $HLTB_5$)[31] bind to GM1 with high affinity and to other gangliosides (e.g., GD1b and GM2) with lower affinities.[32−34]

EXPERIMENTAL SECTION

Proteins and Ligands. Cholera toxin B subunit homopentamer from *Vibrio cholerae* (CTB_5, 58 020 Da), heat labile toxin B subunit expressed in *Pichia pastoris* ($HLTB_5$, 60 020 Da), and horse heart myoglobin (16 951 Da) were purchased from Sigma-Aldrich Canada (Oakville, Canada). The MSP MSP1E1, the plasmid of which was purchased from Addgene (Cambridge, MA), was used to prepare the NDs used in this study. The MSP1E1 was expressed and purified following a protocol described elsewhere.[20] The gangliosides GM1 (1545.8 Da (d18:1−18:0), 1573.9 Da (d20:1−18:0)), GM2 (1383.7, 1411.7 Da), and GM3 (1180.5 Da) were purchased from Axxora, LLC (Farmingdale, NY) and GT1b (2126.4 Da), GD1a (1836.1 Da), GD1b (1836.1 Da) from Sigma-Aldrich Canada (Oakville, Canada), and GD2 (1674.0 Da) from MyBioSource, Inc. (San Diego, CA). Porcine brain extract and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (677.9 Da) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL).

Nanodisc Preparation. Nanodiscs were prepared as previously described.[20,35,36] Briefly, DMPC lipid (dissolved in chloroform) and one or more of the glycolipids GM1, GM2, GM3, GD1a, GD1b, GT1b, GD2 (dissolved in chloroform/methanol 2:1) were mixed in the desired ratios, dried under nitrogen, and placed in a vacuum desiccator overnight to form a lipid film. Lipids were then resuspended in 20 mM TrisHCl, 0.5 mM EDTA, 100 mM NaCl, 25 mM sodium cholate pH 7.4 by sonication for 15 min and MSP1E1 added to give a final molar ratio of 100:1 lipid/MSP1E1. The larger MSP (compared to that used in previous studies of involving ND-incorporated gangliosides)[37] was chosen so as to increase the amount of glycolipid that can be incorporated into the ND and, thereby, enhance the extent of protein−glycolipid binding. After incubation of the cholate/lipid/MSP1E1 mixture for 15 min at room temperature, ND self-assembly was initiated by the addition of Bio-Beads (Bio-Rad Laboratories Ltd., Canada) (0.5 g per mL of reconstitution mixture) and the solution incubated for a further 3 h to ensure all detergent had been removed. Finally, NDs were purified using a Superdex 200 10/300 size exclusion column (GE Healthcare Bio-Sciences, Uppsala, Sweden) equilibrated in 200 mM ammonium acetate pH 6.8. A single peak with an elution volume at approximately 12 mL confirmed ND formation (Figure S2, Supporting Information). The maximum total concentration of ganglioside that could be accommodated by the NDs was found to be ∼15%; higher concentrations were found to reduce the ND yield (presumably due to a reduction in the ND stability). The total number of lipid molecules (DMPC and GSL) per ND was taken to be 205.[37] Nanodiscs were concentrated to approximately 30 μM and stored at −80 °C until needed.

SDS PAGE. To test for the presence of (CTB_5 + GM1) complexes, free from the NDs, under the solution conditions used for the ESI-MS measurements, CTB_5 (5 μM) was incubated with 10% GM1 ND (10 μM) at 22 °C in 200 mM ammonium acetate (pH 6.8) for 30 min. The sample was then placed in a microconcentrators (Millipore Corp., Bedford, MA) with a molecular weight cutoff (MWCO) of 100 kDa, diluted into 200 mM ammonium acetate (pH 6.8) and then reconcentrated to the same initial volume three times. The

