Nanoscale Ion Emitters in Native Mass Spectrometry for Measuring Ligand–Protein Binding Affinities

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ABSTRACT: Electrospray ionization (ESI) mass spectrometry (MS) is a crucial method for rapidly determining the interactions between small molecules and proteins with ultrahigh sensitivity. However, nonvolatile molecules and salts that are often necessary to stabilize the native structures of protein–ligand complexes can readily adduct to protein ions, broaden spectral peaks, and lower signal-to-noise ratios in native MS. ESI emitters with narrow tip diameters (~250 nm) were used to significantly reduce the extent of adduction of salt and nonvolatile molecules to protein complexes to more accurately measure ligand–protein binding constants than by use of conventional larger-bore emitters under these conditions. As a result of decreased salt adduction, peaks corresponding to protein–ligand complexes that differ in relative molecular weight by as low as 0.06% can be readily resolved. For low-molecular-weight anion ligands formed from sodium salts, anion-bound and unbound protein ions that differ in relative mass by 0.2% were completely baseline resolved using nanoscale emitters, which was not possible under these conditions using conventional emitters. Owing to the improved spectral resolution obtained using narrow-bore emitters and an analytically derived equation, $K_I$ values were simultaneously obtained for at least six ligands to a single druggable protein target from one spectrum for the first time. This research suggests that ligand–protein binding constants can be directly and accurately measured from solutions with high concentrations of nonvolatile buffers and salts by native MS.

INTRODUCTION

The interactions between proteins and ligands are crucial to proper cellular function. The structures, functions, and interactions of protein–ligand complexes can be significantly affected by salts. Specific metal ion cofactors can regulate the bioactivity of proteins. In native mass spectrometry (MS), ligand–protein interactions are normally stabilized using volatile salts at high ionic strengths to rapidly and directly measure the mass, binding stoichiometry, and relative ligand–protein binding affinities with high sensitivity. However, most biochemical approaches to probe protein–ligand interactions, including nuclear magnetic resonance spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, and optical spectroscopy, use nonvolatile salts that can more accurately reflect the in vivo environment of the protein–ligand complex. However, nonvolatile salts and common biological buffers readily adduct to proteins ions to result in broad spectral peaks that have deleterious effects on mass spectra by lowering the sensitivity and signal-to-noise ratios and increasing background chemical noise. In addition, the spectral resolution is readily degraded such that peaks corresponding to bound ligand–protein complexes cannot be resolved from the unbound protein using common buffers such as tris(hydroxymethyl)aminomethane (Tris), which hinders the measurement of ligand–protein binding affinities including for more than a few ligands that are competing for a single protein binding site.

Owing to the adverse effects of nonvolatile salts, protein samples for native MS typically need to be desalted and buffer exchanged into ammonium acetate solutions for compatibility with electrospray ionization (ESI) mass spectrometry. However, some proteins and protein complexes require biological buffers (e.g., Tris) and high metal salt concentrations.
for stabilization and noncovalent assembly.\textsuperscript{22,23} Moreover, by use of volatile buffers in native MS, the direct measurement of multiple ligands binding potently to a single protein in one native MS spectrum is limited by the adduction of adventitious nonvolatile salt (e.g., sodium ions), which can make it challenging to resolve ligand–protein complexes that differ by less than 1.0% in relative mass. For example, ESI-MS has been used to probe the direct binding of four ligands simultaneously in a single spectrum,\textsuperscript{24} in which the complexes differed in relative mass by an average of 1.5%. Thus, competitive ligand–protein binding assays in native MS to obtain ligand–protein binding constants are rare and typically limited to two ligands.\textsuperscript{25–29}

An alternative to desalting protein samples prior to ESI-MS is to use electrospray emitters with tips that have inner diameters that are \(\sim 1\ \mu m\) or less.\textsuperscript{30–34} By reducing the emitter tip size, the level of salt adduction to protein ions can be significantly reduced. For example, Schmidt et al. have reported that ESI emitters with tip diameters of \(\sim 1\ \mu m\) or smaller can form ions that have less sodium adduction than those formed with larger tips.\textsuperscript{30,31} Recently, Williams and co-workers\textsuperscript{33,34} have reported that, by using \(\sim 500\ \text{nm}\) diameter tips, the charge-state distributions of proteins and protein–protein complexes formed from buffers with high salt concentration can be resolved. The reduction of salt adduction and salt cluster formation can be attributable to the small initial concentration that can be resolved. The reduction of salt adduction is a challenge to resolve ligand complexes that differ by an average relative mass of 0.09% (and those that differ in relative mass by as low as 0.06%) can now be nearly completely baseline resolved. Moreover, ligand–protein binding constants can be directly, and accurately, measured in solutions containing nonvolatile buffers that are more relevant to those used in many other biochemical assays.

