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Quantifying Carbohydrate–Protein Interactions by Electrospray Ionization Mass Spectrometry Analysis

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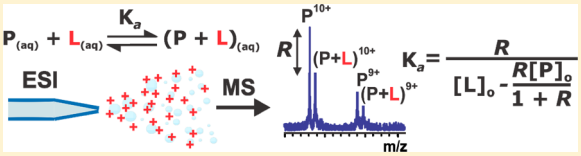
ABSTRACT: The development of analytical methods capable of characterizing carbohydrate–protein interactions, which are critical for many biological processes, represents an active area of research. Recently, the direct electrospray ionization mass spectrometry (ESI-MS) assay has emerged as a valuable tool for identifying and quantifying carbohydrate–protein complexes in vitro. The assay boasts a number of strengths, including its simplicity, speed, low level of sample consumption, and the unique ability to directly probe binding stoichiometry and to measure multiple binding equilibria simultaneously. Here, we describe the implementation of the direct ESI-MS assay for the determination of carbohydrate–protein binding stoichiometries and affinities. Common sources of error encountered with direct ESI-MS analysis of carbohydrate–protein interactions are identified along with strategies for minimizing their effects. The application of ESI-MS and a catch-and-release strategy for carbohydrate library screening are also described. The utility of the direct ESI-MS assay can be extended by combining the technique with competitive protein or ligand binding. An overview of these "indirect" ESI-MS methods is given, as well as examples of recent applications.



Carbohydrates are the most abundant biological molecules.[1] They are commonly found on the surface of cells, in the form of glycopeptides, glycoproteins, and glycolipids, and can bind to suitable protein receptors (e.g., lectins, antibodies, and carbohydrate-processing enzymes) in solution or on cell surfaces.[2] Carbohydrate–protein interactions play critical roles in a wide range of physiological and pathological cell functions, such as inflammation, cell–cell and cell–matrix interactions, signal transduction, fertility, development, and cancer metastasis.[3] They also represent prerequisite first steps for the infection of hosts by many microbes, including viruses, bacteria and their toxins, parasites, and fungi, and are implicated in subsequent immune responses.[3,4] The association of carbohydrate–protein complexes is driven primarily by the formation of hydrogen bond (H-bond) networks and van der Waals contacts.[5] Solvent effects also strongly influence the thermodynamics of carbohydrate–protein binding.[4] In their unbound form, carbohydrate ligands, as well as the protein residues involved in their binding, are typically well-solvated in an aqueous environment. Complete or partial dehydration of the binding partners, which is necessary for formation of the complex, is energetically costly, and as a result, carbohydrate–protein complexes often (but not always) exhibit low association constants (K_a), in the $\sim 10^3$ M^{-1} range.[6] To overcome the low affinities typical of individual carbohydrate–protein interactions, many carbohydrate-binding proteins possess multiple carbohydrate binding sites and exploit multivalent binding to achieve high avidities.[7]

The detection of carbohydrate–protein interactions and their characterization (structure and thermodynamic and kinetic parameters) are both of fundamental importance and facilitate the design of carbohydrate-based therapeutics for the treatment of a variety of diseases and infections. There are a number of established analytical methods for identifying and quantifying carbohydrate–protein interactions in vitro, each with particular strengths and weaknesses. Isothermal titration microcalorimetry (ITC) is generally considered the "gold standard" technique for quantifying the thermodynamic parameters of complex formation and is the only assay that directly provides a measure of the enthalpy of association. Conventional ITC instruments suffer from low sensitivity and generally require large amounts (approximately milligrams) of pure protein and ligand. However, new ITC technologies, such as nano ITC, have improved sensitivity and substantially lower sample requirements.[8]

Surface plasmon resonance (SPR) spectroscopy represents one of the most widely used assays for evaluating the affinities of carbohydrate–protein interactions.[9,10] The technique also allows the direct determination of association and dissociation rate constants. A potential limitation of this approach is the need to immobilize one of the binding partners (usually the ligand) on a sensor chip, which may affect the nature of the binding interaction. Indeed, there are examples in which ITC and SPR spectroscopy yield divergent binding data for the same ligand–protein interaction.[11,12]

Frontal affinity chromatography combined with mass spectrometry detection (FAC–MS) allows for the analysis of mixtures of compounds for specific protein interactions and the determination of the corresponding K_a values.[13] The method involves the continuous infusion of ligands through a column wherein the protein target is immobilized on a solid support. The ligands, which are detected by electrospray ionization MS (ESI-MS), are eluted according to their binding affinities for the target protein, thereby allowing the relative affinities to be easily

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established. A limitation of the FAC−MS assay is the requirement for immobilization of the target protein, which is impractical in some cases and may affect the binding properties of the protein.[13]

The enzyme-linked immunosorbent assay (ELISA) is another widely used method for quantifying carbohydrate−protein interactions.[14] While there are many ways of implementing the ELISA, it requires the immobilization of one of the binding partners, which is incubated with solutions containing the other binding partner, often in the presence of a soluble inhibitor and/or competitive binder. The ELISA method, once set up, is fast and relatively sensitive. However, the assay is quite labor intensive and often requires conjugation of the ligand, which can limit its applicability.

Glycan (carbohydrate) microarrays have become a popular tool for the discovery of interactions of carbohydrates with proteins and protein complexes. The arrays consist of oligosaccharides that are attached to a solid support, usually through a covalent linker.[15,16] Protein targets are incubated with the array, and following a washing step, specific interactions are identified, usually using a fluorescence-based readout.[17] A dense presentation of the oligosaccharides serves to mimic the situation encountered on cell surfaces that allows for multivalent interactions with low-affinity carbohydrate ligands.[18] However, the technique can provide, at best, semiquantitative binding data.