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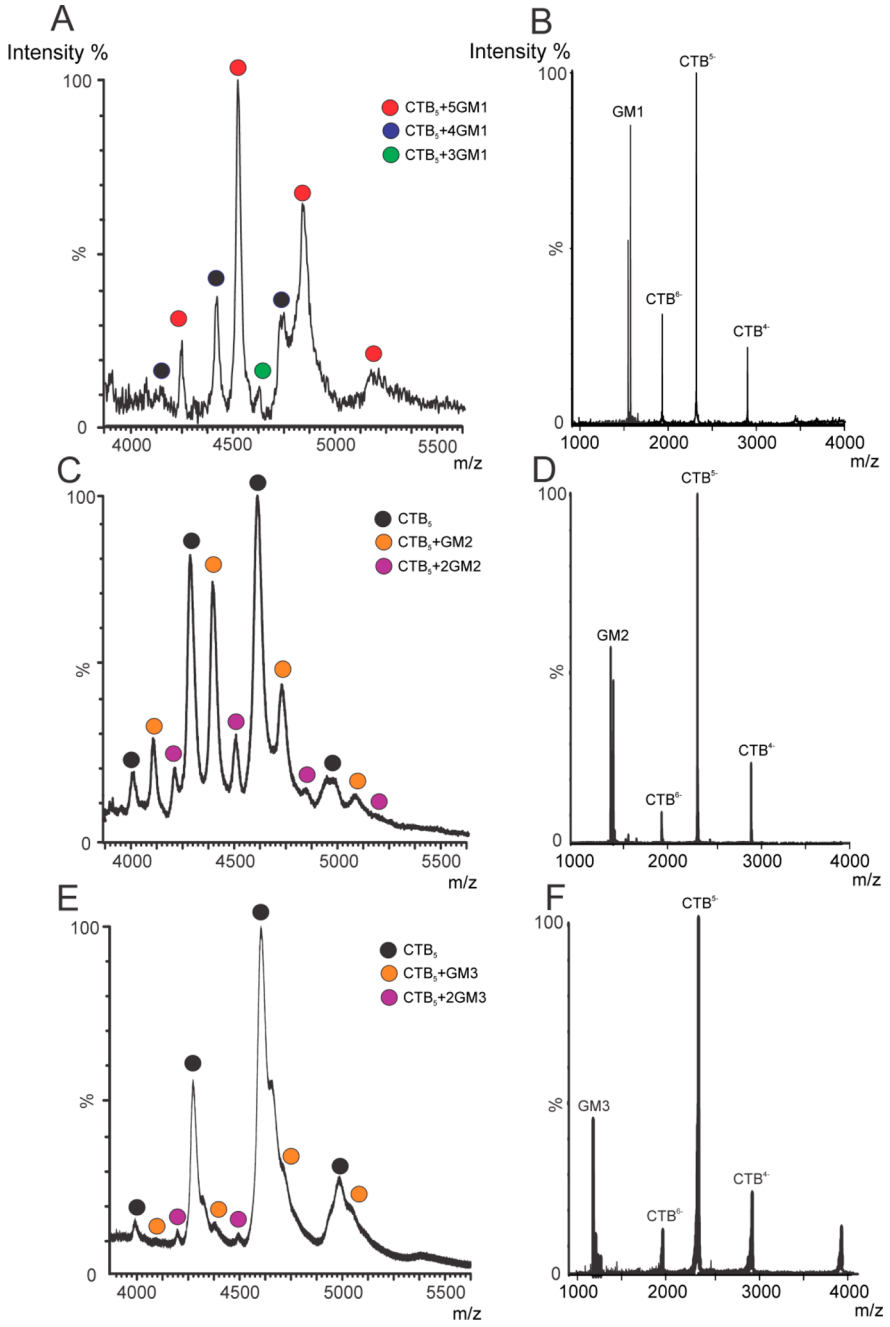

Figure 2. ESI mass spectra of 200 mM aqueous ammonium acetate solutions of CTB_5 (5 μM) with 20 μM 10% GM1 NDs (A), 10% GM2 NDs (C), and 10% GM3 NDs (E). CID mass spectra measured for the $(CTB_5 + 5GM1)^{15-}$ ion (produced from solution A) (B), the $(CTB_5 + 2GM2)^{14-}$ ion (produced from solution C) (D), and the $(CTB_5 + GM3)^{13-}$ ion (produced from solution E) (F) using a Trap collision energy of 100 V.

proteins remaining in the concentrated sample (i.e., >100 kDa) were analyzed by SDS-PAGE along with the flow through (<100 kDa) concentrated to the same initial volume using a 10 kDa MWCO microconcentrator. For SDS-PAGE analysis, all samples were diluted with an equal volume of 2× SDS-PAGE loading buffer (50 mM TrisHCl pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% glycerol). The samples were then heated to 95 °C for 5 min prior to loading onto the Tris-glycine SDS-PAGE gel. All gels were visualized using Coomassie stain (Figure S3, Supporting Information).