\section*{RESULTS AND DISCUSSION

\textbf{Effects of Emitter Tip Diameter on Ligand–Protein Binding Affinities.} The dimensions of nano-electrospray ionization emitter tips can significantly affect the extent of salt adduction to protein ions formed from aqueous buffered solutions.\textsuperscript{34,42} To investigate if such salt adduction can also impact the stability of protein–ligand complexes, nano-electrospray ionization mass spectra of two functionally different proteins with multiple ligands (Table S1) were obtained from "nativelike" solutions using emitter tips that had inner diameters of \(\sim 250, \sim 500, \sim 850, \) and \(\sim 2000\) \text{nm}. Based on scanning electron microscopy measurements, ESI emitters were fabricated with inner tip diameters that were reproducible to within a standard deviation of less than \(\pm 10\%\) (at least five fabrication replicates).

\textbf{Human Carbonic Anhydrase I and Sulfonamide Ligands.} In Figure 1, representative nano-electrospray ionization mass spectra of a buffered aqueous solution containing 5 \(\mu M\) human carbonic anhydrase I (hCAI), 2 \(\mu M\) ethozoxamide, and 70 mM ammonium acetate (pH 7.4) are shown for each tip size. The charge-state distributions for both the protein–ligand complex and the unbound protein were narrow and centered near the 10+ and 9+ charge states (Figure 1), which is characteristic for carbonic anhydrase ions formed from aqueous solutions at near neutral pH.\textsuperscript{43,44} The extent of charging and the widths of the charge-state distributions did not depend significantly on the size of the ESI emitter tips under these conditions (Figure 1). Using 2000 \text{nm} emitter tips, the extent of sodium adduction to the unbound protein ion (58 \(\pm 3\%\)) is about the same or slightly more than the ligand-bound protein ion (49 \(\pm 3\%;\) Figure 1d). Adventitious ionized sodium can originate from proteins purified from solutions with high salt concentrations, the inner surfaces of borosilicate nano-electrospray ionization capillaries, and impurities from solid ammonium acetate (\(\geq 97\%\)).\textsuperscript{45} For the peak corresponding to the unbound protein ion, the acetate adducted protein signal cannot be fully resolved from the sodium adducted signal, which results in the extent of sodium adduction to the unbound protein being slightly overestimated. Moreover, the binding of ethozoxamide results in the disappearance of the peak corresponding to the acetate-bound protein ion, which is consistent with sulfonamide-ligation to the Zn-active site of hCAI preventing the binding of acetate. By reducing the inner diameters of the tips from 2000 to 250 nm, the extent of sodium ion adduction to the bound protein decreased from 49 \(\pm 3\%\) to 14 \(\pm 2\%\) (Figure 1a–d); i.e., the use of small-bore emitters resulted in a decrease in the extent of sodium ion adduction by more than a factor of 3. The significant decrease in the extent of sodium ion adduction by use of narrow-bore...
emitter tips with inner diameters of 2000 to 250 nm. The $K_d$ values obtained using the 850, 500, and 250 nm ESI emitters (7.6–7.8 $\mu$M) are in excellent agreement with values reported in the literature based on measurements using ultraviolet spectroscopy (6.6 $\mu$M), fluorescence spectroscopy (8.6 $\mu$M), and isothermal titration calorimetry (11.1 $\mu$M).

**Binding Affinities of Low-Molecular-Weight Anions to hCA.** In addition to the sulfonamides, inorganic anions are a major class of carbonic anhydrase inhibitors. Previous research demonstrated that the activity of carbonic anhydrases can be inhibited by inorganic anionic binding, which occurs at the cationic zinc active site of carbonic anhydrases. However, owing to the low molecular weight of many anion inhibitors, high-quality mass spectra with minimal salt adduction are required to resolve peaks corresponding to the unbound and ligand-bound protein ions. Thus, the binding strength of anion inhibitors to human carbonic anhydrases using native MS has not been reported in the literature.