Recently, ESI-MS analysis has earned a place among the arsenal of tools available for identifying and quantifying (stoichiometry and affinity) carbohydrate−protein interactions, as well as other ligand−protein complexes. The ESI-MS measurements can be categorized as either "direct" or "indirect" in nature. The direct ESI-MS assay relies on the direct detection of the gas phase ions of the free and ligand-bound protein. One of the earliest examples of direct ESI-MS detection of specific ligand−protein complexes in aqueous solution was reported in 1991 by Ganem, Li, and Henion and involved interaction of lysozyme with *N*-acetylglucosamine substrates.[19] Soon after the first reports of the successful detection of noncovalent ligand−protein complexes by ESI-MS, the potential of the technique for measuring the affinities of ligand−protein interactions began to be exploited.[20,22−39] The first quantitative study of carbohydrate−protein binding using the direct ESI-MS assay was reported by Kitova et al. in 2001 and involved the weak interactions between analogues of the P^k trisaccharide and the B_5 homopentamer of Shiga-like toxin type I.[20] The K_a values measured by ESI-MS were found to be in a good agreement with values measured by ITC.[21] Since then, the direct ESI-MS assay has been used to quantify carbohydrate interactions with a wide variety of carbohydrate-binding proteins (antibodies, bacterial toxins, lectins, and carbohydrate-processing enzymes).[27−39] Direct ESI-MS analysis is also gaining popularity as a convenient and rapid method for screening carbohydrate libraries against target proteins to identify specific ligands.[40−42]

The direct ESI-MS assay possesses a number of strengths, including its simplicity (no labeling or immobilization of the protein or ligand is required), speed (individual K_a measurements can usually be completed within a few minutes), and the unique ability to directly probe binding stoichiometry and to measure multiple binding equilibria simultaneously. Additionally, when performed using nanoflow ESI, which operates at solution flow rates in the range of 10−100 nL/min, the assay normally consumes picomole or smaller amounts of analyte per analysis. However, the ESI-MS assay, like all binding assays, has limitations. The method relies on being able to detect and accurately quantify the free and ligand-bound protein ions, which is not always possible (there are many possible reasons for this (see Sources of Errors)). However, the utility of the direct ESI-MS assay can be extended by combining it with a competitive protein and/or ligand binding strategy. These indirect approaches rely on direct ESI-MS analysis of protein−ligand complexes to deduce the strength of other protein−ligand interactions in the solution. For example, indirect ESI-MS methods have recently been used to quantify carbohydrate interactions with large proteins and protein complexes,[43] which cannot be directly analyzed, and labile carbohydrate−protein interactions that dissociate during ESI-MS analysis.[31]

In this review, we describe the direct ESI-MS assay, its implementation, and the common sources of error and highlight its application in identifying and quantifying a variety of carbohydrate−protein complexes. The use of direct ESI-MS analysis to screen carbohydrate libraries against target proteins is also discussed. Finally, indirect ESI-MS assays and their applications to carbohydrate−protein binding are described.

DIRECT ESI-MS ANALYSIS OF CARBOHYDRATE−PROTEIN INTERACTIONS

The direct ESI-MS assay is based on the direct detection and quantification of the gas phase ions of free and ligand-bound protein. The magnitude of K_a is determined from the ratio (R) of total abundance (Ab) of ligand-bound and free protein ions, as measured by ESI-MS for solutions with known initial concentrations of ligand and protein ($[L]_o$ and $[P]_o$, respectively). For formation of a 1:1 ligand−protein complex (eq 1), K_a is calculated using eq 2:

$$P + L \rightleftharpoons PL \tag{1}$$

$$K_a = \frac{R}{[L]_o - \frac{R}{1+R}[P]_o} \tag{2}$$

where R is given by eq 3:

$$\frac{[PL]_{eq}}{[P]_{eq}} = \frac{Ab(PL)}{Ab(P)} = R \tag{3}$$

Underlying eq 3 is the assumption that PL and P have similar ionization and detection efficiencies (i.e., uniform response factors). This assumption has been shown to be generally valid when L is small, <10% of the molecular weight of P, such that the size and surface properties of the P and PL are similar.[44]

Normally, K_a values are not determined at a single concentration of protein and the ligand but rather from measurements performed at different concentrations or from a titration experiment, where the concentration of the protein is fixed and the concentration of the ligand is varied. With the latter approach, the value of K_a is established from nonlinear regression analysis of the experimentally determined concentration dependence of the fraction of ligand-bound protein, $R/(R + 1)$ (eq 4):

$$\frac{R}{R+1} = \{1 + K_a[L]_o + K_a[P]_o - [(1 - K_a[L]_o + K_a[P]_o)^2 + 4K_a[L]_o]^{1/2}\} / (2K_a[P]_o) \tag{4}$$

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As an example, the results of a titration experiment that aimed to quantify the interaction between a single-chain variable fragment (scFv) of monoclonal antibody (mAb) Se155-4[45] and a pentasaccharide ligand, α-D-Glcp-(1→4)-β-D-Glcp-(1→4)-[α-D-Abep-(1→3)]-α-D-Manp-(1→2)-α-D-Manp-OCH_3, at 25 °C and pH 7 are illustrated in Figure 1. Figure 1a

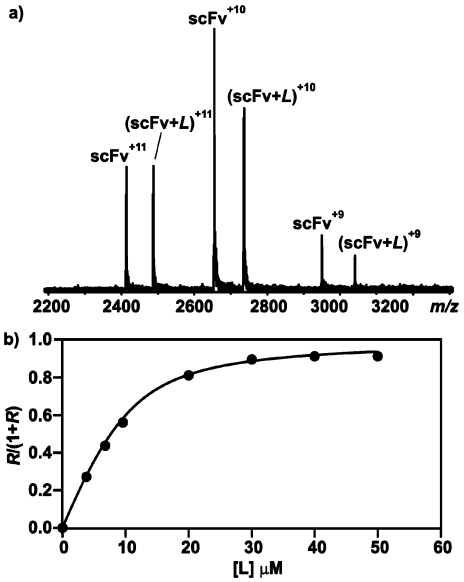

Figure 1. (a) ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate (10 mM) solution of 10 μM Se155-4 scFv and 4 μM pentasacharride ligand {L = α-D-Glcp-(1→4)-β-D-Glcp-(1→4)-[α-D-Abep-(1→3)]-α-D-Manp-(1→2)-α-D-Manp-OCH_3}. (b) Dependence of the fraction of ligand-bound scFv on ligand concentration measured by ESI-MS. The solid line represents the nonlinear fit of eq 4 to the experimental binding data.