Mass Spectrometry. All CaR-ESI-MS measurements were carried out in negative ion mode using a Synapt G2S mass spectrometer (Waters, Manchester, U.K.) equipped with 32k quadrupole mass filter and a nanoESI source. Borosilicate capillaries (1.0 mm o.d., 0.68 mm i.d.) were pulled in-house using a P-97 micropipet puller (Sutter Instruments, Novato, CA). To induce ionization, a platinum wire was inserted into the nanoESI tip and a capillary voltage of between 0.8 and 1.0 kV applied. To release the protein−GSL complex from the NDs in the ion source, a source temperature of 60 °C and a cone voltage 50 V were used; the backing pressure was 3.4 mbar. The pressure in the Trap and Transfer region was maintained at 2.77×10^{-2} mbar and 2.84×10^{-2} mbar, respectively, and the Trap and Transfer voltages were 5 and 2 V, respectively. To release GSL from the protein complexes, ions corresponding to the protein−ligand complex(es) of interest were isolated using the

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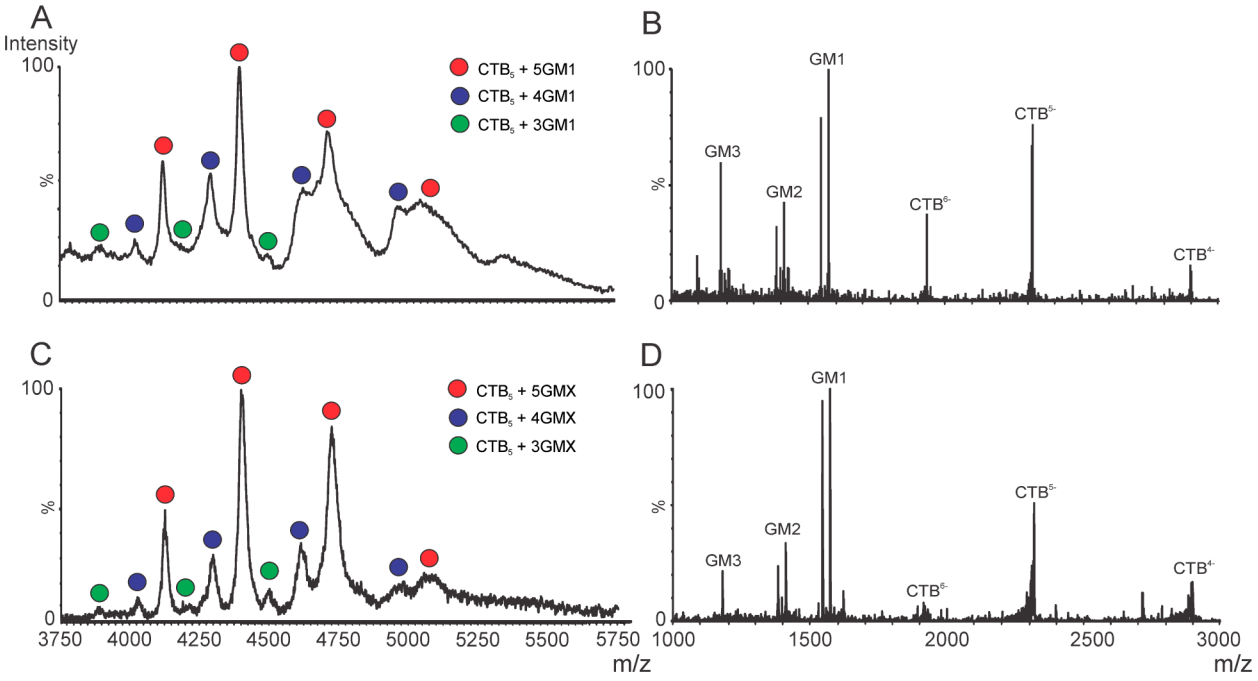

Figure 3. ESI mass spectra of 200 mM aqueous ammonium acetate solutions of CTB_5 (5 μM) and a 1:1:1 mixture of 10% GM1 ND, 10% GM2 ND, and 10% GM3 ND (10 μM) (A) and 5% GM1/2/3 ND (10 μM) (C). CID mass spectra measured using a collision energy of 100 V in Trap region for the m/z region corresponding to $(CTB_5 + 5GM1)^{15-}$ ions produced from solution A (B) and solution C (D).

quadrupole mass filter (LM set to 4.7) and subjected to CID in the Trap region using collision energies ranging from 50 to 200 V. Where necessary, CID of the released ligands was carried out in the Transfer region. All data were processed using MassLynx software (version 4.1).

