Quantifying Labile Protein-Ligand Interactions using

Electrospray Ionization Mass Spectrometry

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Abstract

A new electrospray ionization mass spectrometry (ES-MS) approach for quantifying protein-ligand complexes that are prone to in-source (gas phase) dissociation is described. The method, referred to here as the *reference ligand ES-MS method*, is based on the direct ES-MS assay and competitive ligand binding. A reference ligand (L_{ref}), which binds specifically to the protein (P), at the same binding site as the ligand (L) of interest, with known affinity and forms a stable protein-ligand complex in the gas phase, is added to the solution. The fraction of P bound to L_{ref}, which is determined directly from the ES mass spectrum, is sensitive to the fraction of P bound to L in solution and enables the affinity of P for L to be determined. A mathematical framework for the implementation of the method in cases where P has one or two specific ligand binding sites is given. Affinities of two carbohydrate-binding proteins, a single chain fragment of a monoclonal antibody and the lectin concanavalin A, for monosaccharide ligands are reported and the results are shown to agree with values obtained using isothermal titration calorimetry.

Introduction

The direct electrospray ionization mass spectrometry (ES-MS) assay has emerged as a powerful technique for quantifying binding constants (K_a) for protein-ligand interactions, as well as other noncovalent biological complexes, in solution.[1-3] The assay is based on the direct detection and quantification of the abundance (*Ab*) of ligand-bound and unbound protein ions in the gas phase, e.g., PL^{n+} and P^{n+}, respectively. A key assumption is that the measured abundance ratio (*R*) is equivalent to the equilibrium concentration ratio of ligand-bound and free protein in solution, eq 1:

$$\frac{[PL]_{eq}}{[P]_{eq}} = \frac{\sum_n Ab(PL^{n+})}{\sum_n Ab(P^{n+})} = R \tag{1}$$

From the measured *R* value and initial concentrations of protein ($[P]_o$) and ligand ($[L]_o$), K_a can be calculated,[4] eq 2:

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

The direct ES-MS assay has been used to measure affinities for a range of protein-ligand complexes, including antibody-antigen, lectin-carbohydrate, enzyme-substrate/inhibitor complexes, and in many instances the K_a values agree well with constants obtained by other analytical methods, including isothermal titration calorimetry (ITC), surface plasmon resonance and frontal affinity chromatography MS.[4-10] However, there have also been reports of protein-ligand complexes that could not be detected by ES-MS or, if detected, the relative abundance of ligand-bound and unbound protein ions did not match the distribution expected in solution, with less binding observed in the gas phase.[11-14] These anomalous results are often due the occurrence of in-source dissociation, whereby the gaseous complexes undergo partial or complete

dissociation during ES-MS analysis. If the gas phase PL ions are kinetically labile and undergo dissociation during analysis, the magnitude of the measured R value and, correspondingly, the K_a value will be artificially low. In the extreme case, where no PL ions survive, in-source dissociation will result in a false negative. Recently, it was shown that solution or gas phase additives can, in some instances, protect complexes from in-source dissociation.[12,15] However, this approach does have its limitations and the detection of very labile gas phase complexes, which rapidly dissociate at ambient temperature, by ES-MS remains problematic.

Here, we describe an indirect ES-MS approach to quantify protein-ligand interactions that are highly labile and prone to in-source dissociation. The method, referred to as the *reference ligand ES-MS method*, employs direct ES-MS analysis in conjunction with a reference ligand (L_{ref}). The L_{ref} binds specifically to P, at the same binding site as L, with known affinity and forms a stable protein-ligand complex in the gas phase. The fraction of P bound to L_{ref}, which is determined directly from the ES mass spectrum, is sensitive to the fraction of P bound to L in solution and enables the affinity of the PL complex to be established. A mathematical framework for the implementation of the method in cases where P has one or two specific ligand binding sites is given. To demonstrate the reliability of the method, monosaccharide affinities were measured for two carbohydrate-binding proteins, a single chain fragment of a monoclonal antibody binding and the lectin concanavalin A, and the values compared with data obtained using ITC.