is a representative ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate solution containing scFv (10 μM) and the pentasaccharide ligand (4 μM). At these concentrations, the signal for both free and ligand-bound scFv ions is readily identifiable. Plotted in Figure 1b is the fraction of ligand-bound scFv measured at ligand concentrations ranging from 4 to 50 μM. Fitting eq 4 to the experimental data yields a K_a of (3.4 ± 0.3) × 10^5 M^{-1}, which is in excellent agreement with the value measured using ITC (4 × 10^5 M^{-1}).[46]

The direct ESI-MS assay also lends itself to the analysis of sequential ligand (eq 5a) binding and the determination of both the macroscopic and microscopic K_a values. The equilibrium constants ($K_{a,q}$) for the sequential attachment of the ligand to a protein or protein complex can be expressed as shown in eq 5b:

$$P \overset{K_{a,1}}{\rightleftharpoons} PL \overset{K_{a,2}}{\rightleftharpoons} PL_2 \rightleftharpoons \ldots \overset{K_{a,q}}{\rightleftharpoons} PL_q \tag{5a}$$

$$K_{a,q} = \frac{R_q}{R_{q-1}\left([L]_o - \frac{(R_1 + 2R_2 + \ldots + qR_q)[P]_o}{1 + R_1 + R_2 + \ldots + R_q}\right)} \tag{5b}$$

where the R_q terms represent the corresponding abundance ratios of ligand-bound to free protein ions. For a protein with Q equivalent ligand binding sites, the microscopic association constant, i.e., K_a, is related to $K_{a,q}$ by statistical factors that reflect the number of occupied and unoccupied binding sites (eq 6):

$$K_a = qK_{a,q}/(Q - q + 1) \tag{6}$$

Shown in Figure 2 is an illustrative ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate solution containing wild-type tailspike protein (TSP) of phage P22 and an octasaccharide ligand, {α-D-Galp-(1→2)-[α-D-Abep-(1→3)]-α-D-Manp-(1→4)-α-L-Rhap}$_2$, which was derived from the O-antigen repeats of *Salmonella typhimurium*, at 25 °C and pH 7.[47] The TSP is a 180 kDa homotrimeric complex (TSP_3), which possesses three equivalent ligand binding sites.[48,49] The signal for protonated TSP_3 ions as well as ions corresponding to TSP_3 bound to one, two, and three ligands is clearly evident in the mass spectrum. From the distribution of bound ligand measured at this concentration, the macroscopic $K_{a,q}$ values were found to be (2.1 ± 0.1) × 10^5 M^{-1} ($K_{a,1}$), (7.9 ± 0.1) × 10^4 M^{-1} ($K_{a,2}$), and (2.7 ± 0.4) × 10^4 M^{-1} ($K_{a,3}$). These results serve to confirm that the binding sites are equivalent and independent, with an average microscopic K_a of (7.7 ± 0.7) × 10^4 M^{-1}.

Direct ESI-MS measurements of ligand−protein affinities are normally taken at ambient temperature. However, with the

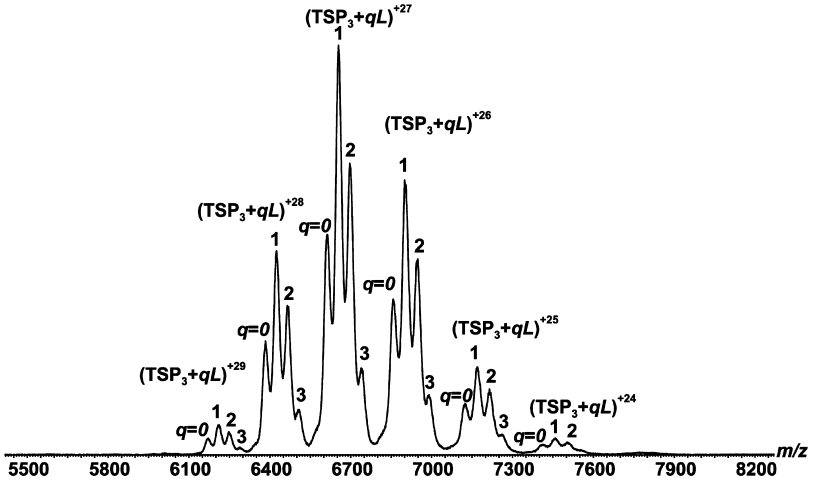

Figure 2. ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate (10 mM) solution of 2 μM wild-type P22 TSP and 10 μM octasaccharide ligand (L = {α-D-Galp-(1→2)-[α-D-Abep-(1→3)]-α-D-Manp-(1→4)-α-L-Rhap}$_2$).

