

**Quantifying Protein-Carbohydrate Interactions Using Liquid Sample Desorption
Electrospray Ionization Mass Spectrometry**

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Abstract

The application of liquid sample desorption electrospray ionization mass spectrometry (liquid sample DESI-MS) for quantifying protein-carbohydrate interactions *in vitro* is described. Association constants for the interactions between lysozyme and β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcNAc and β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcNAc, and between a single chain antibody and α -D-Galp-(1 \rightarrow 2)-[α -D-Abep-(1 \rightarrow 3)]- α -D-Manp-OCH₃ and β -D-Glcp-(1 \rightarrow 2)-[α -D-Abep-(1 \rightarrow 3)]- α -D-Manp-OCH₃ measured using liquid sample DESI-MS were found to be in good agreement with values measured by isothermal titration calorimetry and the direct ESI-MS assay. The reference protein method, which was originally developed to correct ESI mass spectra for the occurrence of nonspecific ligand-protein binding, was shown to reliably correct liquid sample DESI mass spectra for nonspecific binding. The suitability of liquid sample DESI-MS for quantitative binding measurements carried out using solutions containing high concentrations of the non-volatile biological buffer phosphate buffered saline (PBS) was also explored. Binding of lysozyme to β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcNAc in aqueous solutions containing up to 1X PBS was successfully monitored using liquid sample DESI-MS; with ESI-MS the binding measurements were limited to concentrations less than \sim 0.02X PBS.

Introduction

Non-covalent interactions between proteins and carbohydrates on the surfaces of cell, present as either part of membrane glycoproteins or glycolipids, are involved in many normal and pathological cellular processes, including catalysis, signaling and molecular recognition [1]. Studies of protein-carbohydrate interactions *in vitro* can provide fundamental insights into these important processes and guide the development of diagnostics and therapeutics for a variety of infections and diseases. There exist a number of analytical methods for the detection and characterization of protein-carbohydrate interactions. For example, glycan microarrays are now commonly used to screen libraries of carbohydrates for specific interactions with proteins [2], while isothermal titration calorimetry (ITC) [3], surface plasmon resonance (SPR) [4], nuclear magnetic resonance (NMR) spectroscopy [5] and enzyme-linked immunosorbent assays (ELISA) [6] are extensively used to quantify the thermodynamics (and in some instances the kinetics) of protein-carbohydrate binding. In recent years, electrospray ionization mass spectrometry (ESI-MS) has emerged as a powerful method for detecting protein-carbohydrate complexes in solution and measuring the affinities of the interactions [7-18].

In the direct ESI-MS binding assay, the protein-ligand binding equilibrium is determined by quantifying the relative abundances of the free and ligand-bound protein ions in the gas phase [11-13]. The measurements are fast and can often be completed within a few minutes, the amount of sample consumed is low, typically picomoles of protein and nano- to picomoles of ligand, and there is no requirement for labeling or additional reagents, which makes the assay very versatile. Moreover, the direct ESI-MS assay is the only technique that directly measures binding stoichiometry. This feature is particularly beneficial to the study of protein-carbohydrate interactions, as many carbohydrate-binding proteins are composed of multiple subunits and

possess multiple ligand binding sites. The ESI-MS assay also affords the opportunity to measure, simultaneously, the binding of multiple, distinct ligands, and is, therefore, well suited to carbohydrate library screening [16].

A drawback of the ESI-MS assay, which is typically carried out using aqueous ammonium acetate solutions [19], is that it suffers from general incompatibility with nonvolatile “physiological” buffers, such as phosphate buffered saline (PBS), citrate, HEPES and Tris-HCl. Such buffers are often needed to keep the protein stable in solution and to minimize protein aggregation [20]. Several strategies have been proposed to allow ESI-MS analysis of solutions containing physiological buffers at relevant concentrations, including the use of high concentrations of ammonium acetate [21] or carrying out ESI in the presence of a high velocity gas [22]. A possible alternative approach involves separating the sample from the ESI process through the use of desorption electrospray ionization (DESI) [23-25] or liquid sample DESI [26-29]. In liquid sample DESI-MS, the liquid sample is ionized through collisions with charged droplets produced by ESI [26, 27, 30]. The ESI solution is typically a mixture of water and an organic solvent, such as acetonitrile or methanol [28]. Despite this, liquid sample DESI has been shown to produce multiply charged gaseous ions of proteins and non-covalent protein complexes without inducing significant unfolding of the protein [28]. A variation of liquid sample DESI, known as reactive liquid sample DESI, was recently described and used to screen a library of compounds for specific binding to a target protein and to quantify the interactions [27]. In this approach, the ligands are introduced (consecutively) into the ESI spray solvent, rather than to the sample solution, which contained the target protein. The advantage of reactive liquid sample DESI is that the premixing of protein with ligands can be avoided [27].

The goal of the present study was to assess the reliability of liquid sample DESI for the quantification of protein-carbohydrate binding in aqueous ammonium acetate solutions and the tolerance of assay to the presence nonvolatile buffers. The affinities of tri- and tetrasaccharide ligands for lysozyme (Lyz), a glycosyl hydrolase, and a single chain variable fragment (scFv) of a monoclonal antibody were measured by liquid sample DESI-MS and the results compared with those measured using ITC and the direct ESI-MS assay [31-33]. The suitability of liquid sample DESI-MS for quantitative binding measurements carried out using solutions containing high concentrations of PBS was also explored. Binding measurements were performed on solutions of Lyz and a trisaccharide ligand in varying concentrations of PBS and the results compared to those obtained using ITC and ESI-MS.

Experimental Section

Materials

Ubiquitin (Ubq, MW 8565 Da), lysozyme (from chicken egg white, Lyz, MW 14310 Da) and maltotriose (**L1**, MW 504.44 Da) were purchased from Sigma-Aldrich Canada (Oakville, Canada) and β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcNAc (**L2**, MW 627.59 Da) and β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcNAc (**L3**, MW 830.27 Da) were purchased from Dextra Science and Technology Centre (United Kingdom). The single chain variable fragment of Se155-4 (scFv, MW 26539 Da) was produced and purified as described previously [34, 35] and α -D-Galp-(1 \rightarrow 2)-[α -D-Abep-(1 \rightarrow 3)]- α -D-Manp-OCH₃ (**L4**, MW 486.50 Da) and β -D-Glcp-(1 \rightarrow 2)-[α -D-Abep-(1 \rightarrow 3)]- α -D-Manp-OCH₃ (**L5**, MW 486.50 Da) were gifts from Prof. D. Bundle (University of Alberta). Stock solutions of each protein (in 50 mM ammonium acetate) and oligosaccharide (in deionized water) were prepared, and stored at -20 °C until needed. A 10X PBS stock solution (NaCl (1.37 M), KCl (27 mM), Na₂HPO₄ (0.1

M) and KH_2PO_4 (18 mM), pH 7.4) was prepared and stored at 4 °C until needed. Sample solutions for ESI- and liquid sample DESI-MS analysis were prepared from the stock solutions of protein and oligosaccharide. Unless otherwise indicated, the sample solutions contained 20 mM ammonium acetate. In a limited number of experiments, PBS was added, at the concentrations indicated.

Apparatus

Mass Spectrometry

All of the ESI- and liquid sample DESI-MS measurements were carried out in positive ion mode using a Synapt G2 quadrupole-ion mobility separation-time-of-flight (Q-IMS-TOF) mass spectrometer (Waters UK Ltd., Manchester, UK) equipped with a 8k quadrupole mass filter. All data were processed using MassLynx software (v4.1). For the ESI-MS measurements, nanoflow ESI (nanoESI) tips, produced from borosilicate capillaries (1.0 mm o.d., 0.68 mm i.d.) and pulled to ~5 μm using a P-1000 micropipette puller (Sutter Instruments, Novato, CA, USA), were used. A platinum wire was inserted into the nanoESI tip and a Capillary voltage of 1.0 - 1.3 kV was applied to initiate the spray. A Cone voltage of 30 V was used and the source block temperature was maintained at 60 °C. The Trap and Transfer ion guides were maintained at 5 V and 2 V, respectively, and the argon pressure in these regions was 2.22×10^{-2} mbar and 3.36×10^{-2} mbar, respectively. For the liquid sample DESI-MS measurements, a modified OMNI SPRAY Ion Sources 2-D OS-6205 (Prosolia Inc., Indianapolis, IN, USA) was used. The liquid sample solution was delivered through a silica capillary (360 nm o.d., 100 nm i.d.) at a flow rate of 5-10 $\mu\text{L h}^{-1}$ using a syringe pump (Chemyx Syringe Pumps Fusion 100, Chemyx Inc, Stafford, TX, USA). The end of the silica capillary was positioned between the ESI tip and inlet of the mass spectrometer. The ESI solution flow rate was between 2 and 4 $\mu\text{L min}^{-1}$. Capillary and Cone

voltages of 3.0 - 3.5 kV and 30 V, respectively, were used and the pressure of the N₂ nebulizing gas was 60 - 70 psi. The source block temperature was the same as for the ESI-MS measurements.