To determine the binding affinity of anions to human carbonic anhydrases, nano-electrospray ionization mass spectra of a buffered aqueous solution containing human carbonic anhydrases and relatively high concentrations of either sodium thiocyanate or sodium acetate (1 mM) were obtained using emitter tips with inner diameters of ~2000 and ~250 nm. By use of the 2000 nm emitter tips, the resultant individual charge distributions reported by native MS previously. By reducing the tip diameter from 2000 to 250 nm, the $K_d$ value decreased from 9.4 ± 0.3 to 7.6 ± 0.1 $\mu$M (Table S2). The $K_d$ values obtained using the 850, 500, and 250 nm ESI emitters (7.6–7.8 $\mu$M) are in excellent agreement with values reported in the literature based on measurements using ultraviolet spectroscopy (6.6 $\mu$M), fluorescence spectroscopy (8.6 $\mu$M), and isothermal titration calorimetry (11.1 $\mu$M).
Figure 2. Narrow-bore nano-electrospray ionization emitters can be used to identify binding between small anions from sodium salts and human carbonic anhydrases. Nano-electrospray ionization mass spectra of aqueous solutions containing (a, c) 5 μM human carbonic anhydrase I and 1 mM sodium thiocyanate, and (b, d) 5 μM human carbonic anhydrase II and 1 mM sodium acetate obtained using emitters with inner tip diameters of (a, b) ~2000 nm, and (c, d) ~250 nm. L corresponds to (c) thiocyanate and (d) acetate in the respective panels.

Figure 3. Ligand–protein binding constants can be directly measured in native mass spectrometry from aqueous solutions containing high concentrations of salts and biological buffers. Nano-electrospray ionization mass spectra of (a, c, e) 5 μM human carbonic anhydrase I and 3 μM brinzolamide, and (b, d, f) 5 μM human carbonic anhydrase II and 3 μM indapamide formed from (a, b) aqueous 70 mM ammonium acetate (pH 7.4), and (c–f) aqueous 50 mM NaCl and 20 mM Tris-HCl buffer (pH 7.4) using emitter tips with inner diameters of (c, d) ~2000 nm, and (a, b, e, f) ~250 nm. L corresponds to (a, e) brinzolamide and (b, f) indapamide in the respective panels.
Measuring Ligand–Protein Binding Constants in Solutions with Nonvolatile Salts and Biochemical Buffers. Human Carbonic Anhydrase I and Sulfonylamine Ligands. The effects of nonvolatile buffers on the binding affinities of sulfonylamine ligands to human carbonic anhydrases measured using nanoscale ion emitters were investigated using aqueous solutions containing high concentrations of salts and nonvolatile buffers (i.e., 50 mM NaCl and 20 mM Tris-HCl, pH 7.4; or 10 mM Na₂SO₄ and 10 mM 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid, HEPES, pH 7.4). The mass spectra acquired using 2000 nm emitter tips from these solutions resulted in broad, unresolved multimodal peaks between m/z 2000 and 4000 (Figure 3c,d); i.e., essentially no mass information regarding protein−ligand complexes could be obtained from spectra acquired from solutions containing high concentrations of nonvolatile buffers. For the aqueous solution containing 10 mM Na₂SO₄ and 10 mM HEPES (pH 7.4), the individual charge states of protein ions can be resolved but are very broad owing to the addition of sodium ions (Figure S6a,b). Thus, it was not possible to measure the binding affinities of ligands to the proteins under these conditions. In contrast, the use of the 250 nm tips resulted in spectra in which the unbound and ligand-bound protein charge-state distributions can be clearly resolved from each other (Figure 3e,f and Figure S6c,d). Moreover, the adduction of sodium ions and Tris (or HEPES) molecules to the protein and protein−ligand complex ions can be clearly resolved in the resultant mass spectra (Figure S7). For the 250 nm tips, the narrow charge-state distribution corresponding primarily to +8, +9, and +10 human carbonic anhydrase I bound to brinzolamide (Figure 3a,e) is consistent with native mass spectra of carbonic anhydrases reported previously. Moreover, in the case of human carbonic anhydrase II and indapamide, the use of NaCl and Tris (or HEPES) resulted in a reduction in the charge state in comparison to the ions formed from the ammonium acetate solutions. That is, the average charges of the complex ions shifted from 10.5 ± 0.2 for the ammonium acetate solution (Figure 3b) to 9.1 ± 0.1 and 9.3 ± 0.1 for the NaCl−Tris and Na₂SO₄−HEPES solutions (Figure 3f and Figure S6d), respectively, consistent with the formation of slightly more compact ions. In addition, the use of nonvolatile buffers and salts resulted in an increase in the abundances of the ligand−protein complexes compared to the use of ammonium acetate (Figure 3e,f, and Figures S6c,d and S8), which resulted in lower ligand−protein Kₐ values (Table 1). For example, the dissociation constant for brinzolamide to human carbonic anhydrase I that was obtained using the 250 nm tips decreased from 1.05 ± 0.05 µM for ammonium acetate solutions to 0.60 ± 0.02 and 0.76 ± 0.04 µM for the respective Tris and HEPES solutions (Table 1), which agreed well with Kₐ values obtained by CA kinetic inhibition assay (0.73 µM). Likewise, the dissociation constant obtained by use of the nonvolatile buffers and 250 nm tips for indapamide and carbonic anhydrase II were over 30% lower than that obtained using ammonium acetate solutions (Table 1), and are in good agreement with values reported in the literature that were obtained by CA kinetic inhibition assay. These results indicate that nanoscale ion emitters can be used to measure the solution-phase binding equilibria of carbonic anhydrases and ligands in relatively high concentrations of nonvolatile salts and buffers that are commonly used in ligand−protein binding assays. Nanoscale ion emitters can also be used to obtain native mass spectra of human carbonic anhydrase II in aqueous solutions containing higher concentrations of NaCl up to 150 mM (Figure S9). However, the peaks are broad, and the signal corresponding to unbound protein cannot be readily resolved from the protein−ligand complexes under these conditions.