Because of the broad peaks observed for the protein−ganglioside complexes and the similarity in the m/z of ions of multiple different protein−ganglioside complexes, it was not always possible to positively establish ligand composition of the CTB_5−ganglioside complex ions. For example, the $(CTB_5 + 2GM2)^{13-}$ ion has a predicted m/z of 4677 (using a MW of 1398 Da for GM2) and the $(CTB_5 + 5GM1)^{14-}$ ion a predicted m/z of 4700 (using a MW of 1559 Da for GM1). Therefore, to aid with peak assignment it was assumed that each ND contains 205 lipids (phospholipid and GSL). On the basis of the DMPC/ganglioside ratio used to prepare the ND, the number of gangliosides present in a given ND and, as such, the maximum GSL available to bind to the protein was predicted and used as a basis to determine CTB_5−ganglioside complex ion composition.

RESULTS AND DISCUSSION

The main objective of this study was to assess the feasibility of using ND-solubilized GSLs as glycan arrays for CaR-ESI-MS screening. The assay can, in principle, be carried out in two different formats: employing a library of NDs that each contains one GSL or incorporating a mixture of GSL into one (or possibly a few) NDs. Control experiments were carried out using both strategies to identify potential advantages and disadvantages with each and to establish optimal experimental conditions. The interactions between CTB_5 and three gangliosides, GM1, GM2, and GM3, served as model protein−GSL interactions for the study.

Screening NDs Containing a Single GSL. Detection of the CTB_5 interaction with ND-solubilized GM1 using the CaR-ESI-MS assay was shown previously.[24] In-solution experiments carried out in the present study, whereby CTB_5 (5 μM) was incubated with 10% GM1 ND (10 μM) and the solution passed through a 100 kDa MWCO membrane confirmed that CTB_5 is predominantly bound to the ND in solution, as shown by SDS-PAGE (Figure S3, Supporting Information), and that the release of the CTB_5−GM1 complexes occurs in the gas phase. Moreover, attempts to detect intact CTB_5−GM1−ND complexes in the gas phase were unsuccessful (data not shown). Consequently, it is likely that the release of the CTB_5−GM1 complexes from the NDs occurs in the ion source and is driven by collisional activation associated with the introduction of the ND ions into the mass spectrometer and Coulombic repulsion between the ND and the CTB_5−GM1 complexes in the gaseous CTB_5−GM1−ND complexes.

To demonstrate that CaR-ESI-MS could also be used to detect the lower affinity interactions between CTB_5 and GM2 and GM3, the assay was used to analyze 200 mM aqueous ammonium acetate solutions (pH 6.8) of CTB_5 (5 μM) with NDs (20 μM) containing 10% GM2 or 10% GM3 (Figure 2). For comparison purposes, measurements were also carried out using NDs containing 10% GM1 (Figure 2). In all cases, ions corresponding to ganglioside-bound CTB_5 complexes were identified. For the GM1 sample, ions corresponding to CTB_5 bound to five GM1 molecules were detected, i.e., $(CTB_5 + 5GM1)^{n-}$, where n = 13−16 (Figure 2A). Isolation of the $(CTB_5 + 5GM1)^{15-}$ ion, followed by CID in the Trap region, resulted in the appearance of the deprotonated ions of the two major GM1 isoforms found in the commercial sample of GM1 used to prepare the NDs as well as ions corresponding to free B subunit, i.e., CTB^{n-} ions (Figure 2B). For the GM2 and GM3 samples, only partial occupancy of the five CTB_5 binding sites was observed under the same experimental conditions. For the GM2 sample, free CTB_5 was the dominant protein species; ions corresponding to the $(CTB_5 + GM2)$ and $(CTB_5 + 2GM2)$ complexes were also detected but at low abundance (Figure