Prior to carrying out the liquid sample DESI-MS protein-carbohydrate binding measurements, several different spray solvent compositions were tested (deionized water, 20 mM ammonium acetate, 50/50 v/v water/methanol, 20/80 v/v water/acetonitrile, 50/50 v/v water/acetonitrile and 80/20 v/v water/acetonitrile) for the analysis of aqueous ammonium acetate solutions of Lyz or scFv, the two model carbohydrate-binding proteins used in this study. Ultimately, it was found that a 50/50 water/acetonitrile solution gave mass spectra with the highest signal-to-noise ratios. Shown in Figure S1 (Supplementary Information) are representative liquid sample DESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (20 mM) solutions containing Lyz (10 μM) or scFv (10 μM). It can be seen that liquid sample DESI-MS produced abundant signal corresponding to the protonated ions of Lyz (Figure S1a) and scFv (Figure S1b). A 50/50 water/acetonitrile solution was used as the spray solvent for all of the liquid sample DESI-MS binding measurements reported in this study.

Isothermal Titration Calorimetry

The ITC measurements were carried out using a VP-ITC (MicroCal, Inc. USA). For each ITC experiment, the Lyz solution (0.1 - 0.2 mM) in the sample cell was titrated with a solution of **L2** or **L3** (2 mM); both the protein and ligand solutions were aqueous ammonium acetate (50 mM, pH 6.8) or PBS (1X, pH 7.4) at 25 °C.

Data Analysis

The general procedure for determining association constants (K_a) for protein-ligand interactions from ESI mass spectra has been described in detail elsewhere and only a brief description is

given for the case where the protein has single ligand binding site [18,36, 37]. The assay relies on the detection and quantification of the gas-phase ions of free and ligand-bound protein. The concentration ratio (R) of the ligand-bound protein (PL) to free protein (P) in solution is taken to be equal to the total abundance (Ab) of P and PL ions as measured by ESI-MS, eq 1. It follows that K_a can be calculated from eq 2:

$$R = \frac{\sum Ab(PL)}{\sum Ab(P)} = \frac{[PL]_{eq}}{[P]_{eq}} \quad (1)$$

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

where $[P]_o$ and $[L]_o$ are the initial protein and ligand concentrations, respectively.

It was shown previously that, during the ESI process, free L can form so-called nonspecific complexes with P (and specific PL complexes), as the ESI droplets evaporate to dryness [36,37]. The extent of nonspecific ligand binding is sensitive to the concentration of free L and, consequently, is more prevalent when measuring low affinity interactions because high L concentrations are needed to produce detectable concentrations of the PL complexes [37]. The formation of nonspecific PL interactions changes to the measured abundances of the P and PL ions and, thereby, introduces error into the R and K_a values. The reference protein method was developed to quantitatively correct ESI mass spectra for nonspecific binding [37]. The method involves the addition of reference protein (P_{ref}), which does not interact with P or L, to the solution. The presence of nonspecific binding is established from the appearance of ions corresponding to P_{ref} bound to one or more molecules of L, i.e., $P_{ref}L_x$ complexes. As described in detail elsewhere, the contribution of nonspecific binding to the apparent (measured) abundances of P ($Ab_{app}(P)$) and PL ($Ab_{app}(PL)$) can be accounted for using eqs 3a and 3b:

$$Ab(P) = Ab_{app}(P) / f_o \quad (3a)$$

$$Ab(PL^{n+}) = \{Ab_{app}(PL^{n+}) - f_l Ab(P^{n+})\} / f_o \quad (3b)$$

where f_o is the fraction of free P and f_l the fraction of P bound nonspecifically to one molecule of L. These fractions can be determined from the measured abundances of free and ligand-bound forms of P_{ref} , eqs 4a and 4b:

$$f_o = Ab(P_{ref}) / \{Ab(P_{ref}) + Ab(P_{ref}L)\} \quad (4a)$$

$$f_l = Ab(P_{ref}L) / \{Ab(P_{ref}) + Ab(P_{ref}L)\} \quad (4b)$$

To test the reliability of the reference protein method for correcting liquid sample DESI mass spectra for the occurrence of nonspecific carbohydrate-protein binding, control experiments were carried out on solutions containing a pair of proteins (Lyz and Ubq) and **L1**, which does not bind to either protein in solution [31, 32]. Shown in Figure 1 are liquid sample DESI mass spectra acquired in positive ion mode for aqueous 20 mM ammonium acetate solutions of Lyz (10 μ M) and Ubq (10 μ M) and **L1** at concentration of 15 μ M (Figure 1a) or 40 μ M (Figure 1b). It can be seen that, in addition to the protonated ions of Lyz (at charge states +6 to +9) and Ubq (at charge states +4 to +6), ions corresponding to nonspecific complexes with **L1** are evident at both concentrations. Shown in the insets of Figures 1a and 1b are the normalized abundances of Lyz and Ubq in their free and bound (to **L1**) forms. Notably, the distributions of both proteins are identical, within experimental error, at both concentrations of **L1**. These results confirm that the extent of nonspecific carbohydrate binding during the liquid sample DESI process is the same for the two proteins and, further, supports the use of the reference protein method for correcting liquid sample DESI mass spectra for nonspecific carbohydrate-protein binding.

Results and Discussion

To test the reliability of liquid sample DESI-MS for quantifying protein-carbohydrate interactions, the affinities of the tri- and tetrasaccharide ligands, **L2** and **L3** for Lyz, and the trisaccharide ligands **L4** and **L5** for scFv were measured. The affinities of **L4** and **L5** for scFv were previously measured in this laboratory using the direct ESI-MS assay and found to be $1.2 \times 10^5 \text{ M}^{-1}$ [38] and $(5.0 \pm 1.0) \times 10^3 \text{ M}^{-1}$ [39], respectively. The affinities of **L2** and **L3** for Lyz have been measured using several different biophysical techniques. Values of $1.1 \times 10^5 \text{ M}^{-1}$ (**L2**) and $1.8 \times 10^5 \text{ M}^{-1}$ (**L3**) were obtained using fluorescence-based assay [40]. Quantitative ESI-MS studies have also been carried out - Oldham and coworkers measured affinities of $1.0 \times 10^5 \text{ M}^{-1}$ (**L2**) and $1.2 \times 10^5 \text{ M}^{-1}$ (**L3**) [31], while Zenobi and coworkers found somewhat lower values (ranging from $2 \times 10^4 \text{ M}^{-1}$ to $5 \times 10^4 \text{ M}^{-1}$) for **L2** [33]. Given the range of the reported values for **L2**, the affinities of **L2** and **L3** for Lyz in aqueous ammonium acetate (50 mM, pH 6.8 and 25 °C) were measured using ITC, which is widely regarded as the gold standard method for quantifying the thermodynamics of protein-carbohydrate interactions. Shown in Figures S2 and S3 (Supplementary Information) are the raw and integrated ITC data measured for binding of Lyz to **L2** and **L3**, respectively. According to the best fit of a 1:1 binding model to the ITC data, the affinities of **L2** and **L3** for Lyz are $(9.0 \pm 0.3) \times 10^4 \text{ M}^{-1}$ and $(1.1 \pm 0.1) \times 10^5 \text{ M}^{-1}$, respectively. These results are in good agreement with the values obtained using the fluorescence-based assay [40] and those reported by Oldham and coworkers [31].

Binding of Lyz to L2 and L3

The affinities of **L2** and **L3** for Lyz in aqueous ammonium acetate (20 mM, pH 6.8 and 25 °C) were measured at three different ligand concentrations. Shown in Figures 2a and 2c are representative liquid sample DESI mass spectra acquired for solutions of Lyz (10 μM) with **L2** (15 μM) or **L3** (15 μM), respectively. Ubq (5 μM), which served as P_{ref} , was added to both

solutions. For comparison purposes, ESI mass spectra were also acquired for these solutions (Figures 2b and 2d). From Figures 2a and 2c, it can be seen that liquid sample DESI produces ions corresponding to free Lyz and ligand-bound Lyz (i.e., the (Lyz + **L2**) or (Lyz + **L3**) complexes), at charge states +6 to +8, as well as free Ubq at charge states +4 and +5. Ion signal corresponding to the nonspecific (Ubq + **L2**) or (Ubq + **L3**) complexes was negligible. Similar results were obtained for solutions containing **L2** or **L3** at two other concentrations, 5 μM and 10 μM (data not shown). The K_a values, representing the average of the values obtained by liquid sample DESI-MS at the three ligand concentrations, are $(1.0 \pm 0.1) \times 10^5 \text{ M}^{-1}$ (**L2**) and $(9.9 \pm 0.6) \times 10^4 \text{ M}^{-1}$ (**L3**) (Table 1). The ESI mass spectra obtained for aqueous ammonium acetate (20 mM) solutions of Lyz (10 μM), Ubq (5 μM), and **L2** (15 μM) or **L3** (15 μM) (Figures 2b and 2d, respectively) are qualitatively similar to the liquid sample DESI mass spectra, although the average charge states (ACS) of Lyz are slightly higher than those observed with liquid sample DESI (ACS 6.98 (Figure 2a), 7.43 (Figure 2b), 7.02 (Figure 2c), 7.98 (Figure 2d)). The lower ACS values measured with liquid sample DESI-MS may be due to a subtle enhancement in the extent of proton transfer from the protein ions to acetonitrile in the gas phase. Acetonitrile has a relatively low gas phase basicity ($178.8 \text{ kcal mol}^{-1}$), compared to ammonia ($195.7 \text{ kcal mol}^{-1}$) [41], but is present at a high concentration in the spray solvent ($\sim 9.6 \text{ M}$) and is expected to be present at relatively high concentrations in the spray droplets. The resulting acetonitrile vapour could affect proton transfer from the gaseous Lyz ions. Support for this explanation can be found in an observed decrease in ACS measured for Lyz when carrying out ESI in the presence of acetonitrile vapour (data not shown), a phenomenon also observed by Oldham and coworkers [44, 45]. The average K_a values obtained by ESI-MS at the three ligand concentrations are $(8.0 \pm 0.5) \times 10^4 \text{ M}^{-1}$ (**L2**) and $(6.3 \pm 0.5) \times 10^4 \text{ M}^{-1}$ (**L3**) (Table 1). Notably, the absolute affinities measured

by liquid sample DESI-MS for **L2** and **L3** agree within a factor of 2 with the values determined from the ESI-MS measurements. More importantly, the liquid sample DESI-MS values are in excellent agreement with the affinities determined by ITC.