Lysozyme and Tri-N-acetylchitotriose. In Figure S10 native mass spectra of 5 µM lysozyme (Lys) and 7 µM tri-N-acetylchitotriose in an aqueous 50 mM NaCl and 20 mM Tris-HCl buffer (pH 7.4) using a 2000 and 250 nm tip are shown. With the 2000 nm emitter tips, the dominant ions (776, 950, and 1298 m/z) in the spectra correspond to ionic salt clusters, Na⁺(NaCl)ₙ (n up to 23), and no signals corresponding to proteins and protein−ligand complexes could be identified (Figure S10b). In contrast, the charge-state distributions corresponding to the +6, +7, and +8 charge states of the protein and protein−ligand complex are well-resolved using 250 nm tips (Figure S10c). Smaller-molecular-weight clusters are also observed that are lower than 1300 m/z. The dissociation constant of tri-N-acetylchitotriose to lysozyme obtained using the Tris and NaCl solution (6.2 ± 0.1 µM) is in agreement with the literature values obtained using alternative approaches (6.6−11.1 µM).

CYP199A4 and 4-Methoxybenzoic Acid. In Figure 4, nanoelectrospray ionization mass spectra of 5 µM CYP199A4 (45008 Da) and 3 µM of the native substrate 4-methoxybenzoic acid (152 Da) in 10 mM ammonium acetate (pH 7.4) and 10 mM Tris-HCl (pH 7.4) that were obtained using 2000 and 250 nm emitter tips are shown. For 2000 nm tips and both buffers, the individual charge states of protein ions can be resolved but are very broad, and the spectral baselines are elevated owing to the adduction salt, nonvolatile buffer molecules, and/or other impurities from the recombinant protein purification process (Figure 4a,b). By use of the 250 nm tips, individual peaks corresponding to the unbound protein and ligand-bound protein can be readily resolved (Figure 4c,d). The dissociation constant of CYP199A4 and 4-methoxy benzoic acid measured in 10 mM Tris-HCl (0.39 ± 0.02 µM) is significantly lower than that obtained in 10 mM ammonium acetate (0.71 ± 0.03 µM), and the former value is in excellent agreement with that reported in the literature (0.28 µM). The benzoate group in the active site binds through salt bridges and ionic interactions with residues in the binding pocket (Figure S11), which may be affected by changes in

| Buffers | Human Carbonic Anhydrase I−brinzolamide | Human Carbonic Anhydrase II−indapamide |
|---------|----------------------------------------|---------------------------------------|
| 70 mM ammonium acetate pH 7.4 | 1.06 ± 0.05 | 3.22 ± 0.20 |
| 50 mM NaCl and 20 mM Tris pH 7.4 | 0.60 ± 0.02 | 1.85 ± 0.15 |
| 10 mM Na₂SO₄ and 10 mM HEPES pH 7.4 | 0.76 ± 0.04 | 2.05 ± 0.25 |
| Literature | 0.73 | 2.52 |