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development of temperature-controlled ESI devices, it is also possible to evaluate K_a over a range of solution temperatures. The magnitude of the corresponding enthalpy and entropy of association (ΔH_a and ΔS_a, respectively) can be estimated from a van't Hoff analysis of the temperature dependence of the K_a values. Several different designs for temperature-controlled ESI devices have been reported in recent years,[50−53] including one that uses heated or cooled air to accurately (±1 °C) control the temperature of the ESI solution between 0 and 60 °C.[50] Using this device, ΔH_a and ΔS_a values have been determined for a several carbohydrate−protein complexes and the measured thermodynamic parameters shown to be in good agreement with values determined by ITC.[38,50] As an example, shown in Figure 3 is a van't Hoff plot constructed from the temperature

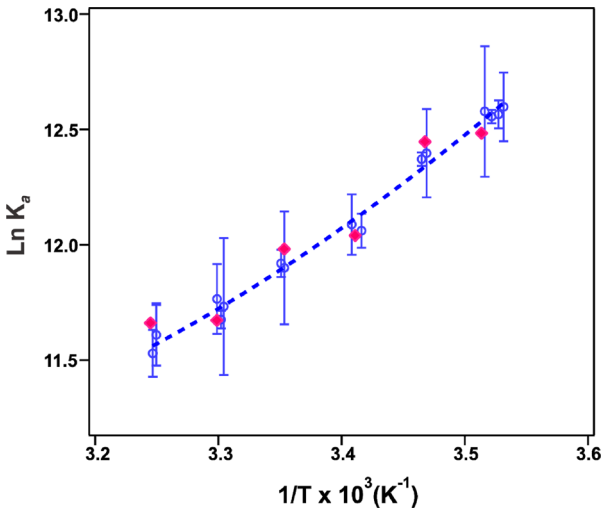

Figure 3. van't Hoff plots for the equilibrium scFv + L ⇌ (scFv + L), where L is β-D-Glc*p*-(1→2)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-OCH_3. The K_a values were determined by ESI-MS (○) and ITC (◆). The ITC values were taken from ref 47. The dashed line corresponds to the nonlinear fit of the van't Hoff equation to the ESI-MS-derived K_a values.

dependence of the K_a values measured by ESI-MS for the interaction between the Se155-4 scFv and the trisaccharide ligand, β-D-Glc*p*-(1→2)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-OCH_3, at pH 7. By fitting an integrated form of the van't Hoff equation that includes a temperature-independent heat capacity change, we obtained ΔH_a and ΔS_a values of 7.0 ± 0.3 kcal mol^{-1} and −0.2 ± 0.2 cal mol^{-1} K^{-1}, respectively. The corresponding values determined by ITC are in close agreement, 6.8 ± 0.4 kcal mol^{-1} and −1.2 ± 1.6 cal mol^{-1} K^{-1}, respectively.[50]

In cases where the solution contains multiple ligands (e.g., L_1, L_2, ..., L_x), with distinct molecular weights, absolute K_a values for individual ligands, i.e., K_{a,L_x}, can be calculated directly from the ESI mass spectrum using eq 7:

$$K_{a,L_x} = \frac{R_{PL_x}}{[L_x]_o - \frac{[P]_o R_{PL_x}}{1 + R_{PL_1} + \ldots + R_{PL_x}}} \quad (7)$$

where R_{PLx} is the ratio of the total ion abundance of protein bound to L_x and free protein. In the case where P possesses multiple (Q) ligand binding sites, the macroscopic association constant ($K_{a,L_x,q}$) corresponding to the attachment of q (1, 2, ..., Q) molecules of L_x can be determined using eq 8:

$$K_{a,L_x,q} = \frac{R_{PL_x,q}}{R_{PL_x,q-1}[L_x]} \quad (8)$$

where $R_{PL_x,q}$ and $R_{PL_x,q-1}$ are the ratios of the total ion abundance of ligand-bound protein (to q or $q-1$ molecules of L_x) and free protein, respectively.

The ability to measure multiple binding equilibria simultaneously makes the direct ESI-MS assay well-suited to the analysis of mixtures of compounds for specific ligand−protein interactions. Indeed, a number of studies utilizing ESI-MS to screen libraries of compounds against target proteins have been reported.[40−42,54−56] The majority of these studies employed a "catch and release" (CaR) strategy,[41] whereby incubation of the target protein with the library is followed by direct ESI-MS analysis to detect the highest-affinity ligands. In cases where the mass of the complex cannot be accurately determined (because of size or heterogeneity) or cases involving isomeric ligands, bound ligands are released (in an ionized form) from the protein using collisional or radiative activation followed by accurate mass measurement, alone or in combination with fragmentation or ion mobility separation of the released ligands for positive ligand identification.[41]

The CaR−ESI-MS approach has been shown to be particularly effective for screening carbohydrate libraries.[40−42] In a recent example, multiple, moderate-affinity (10^4−10^5 M^{-1}) carbohydrate ligands for the Se155-4 scFv were successfully identified in a single ESI mass spectrum acquired for solutions containing scFv and 204 carbohydrates (Figure 4a).[41] Furthermore, the absolute ligand affinities calculated directly from the mass spectra were found to be in good agreement with values determined from individual ESI-MS binding measurements.[41] In this same study, it was shown that the binding of two isomeric pentasaccharide ligands to the antigen binding fragment of mAb CS35 could be differentiated on the basis of ion mobility separation following their release from the protein ions in the gas phase (Figure 4b). The ESI-MS approach has also been used to screen natural product extracts against target proteins.[40,42] Examples include screening of a mixture of highly heterogeneous oligoheparin molecules of low and medium levels of complexity against anti-thrombin III[40] and human milk oligosaccharides, extracted from breast milk, against bacterial toxins (Figure 5).