*Binding of scFv to **L4** and **L5***

The affinities of **L4** and **L5** for scFv in aqueous ammonium acetate (20 mM, pH 6.8 and 25 °C) were also measured at three different ligand concentrations. Shown in Figures 3a and 3c are representative liquid sample DESI mass spectra acquired for solutions of scFv (10 μM) with **L4** (15 μM) or **L5** (40 μM), respectively. Lyz (5 μM), which served as P_{ref} , was added to both solutions. For comparison purposes, ESI mass spectra were also acquired for these solutions (Figures 3b and 3d). In Figures 3a and 3c, ion signal corresponding to protonated free scFv and the (scFv + **L4**) or (scFv + **L5**) complexes, at charge states +8 to +10, as well as free Lyz and the (Lyz + **L4**) or (Lyz + **L5**) complexes, at charge states +6 to +9, is evident. The appearance of ion signal for the (Lyz + **L4**) and (Lyz + **L5**) complexes indicates the occurrence of nonspecific carbohydrate-protein binding during ion formation. Similar results were obtained for solutions at two other concentrations of **L4** (5 and 10 μM) and **L5** (20 and 30 μM). Following correction of the mass spectra for nonspecific binding, average K_a values of $(7.6 \pm 0.1) \times 10^4 \text{ M}^{-1}$ (**L4**) and $(5.7 \pm 0.2) \times 10^4 \text{ M}^{-1}$ (**L5**) were determined (Table 1). The ESI mass spectra measured for solutions of scFv (10 μM), Lyz (5 μM) with **L4** (15 μM) or **L5** (40 μM) (Figures 3b and 3d, respectively) are similar to the corresponding liquid sample DESI mass spectra (Figures 3a and 3c). However, the extent of nonspecific binding is less in the case of ESI - there was no significant signal corresponding to the nonspecific (Lyz + **L4**) complex and significantly less (Lyz + **L5**) detected. The reduced occurrence of nonspecific binding may be due to the small droplets produced with the nanoESI tips, compared to those formed in liquid sample DESI [42].

The smaller nanoESI droplets will contain fewer ligand molecules and, therefore, produce less nonspecific binding, compared to the larger ESI droplets used for liquid sample DESI-MS. Following correction for nonspecific ligand binding, the affinities of **L4** and **L5** are found to be $(6.6 \pm 0.3) \times 10^4 \text{ M}^{-1}$ and $(5.0 \pm 0.1) \times 10^3 \text{ M}^{-1}$, respectively. Importantly, the affinities measured by liquid sample DESI-MS for **L4** and **L5** are in good agreement with the values determined using the direct ESI-MS assay.

Taken together, the results obtained for these model carbohydrate binding proteins demonstrate that absolute affinities for protein-carbohydrate interactions can be accurately quantified using liquid sample DESI-MS. These findings further indicate that the lifetime of the ESI droplets that produce gaseous protein ions in liquid sample DESI-MS are sufficiently short that neither the presence of a high concentration of organic solvent in the ESI spray solution, nor the inevitable dilution of the sample (protein and ligand) solution by the solvent spray results in a measurable shift in the binding equilibrium.

Comparison of liquid sample DESI-MS and reactive liquid sample DESI-MS

It is also interesting to compare the affinity of **L2** for Lyz measured by liquid sample DESI-MS with the value determined by Loo and coworkers using reactive liquid sample DESI-MS [27]. Notably, the value measured using reactive liquid sample DESI-MS, $5.9 \times 10^3 \text{ M}^{-1}$, is eighteen times smaller than the value determined by liquid sample DESI-MS (and ITC). It has been suggested that the short time available for protein and ligand mixing in reactive liquid sample DESI (estimated to be $<2 \text{ ms}$) might be insufficient for equilibration of the binding reaction [43]. To help rule out other alternative explanations, in particular the possibility of in-source dissociation, reactive liquid sample DESI-MS was carried in the present study to measure the affinity of **L2** for Lyz. The experimental and instrumental conditions were identical to those used

for the liquid sample DESI measurements described above, with the exception that **L2** was absent in the sample solution but present in the spray solvent. Shown in Figure S5 (Supplementary Information) is a representative reactive liquid sample DESI mass spectrum acquired for an aqueous ammonium acetate (20 mM) solution of Lyz (10 μ M) and Ubq (5 μ M); the spray solvent was a 50/50 water/acetonitrile solution containing **L2** (50 μ M). Ion signal corresponding to protonated and sodiated **L2** monomer, dimer and trimer was detected, along with protonated ions of Lyz and (Lyz + **L2**), at charge states +6 to +8, and Ubq and (Ubq + **L2**), at charge state +4. Following correction for nonspecific carbohydrate-protein binding, the K_a value was determined to be $(7.9 \pm 0.4) \times 10^3 \text{ M}^{-1}$, which is similar to the value reported by Loo and coworkers [27]. Given that the instrumental conditions were identical to those used for the liquid sample DESI-MS measurements, it can be concluded that the lower affinity is not due to artifacts associated with instrumental conditions, such as in-source dissociation of the protein-carbohydrate complexes. This finding further supports the suggestion that the lower affinity is, in fact, a kinetic artifact owing to the insufficient time in the droplets for the protein-ligand binding equilibrium to be established [20].

Tolerance of liquid sample DESI-MS to non-volatile salts

The influence of non-volatile salts on the performance of liquid sample DESI-MS for protein-carbohydrate binding measurements was assessed by through binding measurements performed on Lyz and **L2** in aqueous solutions containing varying concentrations of PBS. Shown in Figures 4a-4c are representative liquid sample DESI mass spectra acquired for aqueous solutions of ammonium acetate (20 mM), Lyz (40 μ M), **L2** (30 μ M) and 0.1X PBS, 0.5X PBS or 1X PBS. For comparison purposes, ESI mass spectra were also acquired for these solutions (Figures 4d-4f). At PBS concentrations up to 1X, abundant signal corresponding to protonated ions of Lyz

and (Lyz + **L2**) at charge states +7 to +9 was detected. However, adducts corresponding to the attachment of Na⁺, NaCl, K⁺ and KCl were also evident and their abundances increased with increasing PBS concentration. The K_a values determined at each PBS concentration are (1.0 ± 0.1) × 10⁵ M⁻¹ (0.1X), (5.0 ± 0.2) × 10⁴ M⁻¹ (0.5X) and (4.1 ± 0.2) × 10⁴ M⁻¹ (1X). The value at 0.1X PBS is similar to the one measured in aqueous ammonium acetate (20 mM), however, the values at the higher PBS concentrations are about a factor of two smaller. A similar phenomenon was reported by Oldham and coworkers, who proposed that alkali metal ions may destabilize Lyz-ligand complexes in the gas phase [45]. To establish whether the lower values measured at higher PBS concentrations were reflective of an actual decrease in affinity, ITC was used to measure K_a in a solution of 1X PBS (Figure S4, Supplementary Information). Notably, the ITC-derived value of (8.4 ± 0.2) × 10⁴ M⁻¹ confirmed that the interaction between **L2** and Lyz is slightly weakened at high PBS concentrations, although the magnitude of the effect is not as pronounced as suggested by the liquid sample DESI-MS measurements. Nevertheless, the affinity measured by liquid sample DESI-MS for the 1X solution agrees within a factor of two of the ITC value, indicating that liquid sample DESI-MS can be used to quantify protein-carbohydrate interactions in solutions containing relatively high concentrations of nonvolatile salts. The situation is very different in the case of the ESI mass spectra, which reveal the presence of significant nonspecific adducts (Figures 4d-4f). In fact, even for the 0.1X PBS solution, it was not possible to positively identify ions corresponding to the (Lyz + **L2**) complex, making it impossible to quantify the binding interaction. Further investigation revealed that the direct ESI-MS binding measurements were restricted to PBS concentrations of less than ~0.02X.

The differences in the appearances of the liquid sample DESI and ESI mass spectra can be rationalized by considering the differences in the initial composition of the droplets in each case.

In the ESI-MS experiments, the initial droplets will contain concentrations of buffer that are similar to that found in bulk solution, with some enrichment in cations (Na^+ and K^+) expected as a result of the applied electric field [19]. As a result of solvent evaporation, the concentration of buffer components in the droplets will further increase, with the highest concentrations found in the offspring droplets produced late in the ESI process [19]. In contrast, in liquid sample DESI-MS, the initial ESI droplets are devoid of buffer and contain only water and acetonitrile. It is only through collisions with the sample solution that buffer components are transferred to the ESI droplets. Consequently, the overall concentration of PBS in the droplets of liquid sample DESI that lead to the formation of gaseous protein ions is expected to be significantly lower than in the case of the direct ESI-MS measurements.