*See Figure 1 for details. bKₐ values are obtained from the average of triplicate measurements for two different ligand concentrations. This work; measured using a CA kinetic inhibition assay.
the ionic strength of the buffer (i.e., the 10 mM Tris-HCl buffer has an ionic strength that is over 3 orders of magnitude higher than that of 10 mM ammonium acetate). This result provides additional evidence that solution-phase binding affinities measured in native MS using nanometer emitter tips and nonvolatile buffers can more accurately reproduce the dissociation constants measured in biochemical assays using similar buffers. Moreover, this approach can be used to improve the spectra obtained for proteins that are purified in-house from complex mixtures, such as E. coli cultures, utilizing standard biological buffers.

**Competition Experiments: Measuring $K_d$ Values for Multiple Ligands Simultaneously.** In Figure 5, nano-electrospray ionization mass spectra of aqueous solutions containing 20 $\mu$M human carbonic anhydrase; 4 $\mu$M each of ethoxzolamide (258 Da), brinzolamide (383 Da), furosemide (330 Da), dichlorophenamide (222 Da);15 $\mu$M indapamide (365 Da) (Scheme S1); and 70 mM ammonium acetate (pH 7.4) that were obtained using both nanoscale (250 nm) and microscale (2000 nm) emitter tips are shown. By use of the 2000 nm emitter tip, the peaks corresponding to each of the six individual protein−ligand complexes cannot be resolved from one another; i.e., the peaks corresponding to protein−ligand complexes could not be assigned under these conditions (Figure 5a,b). In striking contrast, the use of the 250 nm tip results in the baseline resolution of each of the six protein−ligand complexes in the single spectra for both human carbonic anhydrase I and II (Figure 5c,d). These results indicate that nanoscale ion emitters can be useful for probing the binding of more than two ligands to a single protein target simultaneously, which is normally not possible for many different types of other more common biochemical assays.

A general equation for obtaining the $K_d$ values for more than a few ligands to one protein from ESI mass spectra with different ligand concentrations has not been reported in the literature. For example, Bligh et al.28 reported an equation that can be used to obtain $K_d$ constants for a single protein target with at most two ligands. Thus, we analytically derived a general equation to obtain $K_d$ values for a protein with one ligand-binding site in the presence of multiple, competing ligands that are each at different concentrations (see the SI for derivation):

$$
K_{di} = \frac{\sum_n P^{n+}L_i^{0-}}{\sum_n P^{n+}L_i^{0-} + \sum_n \sum_{i'} P^{n'+}L_{i'}^{0-} + \sum_n [L_i]_0 + \sum_{i'} [L_{i'}]_0 + [P]_0}
$$

where $K_{di}$ corresponds to the dissociation constant of the $i$th ligand ($L_i$); $P^{n+}$ and $PL^{n+}$ correspond, respectively, to the ion abundances of the unbound protein and $L_i$-bound complex; and $[L_i]_0$ and $[P]_0$ correspond to the initial concentrations of the ligands and protein, respectively. An assumption used to obtain this equation is that the ionization efficiencies of the unbound protein and ligand−protein complex are essentially the same, which should hold for low-molecular-weight...
molecules binding to high-molecular-weight proteins. Using eq 1, a program was written (PLbinding) that can be used to automatically integrate the abundances of peaks corresponding to the unbound protein and ligand–protein complexes in mass spectra, and obtain the $K_d$ values based on the integrated abundances, and the initial solution-phase concentrations of the ligands and protein. This is a universal expression that can be used for as many ligands as possible to the extent that the ligand–protein complexes can be sufficiently resolved, and the ligands target a single binding site.