SOURCES OF ERRORS

The direct ESI-MS assay has been applied to a number of carbohydrate−protein interactions, including antibody−antigen, lectin−carbohydrate, and enzyme−substrate/inhibitor complexes, and in many instances, the K_a values agree well with affinities measured using more established methods.[22−24,32,34,37−39,50,57] However, there are situations in which the assay can give incorrect results. The successful implementation of the direct ESI-MS assay requires that the equilibrium ratio of bound to unbound protein, initially present in solution, be preserved throughout the ESI process and in the gas phase. There are a number of processes that can alter the distribution of free and ligand-bound protein, which leads to incorrect K_a values and can obscure the actual binding stoichiometry in solution. These processes are briefly

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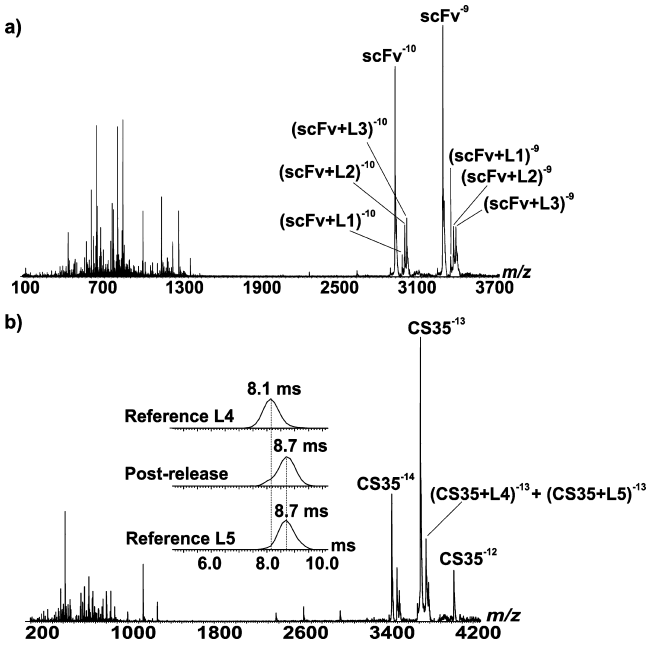

Figure 4. (a) ESI mass spectrum acquired in negative ion mode for an aqueous ammonium acetate (10 mM) solution of 10 μM Se155-4 scFv and 204 carbohydrates (2 μM each), including three specific ligands, L_1 = α-D-Tal*p*-(1→2)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-OCH_3; L_2 = α-D-Abe*p*-(1→3)-2-*O*-methyl-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→4)-β-D-Glc*p*-OCH_3; L_3 = α-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-(1→2)-α-D-Man*p*-OCH_3. (b) ESI mass spectrum acquired in negative ion mode for an aqueous ammonium acetate (10 mM) solution of Fab CS35 (5 μM) and 204 carbohydrates (2.5 μM each), including two specific ligands: L_4 = β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→5)-[α-D-Ara*f*-(1→3)]-α-D-Ara*f*-(1→5)-α-D-Ara*f*-OCH_3; L_5 = β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→3)-[α-D-Ara*f*-(1→5)]-α-D-Ara*f*-(1→5)-α-D-Ara*f*-OCH_3. Also shown are the arrival time distributions measured for the deprotonated ligands (L), L_4 and L_5, released simultaneously from the corresponding $(CS35 + L)^{-13}$ ions (postrelease) and for the deprotonated L_4 and L_5 ions obtained directly from solution (reference).

summarized below together with strategies for eliminating or minimizing their effects.

ESI-MS analysis of ligand–protein complexes can, in some cases, produce false positive results. These arise from so-called nonspecific ligand binding, whereby free ligand molecules associate with proteins or protein complexes in the ESI droplets because of concentration effects.[58−61] In principle, the formation of nonspecific ligand–protein complexes can be minimized by performing the binding measurements at low ligand concentrations. However, for low-affinity interactions (K_a < 10^4 M^{-1}), which are typical for carbohydrate–protein interactions, high concentrations of ligand are generally required to produce detectable levels of the complex.[29] In this case, nonspecific ligand binding is often unavoidable. A straightforward method for correcting ESI mass spectra for the signal arising from nonspecific ligand–protein binding is the reference protein method.[62] This technique involves the addition of a reference protein (P_{ref}), which does not bind specifically to the protein and ligand of interest, to the solution.[62] The "true" abundance of a given PL_q species [$Ab(PL_q)$] can be calculated from the apparent (measured) abundance of the PL_q species [$Ab_{app}(PL_q)$] and the distribution of nonspecific $P_{ref}L_q$ species using eq 9:

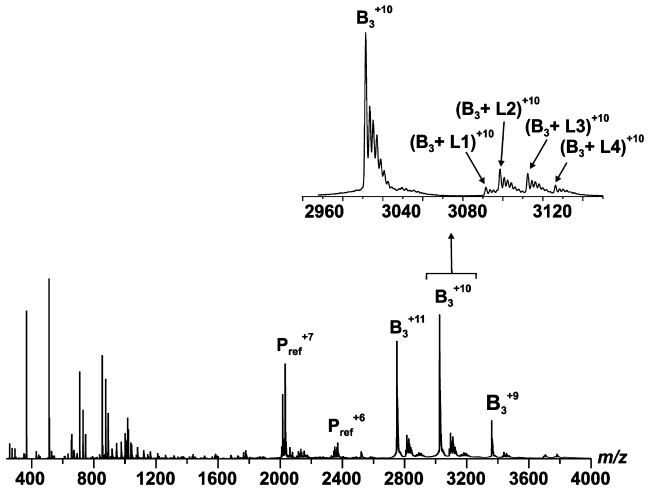

Figure 5. ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate (10 mM) solution containing a fragment (B3) of toxin B of *Clostridium difficile* (15 μM) and a mixture of HMOs extracted from human milk. Possible structures for the HMO ligands: L_1 = β-D-Fuc*p*-(1→2)-α-D-Gal*p*-(1→4)-[β-D-Fuc*p*-(1→3)]-α-D-Glc*p*; L_2 = β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*; L_3 = β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*; L_4 = α-D-Fuc*p*-(1→2)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*; L_5 = α-D-Neu*p*5Ac-(2→6)-α-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*.