Experimental Methods

Proteins and ligands

The carbohydrate-binding antibody single chain fragment, Se155-4 scFv (MW 26 539 Da), was produced using recombinant technology.[16] The scFv was concentrated and dialyzed

against aqueous solution of 50 mM ammonium acetate (pH 7) using microconcentrators (Millipore Corp., Bedford, MA) with a molecular weight cut-off of 10 kDa, and lyophilized prior to MS analysis. The scFv was weighed immediately after removing it from the lyophilizer, dissolved in a known volume of aqueous 50 mM ammonium acetate and stored at –20 ^{o}C if not used immediately. The proteins concanavalin A (ConA, MW 25 600 Da for monomer) and lysozyme (Lyz, MW 14 311 Da) were purchased from Sigma-Aldrich Canada (Oakville, Canada), and used without further purification. The synthetic carbohydrate ligands, abequose methyl glycoside (**1**), methyl 3-deoxyarabinose (**2**), methyl-α-D-glucopyranoside (**3**) and αAbe(2-OCH$_3$-αMan)αGlcβGlc (**4**) were provided by Prof. D. Bundle (University of Alberta), while methyl-α-D-mannopyranoside (**5**) and α-methyl-3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (**6**) were purchased from Toronto Research Chemicals Inc (North York, Canada).

Mass spectrometry

All experiments were performed on an Apex II 9.4 tesla Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers (Bruker, Billerica, MA) equipped with an external nanoflow ES ion source. A description of the instrument and the experimental and instrumental parameters used in the ES-MS binding measurements is given elsewhere.[4]

Results and discussion

Overview of reference ligand method

The influence of in-source dissociation on ES-MS mass spectra acquired for a solution of P and two ligands, L and L$_{ref}$, which bind at the same site, is depicted in Figure 1. In the absence of in-source dissociation (and assuming uniform response factors and an absence of nonspecific ligand binding), the measured abundance ratios of ligand-bound to free P ions (R and R_{ref}) will

reflect the concentration ratios in solution (Figures 1a,b) and K_a for the PL complex can be calculated using eq 3:

$$K_a = \frac{R}{[L]_o - \dfrac{R}{1+R+R_{ref}}[P]_o} \quad (3)$$

where R is given by eq 1 and R_{ref} by eq 4:

$$\frac{[PL_{ref}]_{eq}}{[P]_{eq}} = \frac{\sum\limits_n Ab(PL_{ref}^{\ n+})}{\sum\limits_n Ab(P^{n+})} = R_{ref} \quad (4)$$

If, on the other hand, the gas phase ions of the PL complex are kinetically labile and undergo in-source dissociation, the relative abundance of ligand-bound and free protein ions measured by ES-MS will no longer reflect the original concentration ratios (Figures 1a,c). In the extreme case, where no PL ions survive until detection, the measured abundance ratio of L_{ref}-bound P to free P gas phase ions (i.e., $R_{ref,app}$) will depend on the concentration of both free P and PL originally present in solution, eq 5:

$$\frac{[PL_{ref}]_{eq}}{[P]_{eq} + [PL]_{eq}} = \frac{\sum\limits_n Ab(PL_{ref}^{\ n+})}{\sum\limits_n Ab(P^{n+})} = R_{ref,app} \quad (5)$$

However, if $K_{a,ref}$ is known, R_{ref} (the "true" concentration ratio $[PL_{ref}]/[P]$) can be calculated from $R_{ref,app}$, eq 6:

$$R_{ref} = K_{a,ref}([L_{ref}]_o - [P]_o \frac{R_{ref,app}}{R_{ref,app}+1}) \quad (6)$$

Furthermore, the magnitude of R can be calculated from the corresponding $R_{ref,app}$ and R_{ref} values, eq 7:

$$R = \frac{R_{ref}}{R_{ref,app}} - 1 \quad (7)$$

6

Once R and R_{ref} are known, the K_a value for the PL complex can be calculated using eq 3.

It should be noted that the above treatment can easily be extended to the case where the gas phase PL ions undergo partial dissociation in the source. In this situation, R_{ref} can be determined from the measured abundance ratio of L_{ref}-bound P to free P gas phase ions (i.e., $R_{ref,app}$) and the measured abundance ratio of L-bound P to free P gas phase ions (i.e., R_{app}) using eq 8:

$$R_{ref} = K_{a,ref} ([L_{ref}]_o - [P]_o \frac{R_{ref,app}}{R_{ref,app} + R_{ref,app} + 1})$$ (8)

and R can be found using eq 9:

$$R = \frac{(1 + R_{app})}{R_{ref,app}} R_{ref} - 1$$ (9)

Once R and R_{ref} are known, K_a can be calculated in the usual way.