There are five examples in the literature in which $K_d$ values for multiple ligands have been obtained by ESI-MS measurements (Figure 6).25−29 Four of these involved the simultaneous measurement of two ligands to a single protein target, and another obtained the $K_d$ values from ESI-MS measurements for three ligands all at the same initial solution-phase concentration as that of the protein. Using nanoscale ion emitters and eq 1, the $K_d$ values for at least six ligands can be simultaneously measured using different concentrations for each ligand and the protein. This approach is useful for measuring the dissociation constants for ligands that bind relatively weakly (e.g., indapamide to hCAI, 9.5 ± 1.0 μM) in the presence of ligands that bind more strongly (e.g., ethoxzolamide to hCAI; 0.016 ± 0.004 μM) in ESI-MS (Figure 6a). In addition, carbonic anhydrase–ligand complexes that differ by 18 Da (0.06%) or more in mass can be resolved using the nanoscale ion emitters. Although the minimum resolution required59 to resolve these protein ligand complexes is a factor of 7 higher than reported previously to obtain $K_d$ values (Figure 6b), resolving such complexes for smaller ligands with relative protein–ligand masses that differ by less than 0.06% is anticipated to be challenging based on these results.

By use of eq 1, $K_d$ values can be readily obtained from a competition experiment in native MS by sequentially increasing the ligand concentration to ensure that protein–ligand complex ions are sufficiently abundant to determine that the ligands bind to the protein. The $K_d$ values of six ligands to both human carbonic anhydrase I and II that were obtained from the native MS competition experiment are in excellent agreement with both the literature values for each of the ligands, and the $K_d$ values that were obtained by measuring ESI mass spectra of each ligand individually with each protein (Figure 5, Figures S12–S15, and Table 2). These results

![Figure 5](https://example.com/figure5.png)
and protein chromatography techniques as reported for K₁ binding experiment. (a) Number of the performance of ESI-MS for obtaining multiple ligand in a Competition Experiment (Competition) Tips That Have an Inner Diameter of 250 nm in Individual Ligand Acetazolamide to Human Carbonic Anhydrase I and II Using Nano-Electrospray Ionization Mass Spectrometry with Emitter complexes that are closest in mass (\(M_2\)) are

\[ \text{minimum resolution (R)} = \frac{(m/z)_2 - (m/z)_1}{(m/z)_1} \]

where \((m/z)_1\) and \((m/z)_2\) are \(m/z\) values of the respective complexes.

Figure 6. Nanoscale ion emitters can be used to significantly improve the performance of ESI-MS for obtaining multiple ligand–protein dissociation constants from single mass spectra in a competitive binding experiment. (a) Number of \(K_b\) values obtained simultaneously from a single ESI mass spectrum in a competition experiment, and (b) minimum resolution (R) required to resolve the two protein–ligand complexes that were the closest in mass for each study; i.e., \(R = M_2/(M_2 - M_1) = ((m/z)_2/((m/z)_2 - (m/z)_1))\), where \(M_1\) and \(M_2\) are the average molecular weights of the protein–ligand complexes that are closest in mass \((M_2 > M_1)\), and \((m/z)_2\) and \((m/z)_1\) are \(m/z\) values of the respective complexes.

indicate that nanoscale ion emitters in native MS can be used to measure the binding affinities of six or more ligands to a single protein from one mass spectrum to the extent that the mass of the intact ligand–protein complexes can be resolved.

\[ K_d = \frac{[L][P]}{[LP]} \]

**Experimental Section**

Lysozyme (chicken egg white), human carbonic anhydrase I and II (human erythrocytes), and all small molecules and salts were obtained from Sigma-Aldrich and used without further purification. CYP199A4 was produced recombinantly using *Escherichia coli* and purified using standard biological buffers and protein chromatography techniques as reported for crystallographic studies with this enzyme (Table S3). Aqueous stock solutions of proteins (100 μM) were desalted twice using a centrifugal filter with a 10 kDa cutoff (Amicon Ultra 0.5 mL, Merck, Germany) in which 300 μL of the stock solution was loaded and filtered, and rinsed again with 300 μL of fresh deionized water. The protein concentrations in ESI solutions were obtained using a microvolume spectrophotometer (DeNovix DS-11). Solutions for ESI were prepared by diluting protein into the corresponding buffer at a concentration of 5–20 μM. For the competition and the low-molecular-weight anion binding experiments, \(K_d\) values were obtained from the native mass spectra of a single solution, which was measured in triplicate. For all other experiments, the \(K_d\) values were obtained from the average of triplicate measurements for at least two different ligand concentrations (keeping the protein concentration constant at 5 μM). Refer to the corresponding figures and tables for full details of the solutions that were analyzed. Prior to mass spectrometric analysis, the protein–ligand solution mixtures were centrifuged at 3000 rpm for 3 min (Centrifuge Mini Spin, Eppendorf, Germany) to prevent clogging of nano-electrospray emitters by any particulate matter. Protein–ligand mixtures were incubated at room temperature for at least 30 min to ensure equilibration. For brinzolamide binding to human carbonic anhydrase I, a stopped-flow instrument (SX.18Mv-R Applied Photophysics) was used to obtain the inhibition constant (corresponds to \(K_d\) of this sulfonamide using the CO₂ hydration reaction. Full experimental details are in the SI.