Conclusions

In summary, the application of liquid sample DESI-MS for quantifying protein-carbohydrate interactions in aqueous solutions is described. Notably, the affinities of tri- and tetrasaccharide ligands for Lyz and scFv measured using liquid sample DESI-MS are found to be in good agreement with values measured by ITC and the direct ESI-MS assay. It is also found that the reference protein method, which was originally developed to correct ESI mass spectra for the occurrence of nonspecific ligand-protein binding, can be used to correct liquid sample DESI mass spectra for nonspecific carbohydrate binding. The tolerance of liquid sample DESI-MS for quantitative binding measurements carried out using solutions containing high concentrations of PBS was also explored. The binding between Lyz and a trisaccharide ligand was successfully measured with liquid sample DESI-MS at concentrations up to 1X PBS. In contrast, direct ESI-MS binding measurements were limited to PBS concentrations less than $\sim 0.02\text{X}$ PBS.

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Table 1. Comparison of association constants (K_a) measured by liquid sample DESI-MS, ESI-MS and ITC for the interactions of **L2** and **L3** with Lyz and **L4** and **L5** with scFv in aqueous ammonium acetate solutions at pH 6.8 and 25 °C.^a

Protein	Ligand	K_a (liquid sample DESI-MS)/M ⁻¹	K_a (ESI-MS)/M ⁻¹	K_a (ITC)/M ⁻¹
Lyz	L2	$(1.0 \pm 0.1) \times 10^5$	$(8.0 \pm 0.5) \times 10^4$	$(9.0 \pm 0.3) \times 10^4$
Lyz	L3	$(9.9 \pm 0.6) \times 10^4$	$(6.3 \pm 0.5) \times 10^4$	$(1.1 \pm 0.1) \times 10^5$
scFv	L4	$(7.6 \pm 0.1) \times 10^4$	$(6.6 \pm 0.3) \times 10^4$ $(1.2 \times 10^5)^b$	
scFv	L5	$(5.7 \pm 0.2) \times 10^3$	$(5.0 \pm 0.1) \times 10^3$	

a. Errors correspond to one standard deviation. b. Value taken from ref. 38.

Figure captions

- Figure 1.** Representative liquid sample DESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (20 mM, pH 6.8 and 25 °C) solutions containing Ubq (10 µM), Lyz (10 µM) and **L1** at (a) 15 µM or (b) 40 µM concentrations. The ESI spray solution was 50/50 water/acetonitrile.
- Figure 2.** Representative (a), (c) liquid sample DESI and (b), (d) ESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (20 mM, pH 6.8 and 25 °C) solutions containing Lyz (10 µM), **L2** (15 µM) and Ubq (5 µM) ((a) and (b)) or Lyz (10 µM), **L3** (15 µM) and Ubq (5 µM) ((c) and (d)). For the liquid sample DESI-MS measurements, the ESI spray solution was 50/50 water/acetonitrile.
- Figure 3.** Representative (a), (c) liquid sample DESI and (b), (d) ESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (20 mM, pH 6.8 and 25 °C) solutions containing scFv (10 µM), **L4** (15 µM) and Lyz (5 µM) ((a) and (b)) or scFv (10 µM), **L5** (40 µM) and Lyz (5 µM) ((c) and (d)). For the liquid sample DESI-MS measurements, the ESI spray solution was 50/50 water/acetonitrile.
- Figure 4.** Representative (a), (b) and (c) liquid sample DESI and (e), (f) and (g) ESI mass spectra acquired for aqueous solutions (pH 7.4 and 25 °C) containing Lyz (40 µM) and **L2** (30 µM) in 20 mM ammonium acetate and 0.1X PBS ((a) and (d)), 0.5X PBS ((b) and (e)) and 1X PBS ((c) and (f)). For the liquid sample DESI-MS measurements, the ESI spray solution was 50/50 water/acetonitrile.

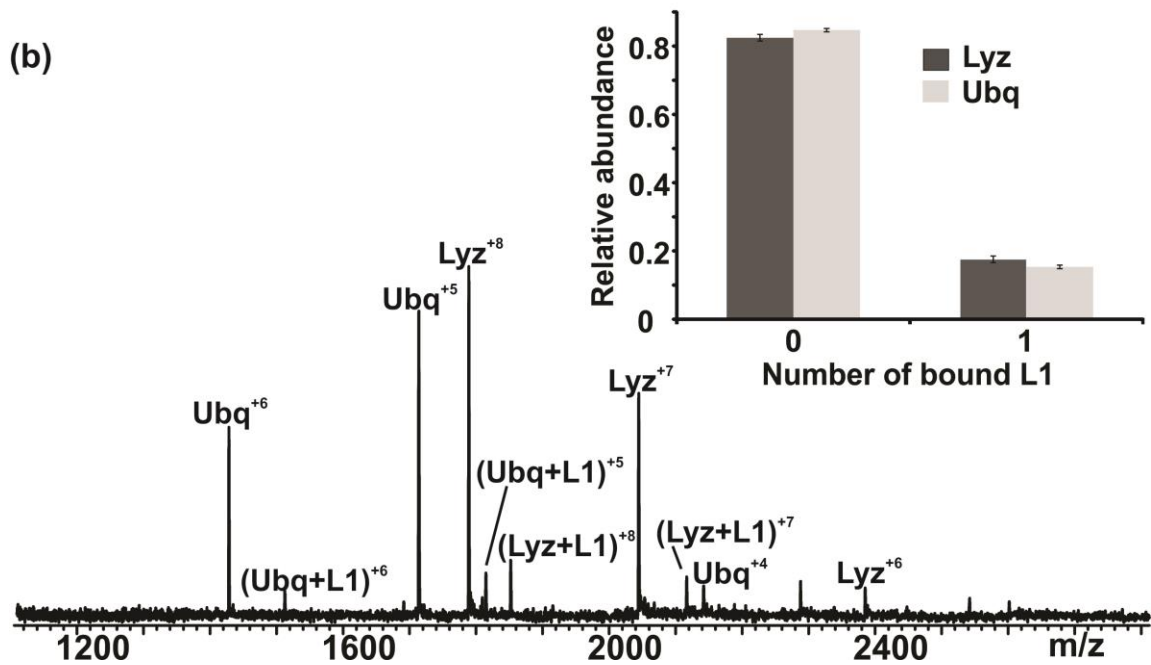
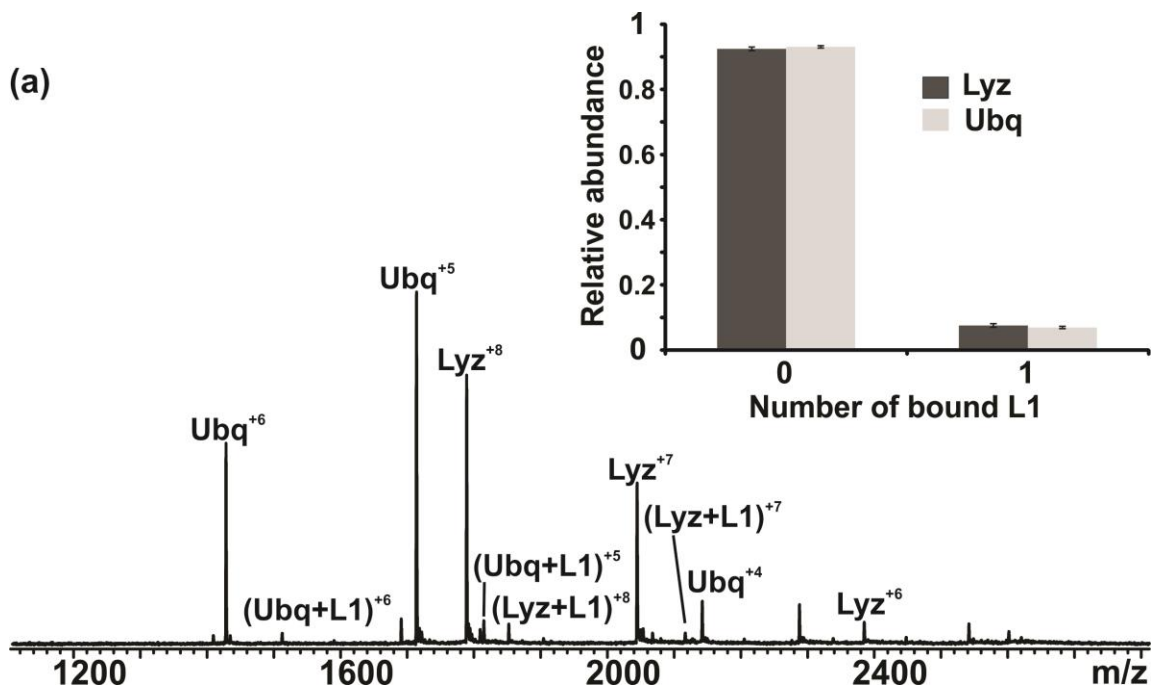
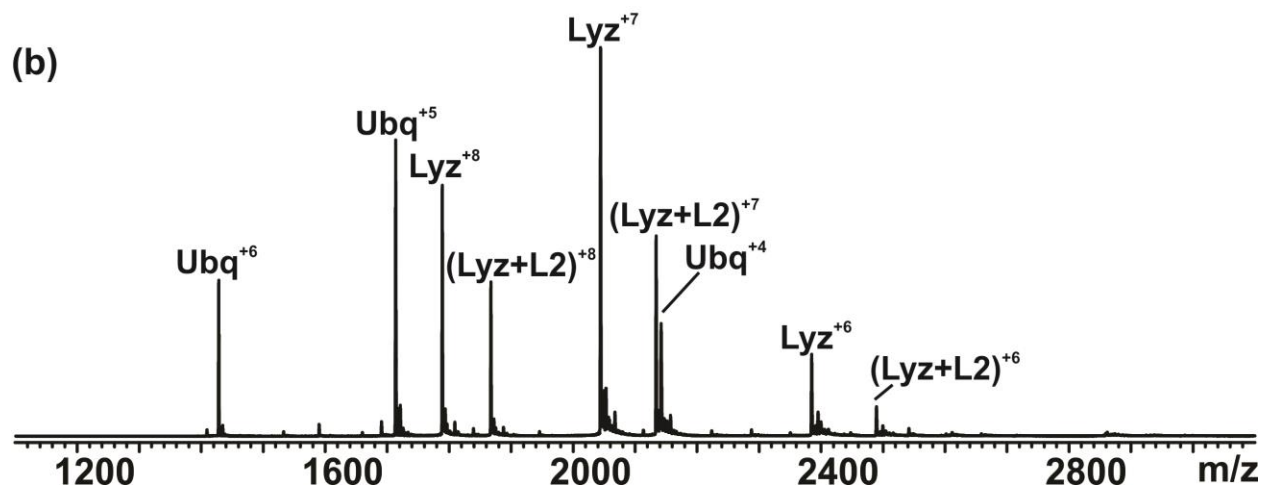
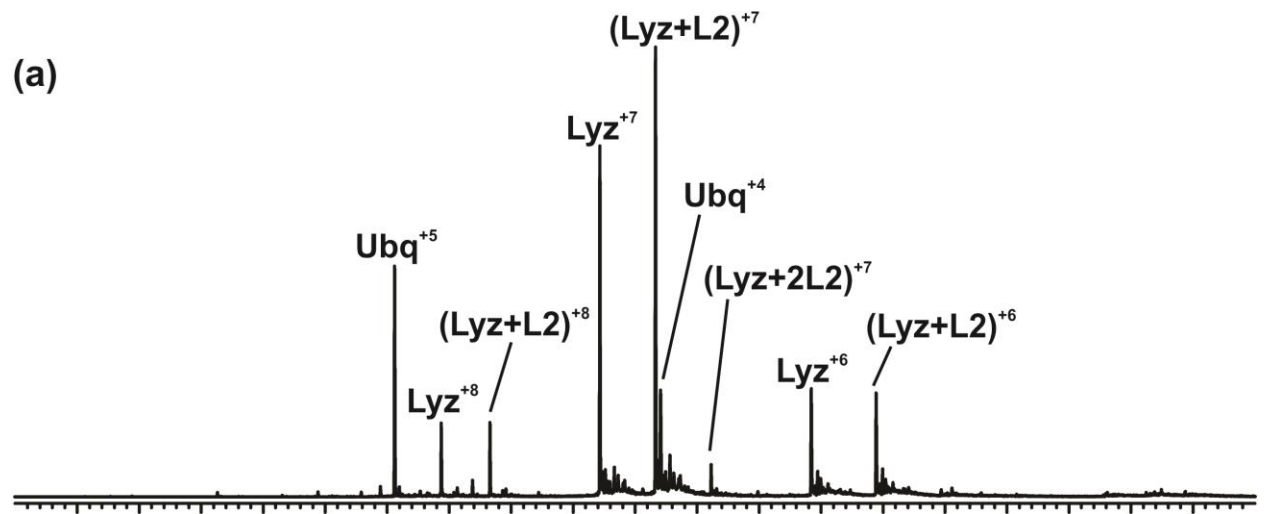