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2C). CID of the $(CTB_5 + 2GM2)^{14-}$ ion resulted in the appearance of the deprotonated ions of the two major GM2 isoforms present in the sample used to prepare the NDs and free B subunits anions (Figure 2D). Similar results were obtained for the GM3 sample, although the ions corresponding to the $(CTB_5 + GM3)$ and $(CTB_5 + 2GM3)$ complexes were poorly resolved (Figure 2E). This observation may be due to the formation of nonspecific DMPC adducts with the CTB_5 complexes, which would cause peak broadening. Notably, the lower occupancies of CTB_5 observed for GM2 and GM3, compared to GM1, are consistent with the lower affinities measured for CTB_5 and the corresponding oligosaccharides.[28]

Having successfully demonstrated the detection of the interactions between CTB_5 and each of the three gangliosides, CaR-ESI-MS measurements were carried out on a mixture of NDs, each containing one of GM1, GM2, or GM3. Shown in Figure 3 are results obtained for a 200 mM aqueous ammonium acetate solution (pH 6.8) of CTB_5 (5 μM) with a 1:1:1 mixture of NDs (10 μM), each containing 10% ganglioside. From the mass spectrum alone it is not possible to fully establish the distribution of bound (to CTB_5) gangliosides. However, the most abundant signal is consistent with CTB_5 bound to four or five GM1 ligands, i.e., $(CTB_5 + 4GM1)^{n-}$ and $(CTB_5 + 5GM1)^{n-}$ ions (Figure 3A). To confirm this assignment, the m/z region corresponding to $(CTB_5 + 5GM1)^{15-}$ was subjected to CID (100 V) and resulted in the appearance of signal corresponding to the deprotonated ions of GM1 as well as GM2 and GM3 (Figure 3B). Notably, the signal corresponding to the GM1 anions (two isoforms) is more abundant than that corresponding to the GM2 and GM3 ions (Figure 3B). The appearance of GM2 and GM3 ions is attributed to the presence of $(CTB_5 + 2GM2)^{14-}$ and $(CTB_5 + 2GM3)^{14-}$ ions, which have m/z values similar to that of the $(CTB_5 + 5GM1)^{15-}$ ion. Taken together, these results confirm that CTB_5 binds preferentially to ND-solubilized GM1, consistent with the results obtained for the solutions of CTB_5 and individual ganglioside containing NDs (Figure 2).

Measurements were also carried out using NDs with different percentages (1%, 2.5%, and 5%) of gangliosides (Figure S4, Supporting Information). As expected, the total number of gangliosides bound to CTB_5 was sensitive to the percentage of ganglioside incorporated into the NDs. For example, at 1% incorporation, the major CTB_5 species detected had a single, bound ganglioside ligand, i.e., $(CTB_5 + GM1)$, while at 10% the major CTB_5 species is $(CTB_5 + 5GM1)$. Interestingly, abundant ions corresponding to the 11− to 15− charge states of the dimer of the membrane scaffold protein (MSP1E1) were observed for the 5% incorporation sample. These ions were also detected for the 1% and 2.5% incorporation samples but at much lower relative abundances. Taken on its own, this observation suggests that higher concentrations of gangliosides destabilize the NDs. That the MSP1E1 ions were not identified in the mass spectrum acquired for the 10% incorporation sample can be explained by the high abundances of the $(CTB_5 + 4GM1)^{n-}$ and $CTB_5 + 5GM1)^{n-}$ ions, which obscure the MSP1E1 dimer ions. Subjecting the m/z region corresponding to $(CTB_5 + 2GM1)^{13-}$ (1%), $(CTB_5 + 2GM1)^{14-}$ (2.5%), and $(CTB_5 + 5GM1)^{15-}$ ions (5%) to CID (125 V) resulted in the release of abundant GM1 anions (Figure S5, Supporting Information). Deprotonated GM2 and GM3 ions were also detected but at very low abundance, particularly for the 1% and 2.5% incorporation samples. These results demonstrate that the CaR-ESI-MS assay can tolerate the use of NDs with a range of ganglioside concentrations.