Because many proteins possess multiple ligand binding sites, it is also useful to consider how the *reference ligand ES-MS method* can be applied to these situations. Given below are the relevant mathematical expressions for the application of the method for the case where P possesses two equivalent and independent ligand binding sites. Expressions for the equilibrium constants ($K_{a,1}$ and $K_{a,2}$) for the sequential binding of L to P in the presence of L_{ref}, written in terms of the concentration ratios of ligand bound (L or L_{ref}) P to free P, are:

$$K_{a,1} = \frac{R_{1,0}}{[L]_o - \frac{(R_{1,0} + 2R_{2,0} + R_{1,1})[P]_o}{1 + R_{1,0} + R_{2,0} + R_{0,1} + R_{1,1} + R_{0,2}}}$$ (10a)

$$K_{a,2} = \frac{R_{2,0}}{R_{1,0}([L]_o - \frac{(R_{1,0} + 2R_{2,0} + R_{1,1})[P]_o}{1 + R_{1,0} + R_{2,0} + R_{0,1} + R_{1,1} + R_{0,2}})}$$ (10b)

where $R_{i,j}$ is the concentration ratio of ligand-bound P (to i molecules of L and j molecules of L_{ref}) to free P. As before, in the absence of in-source dissociation, the $R_{i,j}$ values can be determined directly from the relative abundance of ligand-bound and free P ions measured by ES-MS, eq 11:

$$\frac{[P(L)_i(L_{ref})_j]_{eq}}{[P]_{eq}} = \frac{\sum_n Ab(PL_i L_{ref,j}{}^{n+})}{\sum_n Ab(P^{n+})} = R_{i,j} \qquad (11)$$

However, if in-source dissociation takes place, the measured ratios will no longer reflect solution composition. In the extreme case, where none of the P-L interactions survive the ion source, only the ratios $R_{0,1app}$ and $R_{0,2app}$ can be determined from the ES mass spectrum:

$$\frac{\sum_n Ab(PL_{ref}{}^{n+})}{\sum_n Ab(P^{n+})} = R_{0,1app} \qquad (12a)$$

$$\frac{\sum_n Ab(P(L_{ref})_2{}^{n+})}{\sum_n Ab(P^{n+})} = R_{0,2app} \qquad (12b)$$

Using the known values of $K_{a1,Lref}$, $[L_{ref}]_o$, $[P]_o$, $R_{0,1app}$ and $R_{0,2app}$, the $R_{i,j}$ terms can be calculated using following expressions:

$$R_{0,1} = K_{a1,Lref}([L_{ref}]_o - [P]_o \frac{R_{0,1app} + 2R_{0,2app}}{1 + R_{0,1app} + R_{0,2app}}) \qquad (13a)$$

$$R_{0,2} = (R_{0,1})^2 / 4 \qquad (13b)$$

$$R_{1,1} = \frac{R_{0,1app} R_{0,2}}{R_{0,2app}} - R_{0,1} \qquad (13c)$$

$$R_{1,0} = -2 + 2\sqrt{\frac{R_{0,2}}{R_{0,2app}}} \qquad (13d)$$

$$R_{2,0} = 1 + \frac{R_{0,2}}{R_{0,2app}} - 2\sqrt{\frac{R_{0,2}}{R_{0,2app}}} \qquad (13e)$$

and $K_{a,1}$ and $K_{a,2}$ can be calculated using eqs 10a and 10b.

The two basic requirements for a suitable L_{ref} are that it binds specifically to P in solution, at the same binding site as L, with a known affinity and that it forms a stable protein-ligand interaction in the gas phase, i.e., is resistant to in-source dissociation. In addition there are several other practical considerations. It is desirable to use a reasonably strong binding ligand as L_{ref}, with a $K_{a,ref}$ of $10^5 - 10^7$ M^{-1}, since in this case R_{ref} and $R_{ref,app}$ are more sensitive to the presence of the competing ligand. Additionally, changes in R_{ref} are more pronounced when low protein concentrations (~μM) are used. Finally, depending on the magnitude of K_a and $K_{a,ref}$, the concentrations of P and both ligands (L and L_{ref}) may need to be adjusted so that both complexes are present in solution at significant concentrations.