Figure 1



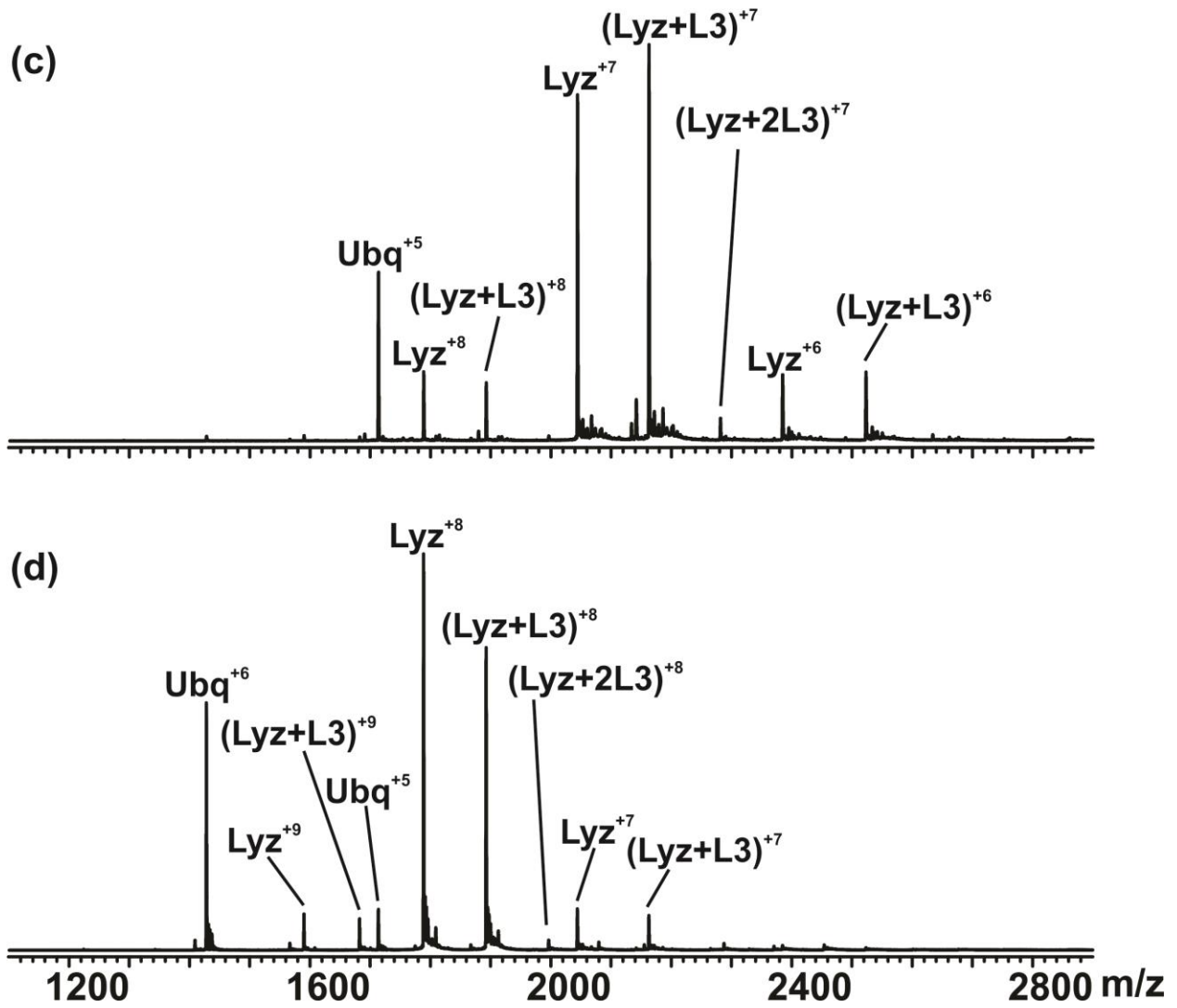
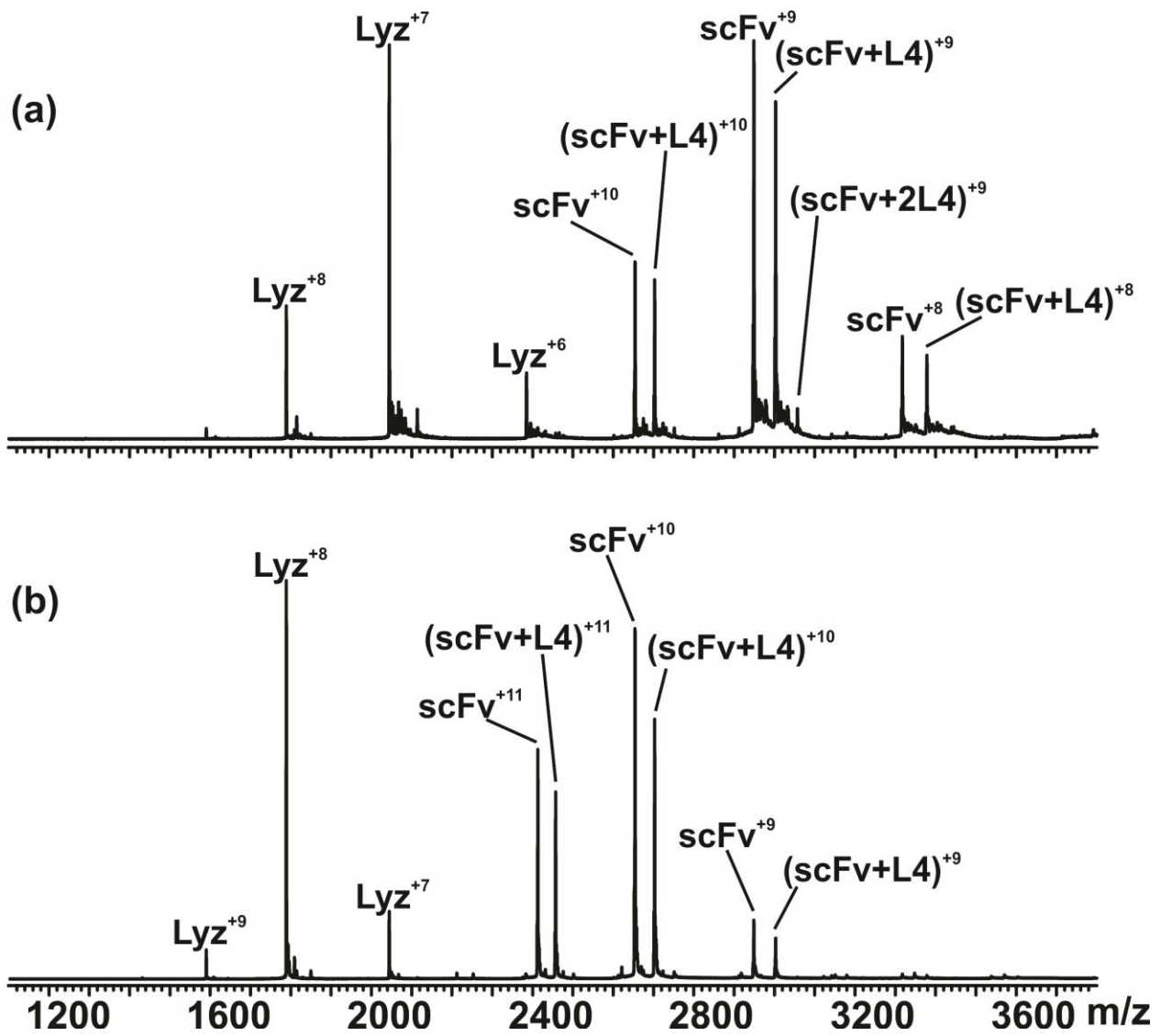