The solutions described above were also analyzed by CaR-ESI-MS using a range of collision energies. Using CID energies ranging from 75 to 125 V produced results very similar to those described above (Figures S5C and S6, Supporting Information). However, the use of higher energies caused fragmentation of the released ganglioside anions and resulted in the appearance of the deprotonated sialic acid ion (290 m/z). Such secondary fragmentation of the gangliosides is undesirable since it can lead to false negatives (i.e., complete conversion of ganglioside anion to sialic acid anion). Moreover, the ease of sialic acid loss is dependent on the structure of the ganglioside. For example, GM3 is known to be less stable (under CID) than GM2 or GM1.[37] Differential fragmentation can, therefore, influence the relative abundances measured for the released gangliosides. It should also be noted that, in the case of disialylated (and trisialylated) gangliosides, sialic acid loss can also lead to formation of monosialylated (and disialylated) gangliosides, potentially resulting in false positives.

Finally, the possibility of false positives resulting from the formation nonspecific protein−ganglioside interactions during the ESI process was explored.[39] Myoglobin, which to the best of our knowledge does not bind to gangliosides, was chosen as a model system for these measurements. Importantly, application of the CaR-ESI-MS to solutions containing myoglobin (5 μM) and a mixture of NDs (10 μM), each containing the 5% of GM1, GM2, or GM3 failed to detect any interactions between the protein and the gangliosides. Instead, the only protein signal detected corresponded to free myoglobin (Figure S7, Supporting Information). Similar results were obtained from measurements performed on the 54 kDa homotetramic protein complex streptavidin (data not shown).

Screening NDs Containing Multiple GSL. The CaR-ESI-MS assay was also applied using NDs containing equimolar amounts of GM1, GM2, and GM3 (these NDs is referred to as the GM1/2/3 NDs) in order to establish whether similar results could be achieved when multiple gangliosides are incorporated into the same ND. Application of the assay to a 200 mM aqueous ammonium acetate solution (pH 6.8) of CTB_5 (5 μM) and GM1/2/3 ND (10 μM), where each GSL was present at 5%, yielded abundant signal consistent with the 16−, 15−, and 14− charge states of CTB_5 bound to four and five GM1 molecules (Figure 3C). However, given the possibility of the simultaneous binding of GM1, GM2, and GM3 to CTB_5, it was not possible to positively establish the composition of the complexes based on the mass spectrum. Consequently, it is more appropriate to label the complex ions according to the number of bound gangliosides, e.g. $(CTB_5 + 4GMX)^{n-}$ and $(CTB_5 + 5GMX)^{n-}$, which represent CTB_5 bound to four and five gangliosides, respectively. To establish the composition of the complexes, release of the ganglioside anions by CID is needed. For example, subjecting the m/z region corresponding to the $(CTB_5 + 5GMX)^{15-}$ ions to CID led predominantly to the release of GM1 anions, with GM2 and GM3 anions detected at lower abundance (Figure 3D). These results agree qualitatively with those obtained when GM1, GM2, and GM3 were present in separate NDs (Figure 3A), suggesting that the $(CTB_5 + 5GMX)^{15-}$ ions were composed primarily of GM1. Similar results were obtained using lower ganglioside incorporation percentages (1% and 2.5%) (Figure S8, Supporting Information) and a range of collision energies (75−125 V) for CID (Figure S9, Supporting Information).

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Interestingly, at lower concentrations of gangliosides, differences in the extent of ligand binding to CTB_5 were observed when the gangliosides were incorporated into separate NDs compared to when they were incorporated into the same ND (Figure S4, Supporting Information). For example, at 1% ganglioside incorporation, the (CTB_5 + GMX) complexes are the major species observed when separate NDs are used (Figure S4A, Supporting Information), while the (CTB_5 + 2GMX) complexes dominate when the three gangliosides were incorporated into the same ND (Figure S4B, Supporting Information). In contrast, at 5% ganglioside incorporation, more bound ganglioside is observed when separate NDs are used compared with incorporating all gangliosides into the same ND. The reason for the differential dependence on percent incorporation is not fully understood but is believed to reflect the combined effect of the relative affinities of the different gangliosides and their distributions in the NDs.