Determination of protein-ligand affinities

To demonstrate the reliability of the *reference ligand ES-MS method* for quantifying protein-ligand interactions that are not readily detected by ES-MS, binding measurements were carried out on two carbohydrate-binding proteins, Se155-4 scFv and ConA. Association constants have been determined by ITC for the binding of Se155-4 scFv to the monosaccharides **1** (1.5 x 10^3 M^{-1}) and **2** (1.2 x 10^2 M^{-1}) at pH 7 and 25 ^{o}C.[17] The monosaccharide **3**, which was shown by ITC not to bind to the scFv,[18] served as a negative control. Con A is a tetramer above pH 7 and a dimer below pH 6. Each subunit possesses a single carbohydrate binding site with specificity for the α-pyranose forms of Glc or Man. The affinity of dimeric ConA for the monosaccharide **5** was determined to be (7.9 ± 1.0) x 10^3 M^{-1} at pH 5.2 and 25 ^{o}C by ITC.[19]

a. scFv-monosaccharide binding

Shown in Figure 2a is a representative ES mass spectrum acquired for a solution of scFv (10 μM) and **1** (1 mM) at pH 7 and 25 $^{\circ}$C. A reference protein (Lyz) was also added to monitor for the occurrence of nonspecific ligand binding to scFv during the ES process.[20] At these concentrations, 60% of the scFv is expected to be bound to **1** in solution. However, no gas phase ions corresponding to the specific (scFv + **1**) complex were detected, indicating the occurrence of in-source dissociation. Attempts to stabilize the complex during ES-MS analysis using a high concentration (10 mM) of imidazole, a stabilizing additive,[12] were unsuccessful (data not shown). The instability of the gas phase ions of the (scFv + **1**) complex can be explained by the small number of intermolecular hydrogen bonds that **1** is capable of making. Direct ES-MS analysis of solutions of scFv (10 μM) with **2** (2 mM) or **3** (1 mM) also failed to detect ions of the (scFv + **2**) or (scFv + **3**) complex (data not shown). In contrast, ES-MS analysis of a solution of scFv (10 μM) and **4** (59 μM) clearly identified the presence of gas phase ions corresponding to the (scFv + **4**) complex, Figure 2b. The K_a value determined directly from the ES mass spectrum, following correction for nonspecific ligand binding,[20] was $(1.6 \pm 0.5) \times 10^5$ M^{-1}. Shown in Figure 2c is a representative ES mass spectrum acquired for a solution of scFv (10 μM), **1** (1 mM) and **4** (59 μM). Although ions corresponding to the (scFv + **1**) complex were not detected, the addition of **1** to the solution resulted in a decrease in the fraction of scFv bound to **4**, indicating the presence of specific binding between scFv and **1** in solution. Plotted in Figure 3 is the fraction of bound (to **4**) and unbound scFv determined by direct ES-MS measurements at fixed concentrations of scFv (10 μM) and **4** (59 μM) and varying concentrations of **1** (0, 500, 1000 and 2000 μM). Notably, the fraction of scFv bound to **4** decreased with increasing concentration of **1**. Analysis of the ES-MS data using the approach described above leads to an average K_a value of $(1.4 \pm 0.3) \times 10^3$ M^{-1} for the (scFv + **1**) complex. This value is indistinguishable, within

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experimental error, from the ITC-derived value, of $(1.5 \pm 0.4) \times 10^3$ M^{-1}.[17] Following the same approach, a K_a value of $(1.7 \pm 0.9) \times 10^2$ M^{-1} was determined for the (scFv + **2**) complex, which is also in good agreement with the ITC value of $(1.2 \pm 0.5) \times 10^2$ M^{-1}.[17] In contrast, the assay did not detect any binding between the scFv and **3**, consistent with the results of ITC measurements.[18]

b. ConA-monosaccharide binding

To demonstrate that the *reference ligand ES-MS method* is generally applicable to the quantification of labile protein-ligand interactions, the method was also used to measure the affinity of the lectin ConA for the monosaccharide **5**. The binding measurements were performed at pH 5.2, where ConA exists predominantly as a homodimer. At this pH, ConA is known to bind the monosaccharide **5**, as well as oligomannose ligands such as **6**, which served as L_{ref} for these measurements.[21] Importantly, it was previously shown that **5** and **6** bind in the same binding site.[22] In an earlier study, it was reported that the ConA-**5** interaction is quite labile in the gas phase and can be, depending on the ES-MS instrumentation used, difficult to detect.[11] In the present work, it was found that the complex could be directly detected by ES-MS. However, the relative abundance of the ligand-bound forms of ConA dimer was sensitive to ion source conditions, such as hexapole accumulation time. In order to demonstrate the reliability of the assay in cases where the complex could not be directly detected, source conditions that lead to complete dissociation of the ConA-**5** interactions were used.