Figure 2



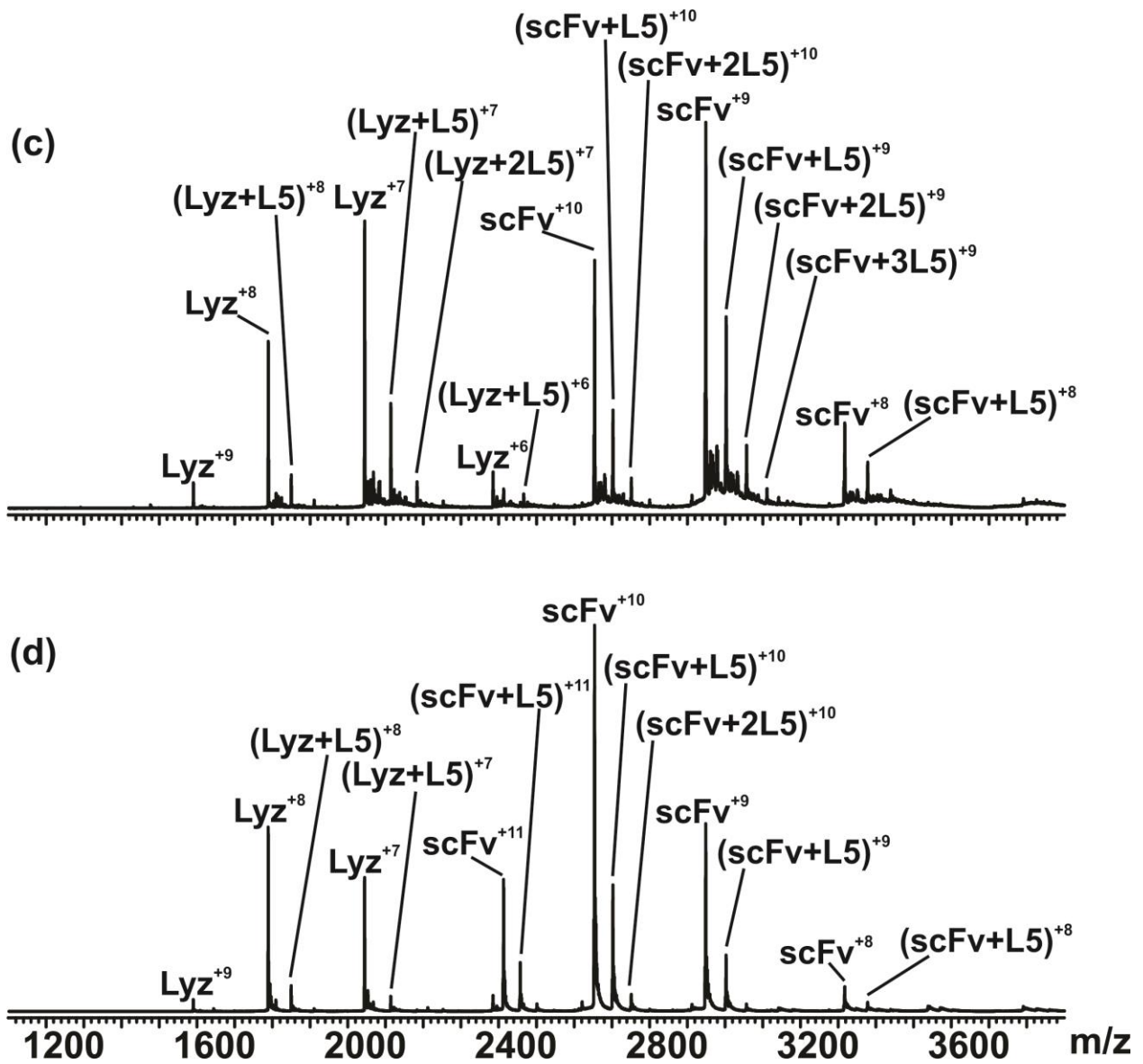


Figure 3

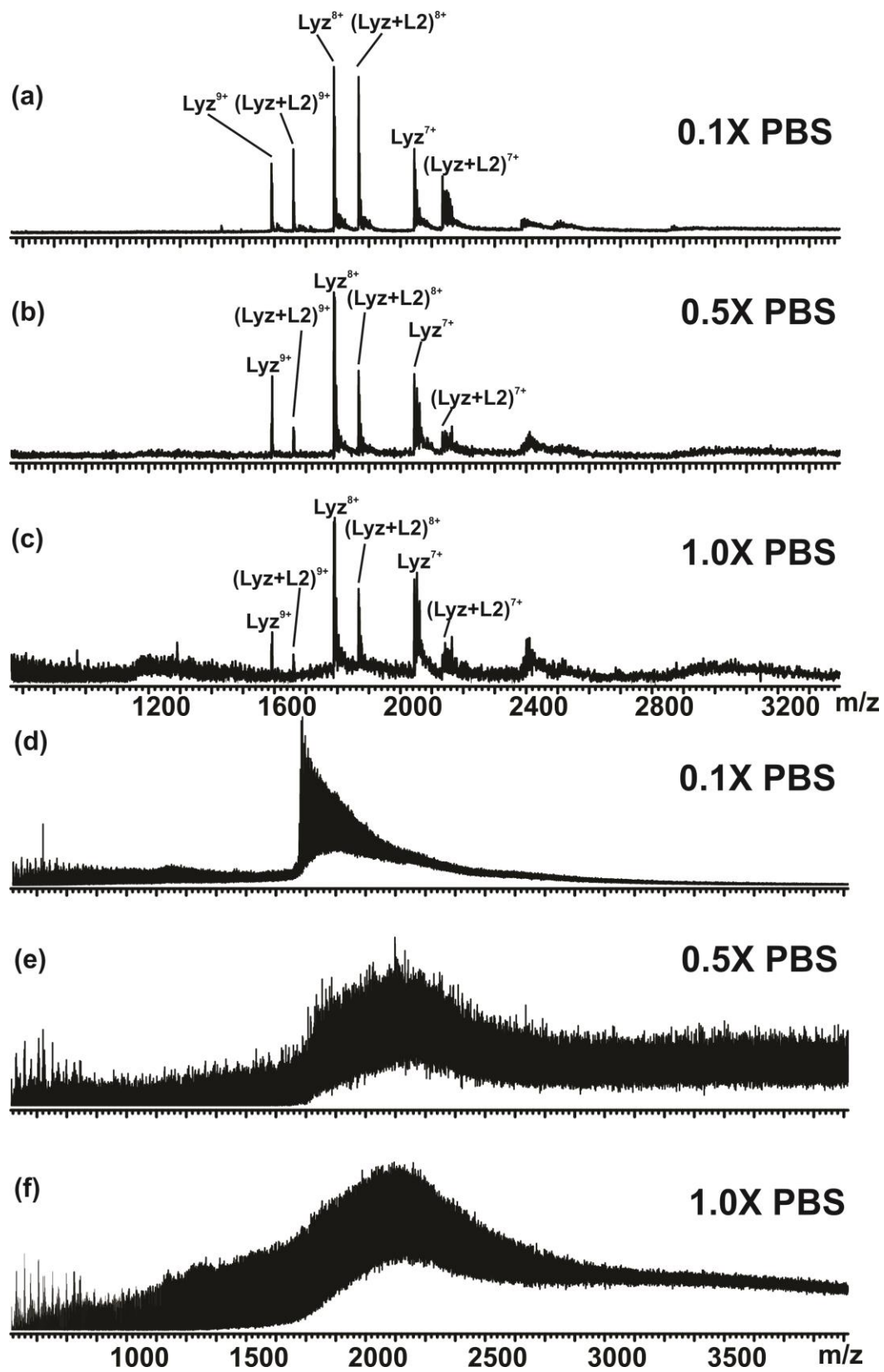


Figure 4

Supplementary Information for:
Quantifying Protein-Carbohydrate Interactions Using Liquid Sample Desorption
Electrospray Ionization Mass Spectrometry