Taken together, the aforementioned results demonstrate that the CaR-ESI-MS assay can simultaneously detect both high and low affinity GSL ligands using either a library of NDs that each contains one GSL or incorporating a mixture of GSLs into a single ND. Moreover, the relative abundances of the released ligands appear to reflect their relative affinities in solution. The application of CaR-ESI-MS assay to NDs containing more complicated GSL mixtures was also investigated. Shown in Figure 4 are CaR-ESI-MS assay data acquired for a 200 mM

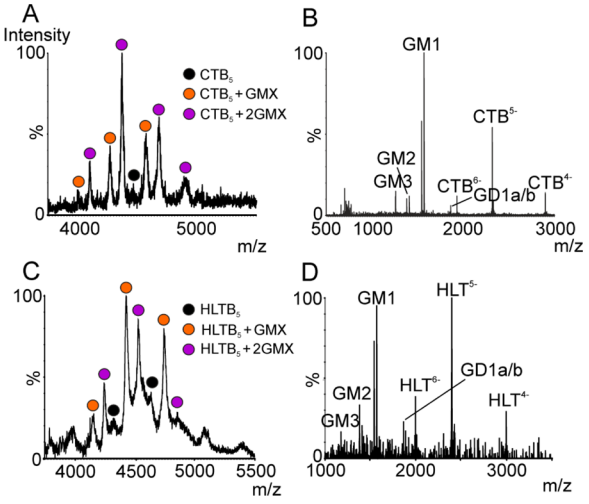

Figure 4. ESI mass spectra of 200 mM aqueous ammonium acetate solutions of CTB_5 (5 μM) and 5 μM 7G ND (A) and $HTLB_5$ (5 μM) with 10 μM 7G ND (C). The 7G ND contained GM1, GM2, GM3, GD1a, GD1b, GT1b, and GD2. CID mass spectra measured for the $(CTB_5 + 2GMX)^{14-}$ (B) and $(HLTB_5 + 2GMX)^{14-}$ ions (D) using a collision energy of 100 V in the Trap region.

aqueous ammonium acetate solution (pH 6.8) of CTB_5 (5 μM) and seven different gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD2, and GT1b) incorporated (1% each) into a single ND (≡ 7G ND) (10 μM). Notably, CTB_5 was found to be bound predominantly to two molecules of ganglioside (Figure 4A). Dissociation of the $(CTB_5 + 2GMX)^{14-}$ ions resulted primarily in the appearance of deprotonated GM1 ions. Anions corresponding to GM2, GM3, as well as GD1a and/or GD1b, were also detected but at low abundance (Figure 4B,D). Because GD1a and GD1b are structural isomers, they cannot be distinguished based simply on molecular weight. The appearance of signal corresponding to GD1a/b is consistent with the results of previously reported binding experiments that identified GD1b as a low affinity ligand for CTB_5.[34,40,41] Direct ESI-MS binding measurements performed on solutions of CTB_5 with GD1a or GD1b revealed that GD1b binds with a measurable affinity, while GD1a does not bind (data not shown).

To demonstrate that the ND-based CaR-ESI-MS approach described above can generally be used to screen GSL libraries against proteins, measurements were also carried out on a 200 mM aqueous ammonium acetate solution (pH 6.8) of $HLTB_5$ (5 μM) and the 7G ND (10 μM). Abundant signal corresponding to the 13−, 14−, 15−, and 16− charge states of the ($HLTB_5$ + GMX) and ($HLTB_5$ + 2GMX) complexes (Figure 4C) was detected. CID of the $(HLTB_5 + 2GMX)^{14-}$ ions resulted in the release GM1 GM2, GM3, and GD1a/b ions, with the GM1 ions being the most abundant (Figure 4D). Similar results were obtained from measurements performed on solutions containing the 5% GM1/2/3 ND (data not shown). These results suggest that GM1 binds preferentially over GM2, GM3, and GD1a/b to $HLTB_5$, and are, in part, consistent with the results of direct ESI-MS binding measurements, which reveal that the GM1 pentasaccharide exhibits a greater than 10-fold higher affinity for $HLTB_5$ compared with the oligosaccharides of GM2 and GM3 (Figure S10, Supporting Information).