Shown in Figure 4a is an ES mass spectrum measured for a solution of ConA (30 μM for monomer) at pH 5.2. Notably, only ions corresponding to ConA homodimer (ConA$_2$) were identified; no ions corresponding to monomer or homotetramer were detected. Upon addition of **6** (15 μM) to the solution, ions corresponding to ConA$_2$ bound to one and two molecules of **6**

were also detected (Figure 4b). The intrinsic affinity of $ConA_2$ for **6**, based on the $K_{a,1}$ and $K_{a,2}$ values determined directly from the ES mass spectra, is (5 ± 2) x 10^5 M^{-1}. This value is in excellent agreement with a value of (5.1 ± 0.2) x 10^5 M^{-1} that was determined by ITC for homodimer of ConA at pH 5.2.[21] Under the experimental conditions used, ES-MS analysis of a solution of $ConA_2$ (13 μM) with **5** (50 or 100 μM) and **6** (15 μM) led to the detection of ions corresponding to $ConA_2$ bound to one and two molecules of **6** but not ions corresponding to $ConA_2$ bound to **5** (Figure 4c). However, the addition of **5** to the solution did result in a small but measurable reduction in the fraction of $ConA_2$ bound to **6**. Following the procedures outlined above, the intrinsic K_a for the $ConA_2$-**5** interaction was determined to be (1.0 ± 0.4) x 10^4 M^{-1}, which is in reasonable agreement with the ITC-derived value of (7.9 ± 1.0) x 10^3 M^{-1}.[19]

Conclusions

In summary, an ES-MS approach for quantifying protein-ligand interactions that are prone to in-source dissociation and, therefore, difficult to detect directly is described. The *reference ligand ES-MS method* employs the direct ES-MS assay in conjunction with a L_{ref}, which binds competitively to protein of interest with known affinity and forms a stable complex in the gas phase. The relative abundance of L_{ref}-bound protein to free protein, which can be measured directly by ES-MS, is sensitive to the presence of other ligands in solution that compete for the same binding site. As a result, it is possible to quantify protein-ligand interactions that are unstable in the gas phase by measuring the relative abundance of L_{ref}-bound protein using the direct ES-MS assay. The relevant mathematical expressions for the implementation of the method for proteins with a single ligand binding site or two equivalent binding sites are given. To demonstrate the reliability of the method, the binding of monosaccharide ligands to two carbohydrate-binding proteins was quantified. Importantly, the

carbohydrate affinities were found to be in good agreement with values measured using ITC. It is anticipated that this method will prove particularly useful in extending the application of ES-MS binding measurements to protein interactions with small or hydrophobic ligands and for implementing a small fragment approach to the elucidating details of protein-oligosaccharide interactions.[23]

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Figure captions

Figure 1.   Influence of in-source dissociation on the relative abundance of gas phase protein-ligand complexes. (a) Hypothetical distribution of species present in solution when ligands L and L_{ref} interact with a single binding site of protein P. (b) In the absence of in-source dissociation, the relative abundance of gas phase PL^{n+} and PL_{ref}^{n+} ions will reflect the original distribution in solution. (c) In-source dissociation of the PL^{n+} ions (but not the PL_{ref}^{n+} ions) will result in a decrease in the measured abundance ratio of PL_{ref}^{n+} to P^{n+} ions.

Figure 2.   ES mass spectra obtained for the solutions of Se155-4 scFv (10 μM) and (a) **1** (1000 μM), (b) **4** (59 μM) and (c) **4** (59 μM) and **1** (1000 μM). The number of molecules of **4** bound to the protein ions is indicated by q.