Yuyu Yao, Km Shams-Ud-Doha, Rambod Daneshfar, Elena N. Kitova and

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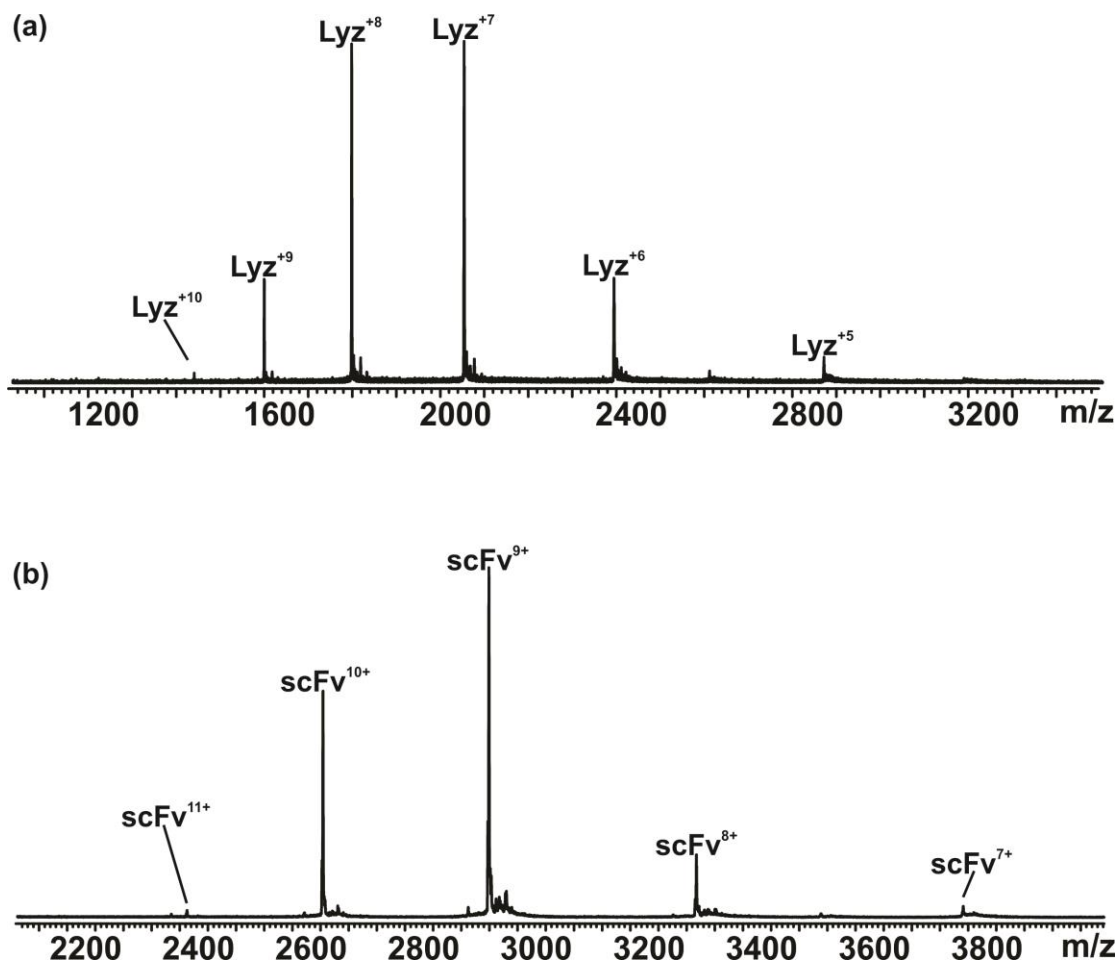


Figure S1. Representative liquid sample DESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (20 mM) solutions containing (a) Lyz (10 μ M), and (b) scFv (10 μ M). The ESI spray solution was 50/50 water/acetonitrile.

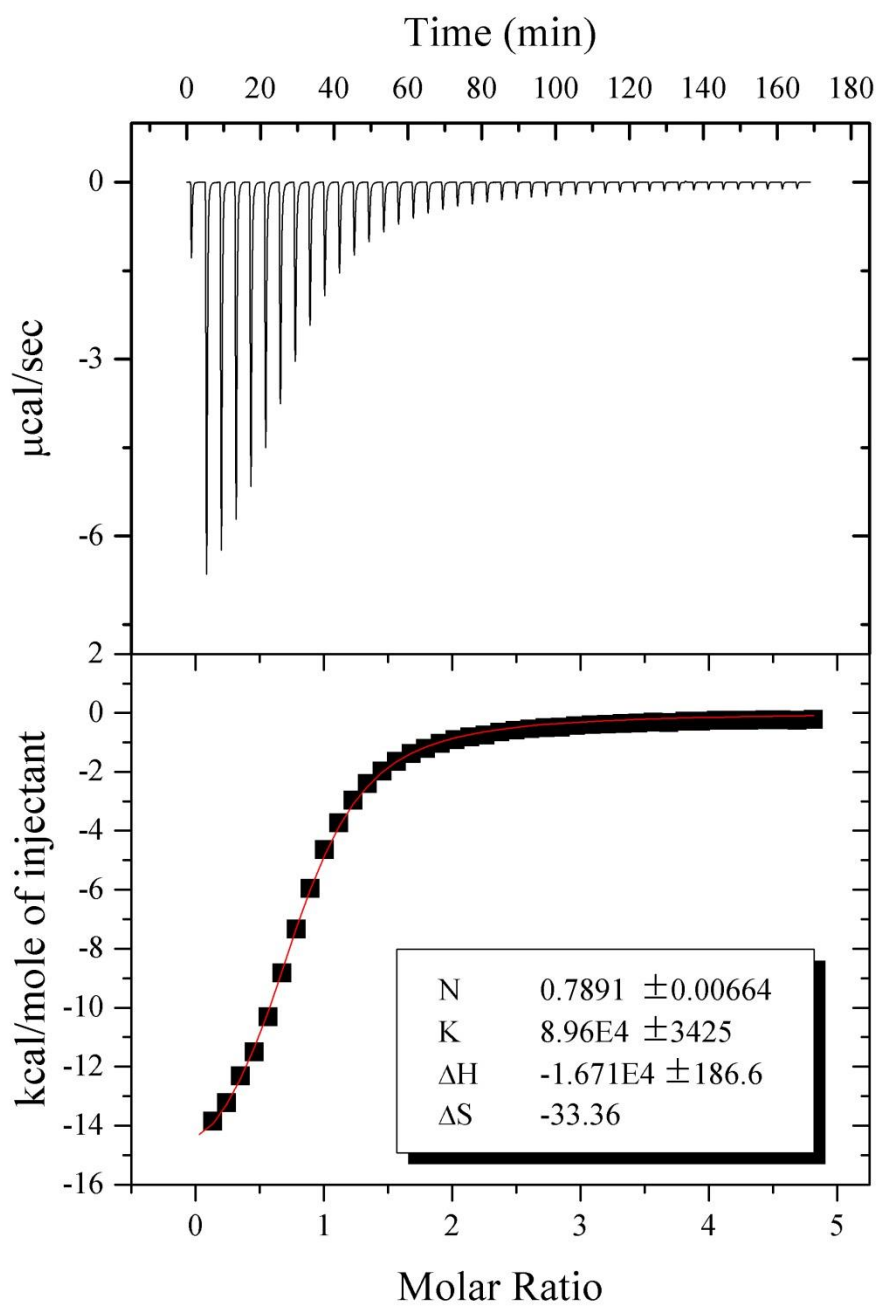


Figure S2.

ITC data measured for the binding of Lyz (0.087 mM) to L2 (2.0 mM) in aqueous ammonium acetate (50 mM, pH 6.8 and 25 °C) solutions.