$$Ab(PL_q) = [Ab_{app}(PL_q) - f_{1,P_{ref}}Ab(PL_{q-1}) - f_{2,P_{ref}}Ab(PL_{q-2}) - ...f_{q,P_{ref}}Ab(P)]/f_{0,P_{ref}} \quad (9)$$

where $f_{q,P_{ref}}$ is the fractional abundance of P_{ref} bound to q molecules of L. An underlying assumption with the method is that nonspecific ligand binding is a random process and affects equally all proteins in solution, regardless of their size or structure.[62] This hypothesis has been rigorously tested and shown to be generally valid.[62−65]

The P_{ref} method has been used in a variety of ESI-MS studies of carbohydrate–protein binding.[29−38,41] Shown in Figure 6a is a representative ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate solution of Se155-4 scFv (10 μM) and a pentasaccharide ligand (50 μM), α-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-(1→2)-α-D-Man*p*-OCH_3. Lyzosyme was also added (4 μM) to serve as P_{ref} to monitor for the occurrence of nonspecific ligand binding to scFv during the ESI process. The ion signal corresponding to free scFv and scFv bound to one or two ligand molecules is clearly evident in the mass spectrum. However, the signal corresponding to free and pentasaccharide-bound P_{ref} ions is also observed, indicating that nonspecific binding of the pentasacharide to scFv occurred during the ESI. The distributions of ligands bound to scFv before and after correction for nonspecific binding are shown in Figure 6b. It can be seen that, after correction for nonspecific binding, scFv binds to a single pentasaccharide molecule, which is consistent with the fact that scFv has only one binding site.[45] Also, the K_a of (3.4 ± 0.3) × 10^5 M^{-1}, which was determined from the abundances following correction for nonspecific binding, is in good agreement with the ITC value (vide supra).

Collisional heating of gaseous ions of ligand–protein complexes in the ESI source of the mass spectrometer can influence the relative abundance of bound and unbound protein

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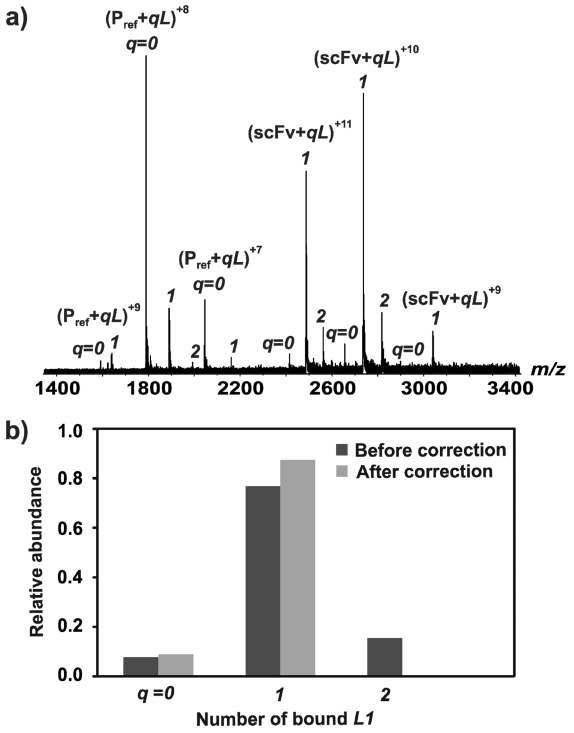

Figure 6. (a) ESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate (10 mM) solution of 10 μM Se155-4 scFv and 50 μM pentasaccharide ligand {L = α-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-(1→2)-α-D-Man*p*-OCH_3}. A P_{ref} (4 μM) was used to correct the mass spectrum for the contribution of nonspecific ligand–protein binding. (b) Normalized distributions of L bound to scFv determined from the ESI mass spectrum before and after correction for nonspecific binding.

ions by inducing dissociation of the noncovalent interactions.[66−68] In the extreme case, this so-called "in-source dissociation" can lead to the loss of all of the complex ions, resulting in a false negative result. Carbohydrate–protein complexes consisting of mono- or disaccharides, which can engage in only a small number of intermolecular interactions (H-bonds or van der Waals interactions) in the gas phase and have low dissociation activation energies, tend to be susceptible to dissociation during ESI-MS analysis.[31,69,70]

The first line of defense against in-source dissociation is the use of "gentle" sampling conditions (e.g., low drying gas and sampling capillary temperatures, low potentials across lens elements, and short accumulation times), which minimize the extent of collisional heating. However, in some cases, in-source dissociation cannot be eliminated, even with the use of gentle conditions. In these instances, the extent of in-source dissociation may be reduced by introducing stabilizing additives into the solution or the gas phase. For example, it has been shown that the presence of imidazole at an elevated concentration (typically 10−30 mM) helps to stabilize noncovalent protein complexes during ESI-MS analysis. The origin of the stabilizing effects of imidazole is not fully understood but is believed to be due, at least in part, to enhanced evaporative cooling resulting from the dissociation of nonspecifically bound imidazole molecules from the gaseous complexes.[70] The presence of imidazole in solution often results in a reduction in the charge states of the protein complex ions formed by ESI. The lower-charge state ions may be more stable (kinetically) and, consequently, more resistant to in-source dissociation.[71] The stabilizing effect of imidazole is illustrated in Figure 7 for the interaction of the Se155-4 scFv

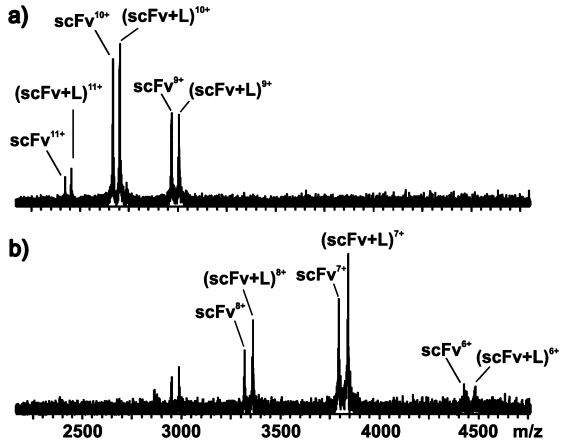

Figure 7. ESI mass spectrum measured in positive ion mode for an aqueous ammonium acetate (10 mM) solution of 10 μM Se155-4 scFv and 10 μM disaccharide ligand (L), α-D-Abe*p*-(1→3)-2-*O*-methyl-α-D-Man*p*-OCH_3, with (a) no imidazole or (b) 10 mM imidazole.