Figure 3.   Distribution of the relative abundance of scFv ($\equiv$ P) and (scFv + **4**) complex ($\equiv$ PL) measured by ES-MS for solutions of scFv (10 μM), **4** (59 μM) and **1** at concentrations of 0, 500, 1000 and 2000 μM.

Figure 4.   ES mass spectra obtained for the solutions of (a) $ConA_2$ (15 μM) alone and with (b) **6** (15 μM) and (c) **6** (15 μM) and **5** (100 μM). The number of molecules of **6** bound to the protein ions is indicated by q.
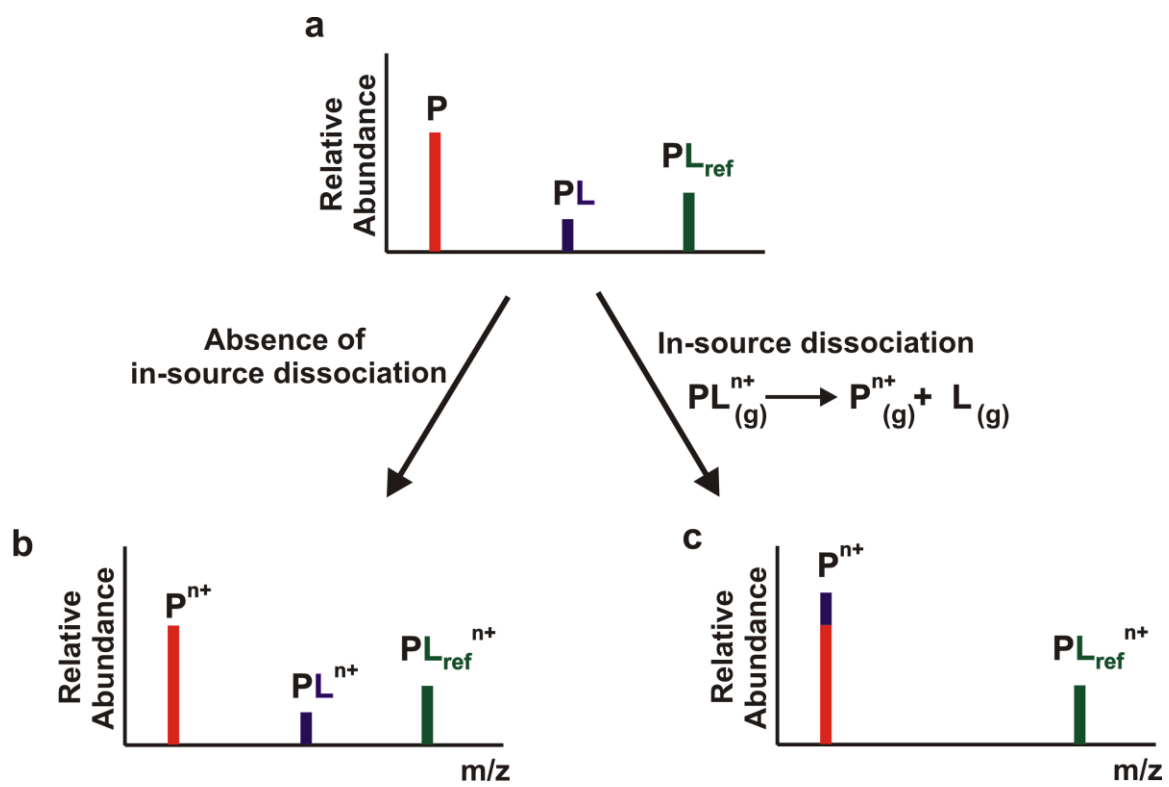
a

Relative Abundance

P   PL   PL$_{ref}$

Absence of in-source dissociation

In-source dissociation

$$PL^{n+}_{(g)} \longrightarrow P^{n+}_{(g)} + L_{(g)}$$

b

Relative Abundance

P^{n+} PL^{n+} PL_{ref}^{n+}

m/z

c

Relative Abundance

P^{n+} PL_{ref}^{n+}

m/z

Figure 1

19

a

P_{ref}^{+9} P_{ref}^{+8} P_{ref}^{+7} P_{ref}^{+6} $scFv^{+11}$ $scFv^{+10}$ $scFv^{+9}$