Screening NDs Containing GSL Mixtures Extracted from Cell Cultures. The use of ND-based glycan libraries composed of purified GSL is limited by the availability of suitable GSL samples. Moreover, the precise distributions of GSLs in human tissue and organs have not been accurately established. Consequently, implementation of the CaR-ESI-MS assay using NDs composed of GSLs extracted from cell cultures or tissue is likely to be a more effective strategy for the discovery of new GSL receptors. Here, we illustrate this approach with NDs prepared using a ganglioside extract from porcine brain (≡ pig ND). This mixture is known to consist of 10 different gangliosides (each with multiple ceramide types/acyl chain lengths) and 17 sulphatides.[38] CaR-ESI-MS performed on a 200 mM aqueous ammonium acetate solution (pH 6.8) of CTB_5 (5 μM) and the pig ND (10 μM) resulted in abundant signal corresponding to (CTB_5 + GMX) complexes (Figure 5A). CID of the $(CTB_5 + GMX)^{13-}$ ions resulted in the appearance of GM1 ion, along with CTB monomer (Figure 5B). Additionally, signal corresponding to the doubly deprotonated ion of GD1a/b and a singly charged neolacto GSL (Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer) is evident (Figure S11, Supporting Information). These observations are independent of the collision energy used and the percentage of brain extract incorporated into the ND. To our knowledge, this is the first report that CTB_5 binds to this neutral GSL. However, it should be noted that the Galβ1-4GlcNAc moiety of GM1, which participates in intermolecular interactions with CTB_5, is also present in the newly identified neolacto GSL ligand. These results nicely highlight the ability of the CaR-ESI-MS assay for screening GSL mixtures extracted from tissue or cell membranes for the discovery of new receptors.

The CaR-ESI-MS assay was also performed using $HLTB_5$ and the pig ND. In contrast to CTB_5, $HLTB_5$ exhibited less ganglioside binding under identical conditions (Figure 5C). CID of the $(HLTB_5 + GMX)^{15-}$ ions resulted in the appearance of GM1 anions, with no evidence of other ligands (Figure 5D). These results suggest that the affinities of $HLTB_5$ for gangliosides are generally lower than those for CTB_5.

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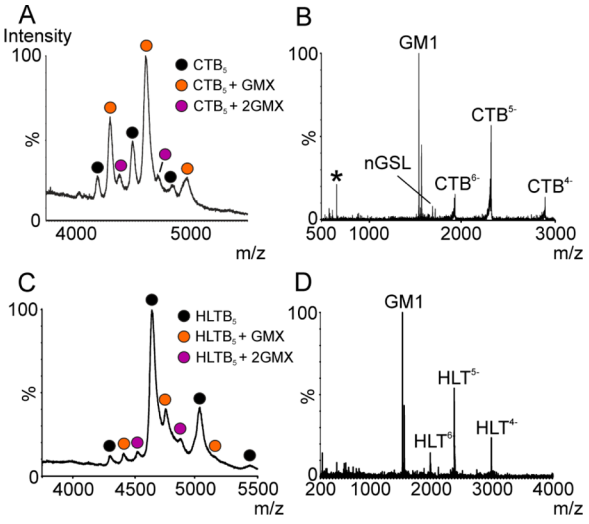

Figure 5. ESI mass spectra of 200 mM aqueous ammonium acetate solutions of CTB_5 (5 μM) and 10 μM pig ND (A) and $HTLB_5$ (5 μM) and 10 μM pig ND (C). CID mass spectra measured for the $(CTB_5 + GMX)^{13-}$ ions produced from solution A (B) and the $(HLTB_5 + GMX)^{15-}$ ions produced from solution C (D) using a collision energy of 100 V in the Trap region. ∗ denotes ions corresponding to DMPC and nGSL is a neolacto glycosphingolipid that is released from the $(CTB_5 + GMX)^{13-}$ ions.

CONCLUSIONS

The detection of protein−glycolipid interactions, whereby the glycolipids are present in a native-like environment, is experimentally challenging. The present work illustrates the power of a CaR-ESI-MS assay, implemented using NDs to solubilize the glycolipids, for screening libraries of GSLs against proteins to identify specific interactions and to rank their relative affinities. Application of the assay for the detection of protein−ganglioside interactions and establishing relative affinities was demonstrated using two different approaches, using a library of NDs (where each ND contains one GSL) or incorporating a mixture of GSL into a single ND. The assay was also demonstrated using NDs made up using a mixture of glycolipids extracted from tissue. This assay also enables screening of glycolipids that are naturally present in a given tissue sample or cell culture, and is, therefore, ideal for the discovery of biologically relevant GSL receptors. Notably, screening NDs made up from glycolipids extracted from pig brain against CTB_5 identified a novel GSL ligand, the neolacto GSL Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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