and a disaccharide ligand, α-D-Abe*p*-(1−3)-2-*O*-methyl-α-D-Man*p*-OCH_3. It can be seen that, under identical instrumental conditions, the addition of imidazole results in a marked increase in the fraction of ligand-bound scFv. Recently, it was shown that a high partial pressure of SF_6 in the ESI ion source also protects complexes against in-source dissociation.[69] In cases where it is not possible to eliminate in-source dissociation, indirect ESI-MS assays may be used (vide infra).

In addition to the problems of nonspecific ligand binding and in-source dissociation, there are other practical issues that may hinder the application of the direct ESI-MS assay for quantifying carbohydrate–protein interactions. Notably, electrochemical reactions, which occur at the electrode in the ESI tip, can alter the pH of the solution because of the oxidation (positive ion mode) or reduction (negative ion mode) of H_2O.[39,72] At low solution flow rates (<100 nL/min), the resulting pH changes can be quite large, >1 pH unit after samples had been sprayed for 30 min.[39] The use of ESI solutions with a high buffer capacity or short spraying times (<10 min) is usually sufficient to minimize errors in K_a introduced by pH changes.[39] Another consideration is the inadvertent heating of the ESI solution. Commercial ESI sources generally rely on heating to accelerate or /assist with the desolvation of ions to improve sensitivity. This is commonly achieved by the introduction of heated nitrogen gas to the ESI source or by sampling the ESI droplets in the mass spectrometer through a heated metal capillary. Exposure of the ESI solution to heated gas or having the tip in the proximity of a heated metal capillary can lead to an increase in temperature of several degrees.[50] Another practical challenge is the general incompatibility of the ESI-MS assay with "physiological" buffers (e.g., PBS, citrate, HEPES, or TRIS), which are often used to stabilize proteins and ensure relevance to physiological conditions. However, ESI-MS studies typically employ aqueous ammonium acetate solutions (1−200 mM), with minimal amounts of nonvolatile salts or detergents added to the solution. A variety of strategies have been developed recently,[73−76] including the use of desorption electrospray ionization (DESI) to decouple the sample solution from the

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ESI process,[76] to allow ESI-MS analysis of solutions containing physiological buffers.

INDIRECT ESI-MS ASSAYS

While many carbohydrate−protein interactions can reliably be studied using the direct ESI-MS assay, the technique has limitations. As noted above, it is not generally applicable to interactions for which the corresponding gaseous ions readily dissociate at room temperature. Binding of a ligand to very large or heterogeneous proteins or protein complexes also poses a significant challenge because of instrumental limitations (mass range and resolution). Additionally, the technique is generally incompatible with the analysis of interactions involving membrane proteins or insoluble cellular receptors. In cases where direct ESI-MS analysis is not feasible, it is sometimes possible to quantify the interactions of interest by combining the direct ESI-MS assay with competitive protein or ligand binding. Several such strategies have been developed, and a brief overview of these methods is given below.

Reference Ligand ESI-MS Method. Recently, an indirect ESI-MS method for quantifying ligand−protein complexes that are prone to in-source dissociation was described.[31] The assay, termed the reference ligand ESI-MS method, is based on the direct ESI-MS measurements and competitive ligand binding. To implement the method, a suitable reference ligand (L_{ref}), which binds specifically to the protein, at the same binding site as the ligand of interest, with known affinity and forms a stable ligand−protein complex in the gas phase, is added to the solution together with the protein and ligand of interest. The fraction of protein bound to L_{ref}, which is determined directly from the ESI mass spectrum, is sensitive to the fraction of protein bound to the ligand of interest and allows the affinity of the ligand−protein interaction to be determined. This assay has been used effectively for a variety of complexes,[26,31] including interactions between proteins and their monosaccharide ligands.[31]

As an illustration of the method, a representative ESI mass spectrum acquired for a solution of Se155-4 scFv and α-D-Abe*p*-OCH_3 at pH 7 and 25 °C is shown in Figure 8a. Under these solution conditions, approximately 60% of the scFv is expected to be bound to the monosaccharide. However, no gas phase ions corresponding to the complex were detected, indicating the occurrence of in-source dissociation. Attempts to stabilize the complex using a high concentration of imidazole were unsuccessful.[31] To quantify the interaction, the tetrasaccharide ligand α-D-Abe*p*-(1→3)-2-*O*-methyl-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→4)-β-D-Glc*p*-OCH_3, was introduced into the solution to serve as L_{ref}. As one can see in Figure 8b, the ion signal corresponding to scFv bound to the tetrasaccharide ligand is clearly evident. Analysis of the abundances of the bound and unbound scFv ions, together with the known affinity of scFv for the tetrasaccharide ligand, yielded a K_a of $(1.4 \pm 0.3) \times 10^3$ M^{-1} for the interaction of scFv with abequose methyl glycoside. This value is indistinguishable, within experimental error, from the ITC-derived value of $(1.5 \pm 0.4) \times 10^3$ M^{-1}.[77]