b

$(scFv+q4)^{+10}$

$(P_{ref}+q4)^{+8}$
$q=0$

$(P_{ref}+q4)^{+7}$
$q=0$

$(scFv+q4)^{+11}$

$(scFv+q4)^{+9}$

1

1

1

1
$q=0$

2

$q=0$

2

$q=0$

1

2

c

$(scFv+q4)^{+10}$
$q=1$

$(scFv+q4)^{+11}$

$(P_{ref}+q4)^{+8}$
$q=0$

$(scFv+q4)^{+9}$

1

P_{ref}^{+7}

$q=0$

1

2

0

1

2

$q=0$

1

1500   2000   2500   3000   3500

Figure 2

20

Figure 3

a

ConA$_2^{+15}$

ConA$_2^{+16}$

ConA$_2^{+14}$

b

(ConA$_2$+q6)$^{+15}$

1

(ConA$_2$+q6)$^{+16}$

2

(ConA$_2$+q6)$^{+14}$

q=0

1

2

q=0

1

q=0

1

2

c

(ConA$_2$+q6)$^{+14}$

1

(ConA$_2$+q6)$^{+15}$

q=0

1

2

q=0

2
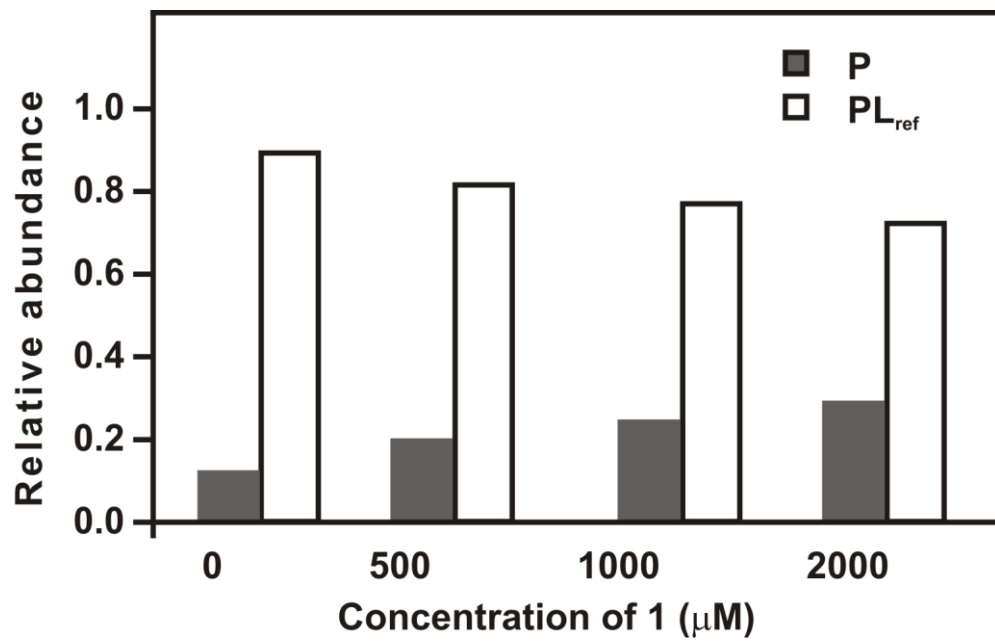
q=0

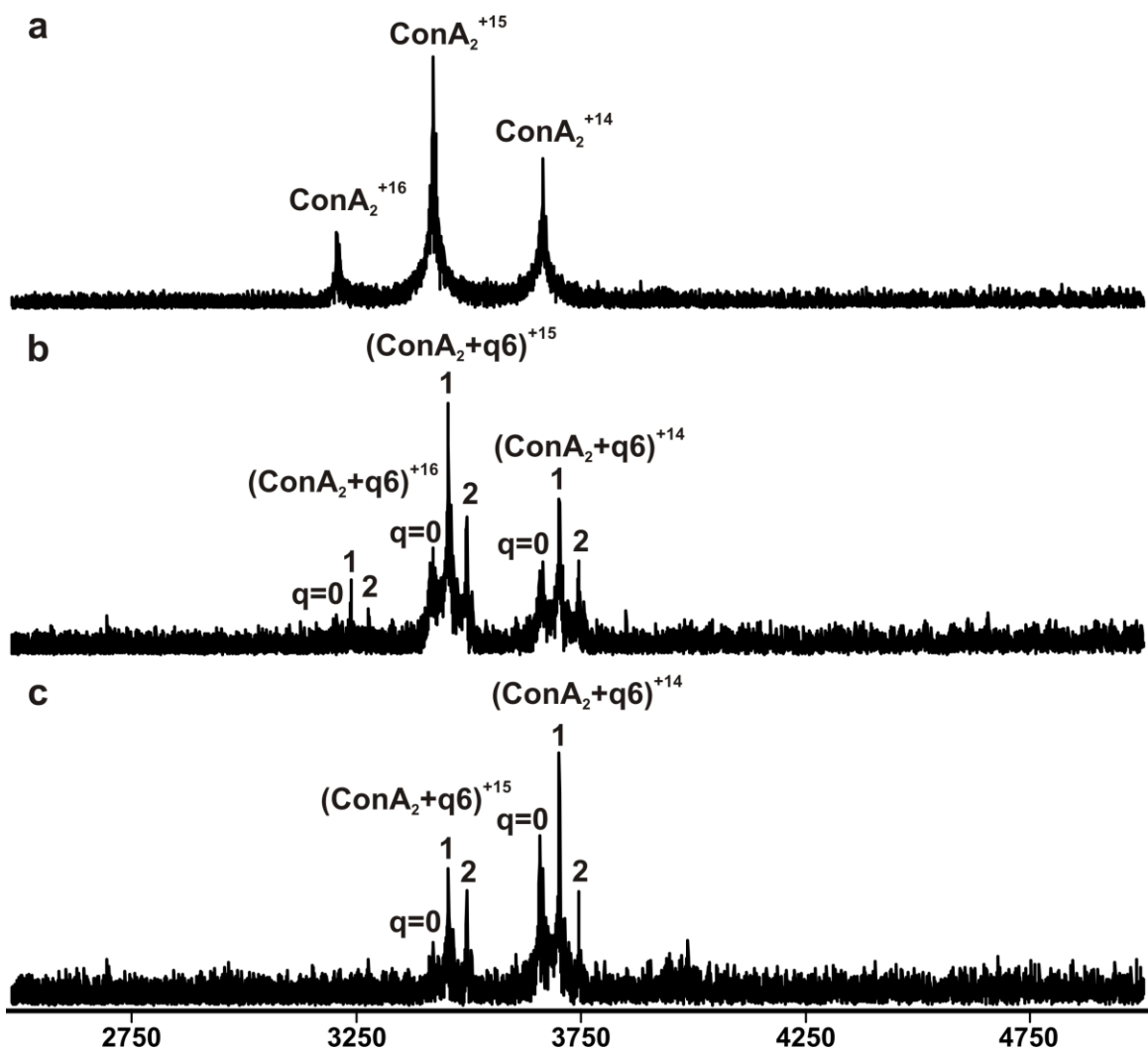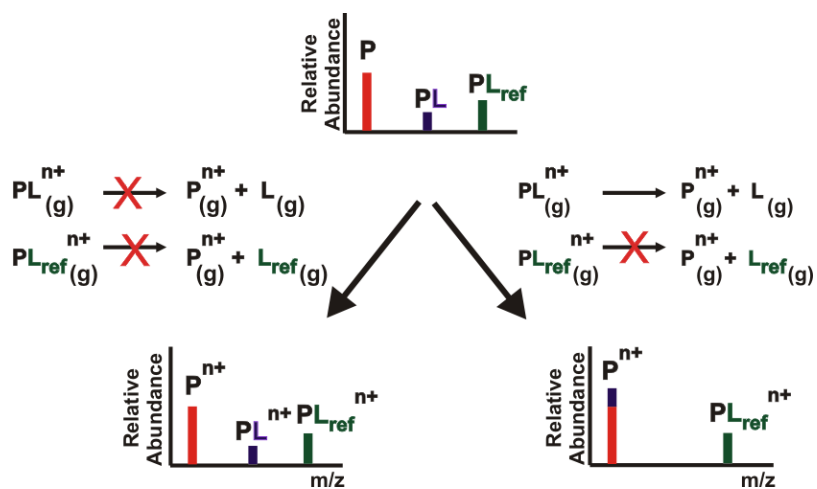2750   3250   3750   4250   4750

Figure 4

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Graphical Abstract

Synopsis: Affinities of labile protein-ligand complexes are quantified using the direct ES-MS assay and competitive binding with a reference ligand.