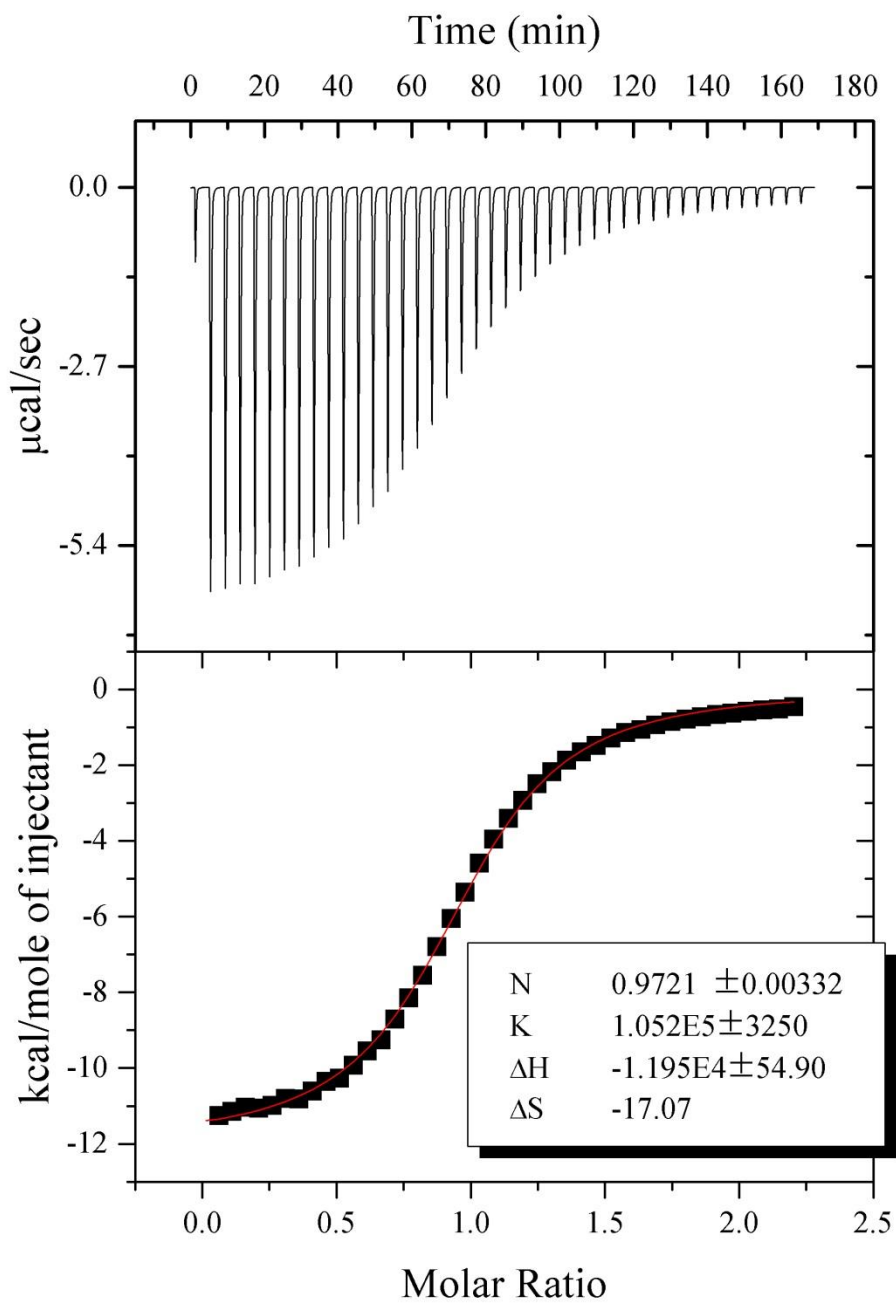


Figure S3. ITC data measured for the binding of Lyz (0.202 mM) to L3 (2.0 mM) in aqueous ammonium acetate (50 mM, pH 6.8 and 25 °C) solutions.

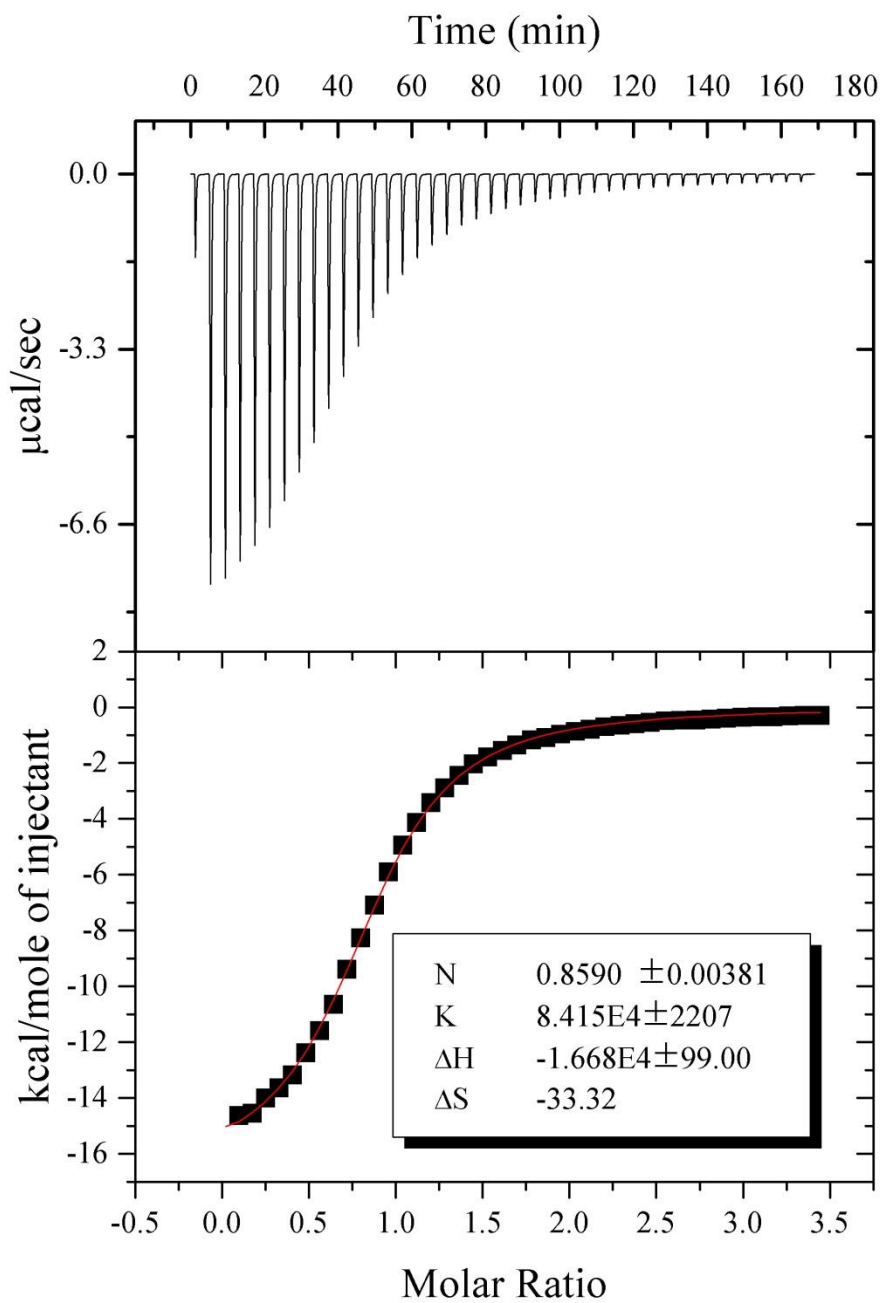


Figure S4. ITC data measured for the binding of Lyz (0.128 mM) to L2 (2.0 mM) in 1X PBS (pH 7.4 and 25 °C).

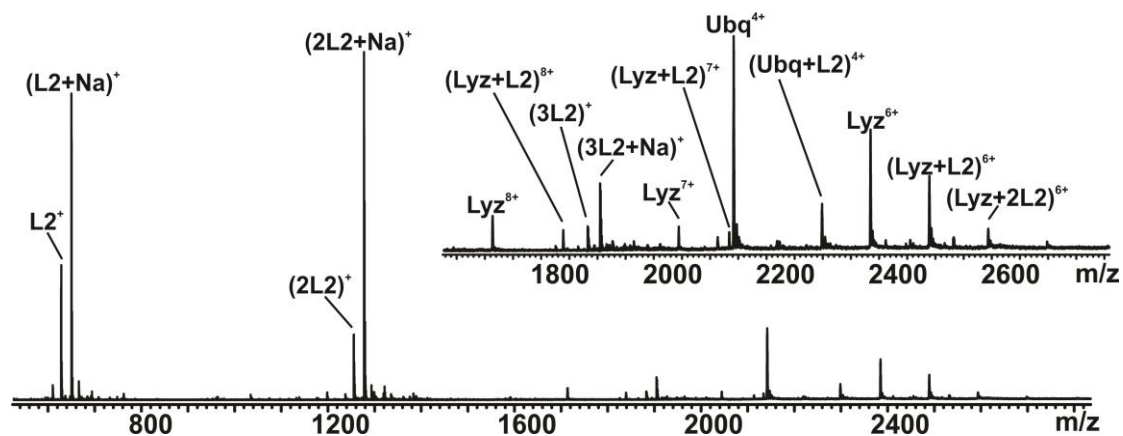


Figure S5. Representative reactive liquid sample DESI mass spectrum acquired in positive ion mode for an aqueous ammonium acetate (20 mM, pH 6.8 and 25 °C) solution containing Lyz (10 μ M) and Ubq (5 μ M) and an ESI spray solution (50/50 water/acetonitrile) that contained **L2** (50 μ M). The flow rates of both the sample and ESI spray solution were 5 μ L min^{-1} . All other instrumental conditions were identical to those used for the liquid sample DESI measurements.