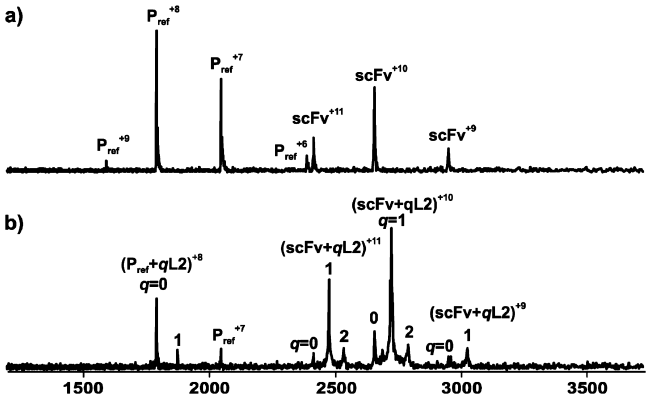

Figure 8. ESI mass spectrum measured in positive ion mode for an aqueous ammonium acetate (10 mM) solution of 10 μM Se155-4 scFv and (a) L_1 (1 mM) and (b) L_2 (59 μM) and L_1 (1 mM). The number of molecules of L_2 bound to the protein ions is indicated by *q*. L_1 = α-D-Abe*p*-OCH_3; L_2 = α-D-Abe*p*-(1→3)-2-*O*-methyl-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→4)-β-D-Glc*p*-OCH_3.

Proxy Protein ESI-MS Assay. Because of mass range and resolution considerations, direct ESI-MS affinity measurements involving relatively small ligands, with molecular masses of a few hundred daltons, are generally restricted to proteins and protein complexes with molecular masses of <100−200 kDa. In cases where direct analysis is not feasible, it may be possible to quantify ligand−protein interactions indirectly using the proxy protein ESI-MS assay.[43] This assay combines direct ESI-MS binding measurements and competitive protein binding. To implement the method, a proxy protein (P_{proxy}), which interacts specifically with the ligand of interest with known affinity and can be detected directly by ESI-MS, is used to quantitatively monitor the extent of binding of the ligand to the protein of interest.

The first example of the application of the proxy protein ESI-MS assay to carbohydrate−protein binding was recently described.[43] The study focused on the interactions between the wild-type P22 TSP and a single-point mutant (D392N) with octasaccharide ({α-D-Gal*p*-(1→2)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-(1→4)-α-L-Rha*p*}$_2$) and dodecasaccharide ({α-D-Gal*p*-(1→2)-[α-D-Abe*p*-(1→3)]-α-D-Man*p*-(1→4)-α-L-Rha*p*}$_3$) ligands.[43] With the mass spectrometer used for these measurements, a 9.4 T Fourier-transform ion cyclotron resonance instrument, it was not possible to directly detect the TSP_3 ions. However, using Se155-4 scFv as P_{proxy}, the affinities of TSP for the octasaccharide and dodecasaccharide ligands were measured at 10 and 25 °C, and the resulting K_a values were found to be in excellent agreement with reported values.[49] Notably, the proxy protein ESI-MS assay is general and can, in principle, be applied to quantify binding of a carbohydrate to any soluble protein or protein complex, provided a suitable P_{proxy} is available. It is expected that this method will find widespread applications in the analysis of interactions of carbohydrate ligands with large protein assemblies, such as virus particles.

In addition to the proxy protein and reference ligand methods, several other indirect ESI-MS assays have been developed. For example, Konermann and co-workers have shown that ligand−protein interactions can be quantified on the basis of changes in the diffusion coefficient of a ligand in the presence and absence of protein, as measured by ESI-MS.[67] An alternative strategy for detecting specific ligand−protein interactions and measuring their affinities involves the quantification of the concentration of free ligand in solution. For example, Leary and co-workers demonstrated such an approach, which is based on ESI-MS analysis of a library of ligands before and after incubation with the immobilized enzyme, for identifying potential inhibitors.[78] Another strategy, which is suitable for the analysis of high-affinity ligand−protein

interactions, relies on the use of a L_{ref}, with known affinity for the protein of interest, and the determination of the relative abundance of the ligand and L_{ref} by ESI-MS.[79] A related method uses the changes in the abundance ratio of ligands with changes in protein concentration to establish relative affinities.[80]

CONCLUDING REMARKS

The direct ESI-MS assay has emerged as an important addition to the arsenal of tools for the identification and quantification of carbohydrate−protein interactions in vitro. Arguably, the greatest strengths of the technique are the ability to directly probe binding stoichiometry, to measure multiple binding equilibria simultaneously, and, when combined with gas phase techniques such as collision-induced dissociation or ion mobility separation, to interrogate the composition and structure of the complexes. The speed of analysis and low sample consumption, together with the absence of labeling or immobilization requirements, are also important features. The direct ESI-MS assay has been applied to a variety of carbohydrate−protein interactions, and the measured affinities generally agree well with values measured using more established binding assays, such as ITC. Furthermore, the CaR−ESI-MS approach has been shown to be very effective for screening carbohydrate libraries for specific interactions with target proteins.

As with all binding assays, the direct ESI-MS assay has its shortcomings. In some cases, the technique produces false positive or false negative results. False positives, which result from nonspecific ligand binding during the ESI process, can be effectively corrected for using the reference protein method. False negatives, which result from in-source dissociation, can often be avoided using solution or gas phase additives that protect the complexes against gas phase dissociation. The utility of the direct ESI-MS assay can also be extended by combining it with a competitive protein or ligand binding strategy. For example, the reference ligand ESI-MS method has proven to be effective for quantifying very labile carbohydrate−protein complexes, while the proxy protein ESI-MS method allows affinity measurements for interactions of carbohydrates with very large protein complexes that cannot be directly detected.

Admittedly, a number of outstanding technical challenges that limit the implementation of the direct ESI-MS assay for the analysis of carbohydrate−protein complexes remain, such as the incompatibility of the technique with many of the widely used biological buffers and the analysis of complexes that tend to rapidly dissociate in the gas phase at ambient temperature. However, given the rapid pace of development of the ESI method, together with continuing improvements in MS instrumentation, the ESI-MS assay may soon emerge as one of the dominant analytical methods for the detection and quantification of carbohydrate−protein interactions.

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