Nano-electrospray ionization emitters were fabricated with different inner tip diameters from borosilicate glass capillaries (Harvard Apparatus, 1.2 mm o.d., 0.68 mm i.d.) using a microcapillary puller (Model P-97, Sutter Instruments). The inner diameters of the nano-electrospray ionization tips were measured using scanning electron microscopy (FEI Nova NanoSEM 450 FE-SEM, Thermo Fisher Scientific) (Figure S16). Nano-electrospray ionization emitters were coated with a mixture of gold and palladium (Scancoat Six, Edwards). All mass spectrometry experiments were performed using a hybrid linear trap quadrupole and Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific). For ESI, a voltage of +0.7–1.5 kV was applied to the nano-electrospray ionization emitters relative to the heated capillary entrance to the mass spectrometer (180 °C). A maximum ion injection time of 500 ms was used throughout. Mass spectra were acquired for 2–3 min in triplicate using three different nano-electrospray ionization emitters. For each mass spectrum, peak areas

| Table 2. Measured \(K_d\) (μM) Values of Ethozolamide, Brinzolamide, Furosemide, Dichlorophenamidine, Indapamide, and Acetazolamide to Human Carbonic Anhydrase I and II Using Nano-Electrospray Ionization Mass Spectrometry with Emitter Tips That Have an Inner Diameter of 250 nm in Individual Ligand–Protein Binding Experiments (Single) and Simultaneously in a Competition Experiment (Competition) |
|-----------------|-----------------|-----------------|
|                  | human carbonic anhydrase I | human carbonic anhydrase II |
|                  | single \(^a\) | competition \(^b\) | literature | single \(^a\) | competition \(^b\) | literature |
| ethozolamide     | 0.014 ± 0.002 | 0.016 ± 0.004 | 0.009, 0.025 \(^{50}\) | 0.010 ± 0.001 | 0.014 ± 0.002 | 0.008 \(^{50}\) |
| brinzolamide     | 1.06 ± 0.05   | 1.12 ± 0.06   | 0.73 ± 0.04 \(^{43}\) | 0.005 ± 0.001 | 0.007 ± 0.001 | 0.003 \(^{43}\) |
| furosemide       | 0.055 ± 0.005 | 0.048 ± 0.006 | 0.062, 2.38 \(^{44}\) | 0.098 ± 0.009 | 0.110 ± 0.010 | 0.065 \(^{47}\) |
| dichlorophenamidine | 1.3 ± 0.1 | 1.4 ± 0.1 | 1.58 \(^{43}\) | 0.027 ± 0.002 | 0.035 ± 0.002 | 0.038 \(^{43}\) |
| indapamide       | 9.2 ± 0.3     | 9.5 ± 1.0     | 51.9 \(^{52}\) | 3.22 ± 0.20   | 3.32 ± 0.31   | 2.52 \(^{52}\) |
| acetazolamide    | 0.24 ± 0.02   | 0.29 ± 0.03   | 0.25, 0.48 \(^{44}\) | 0.015 ± 0.001 | 0.022 ± 0.002 | 0.012 \(^{50}\) |

\(^a\)\(K_d\) values obtained from triplicate measurements of solutions that contain different ligand concentrations (Table S1). \(^b\)\(K_d\) values obtained from triplicate measurements of a single solution (see Figure S5). \(^c\)This work; measured using a CA kinetic inhibition assay.

DOI: 10.1021/acscentsci.8b00787

ACS Cent. Sci. 2019, 5, 308–318
corresponding to the unbound and ligand-bound protein were automatically integrated by an in-house software program entitled PLbinding, which was written in MATLAB (2017a, The MathWorks). This software was also used to calculate ligand–protein dissociation constants, including for multiple ligands competing for a single binding site of a given protein. Full details of the ESI tip fabrication procedures (Table S4) and the data analysis methods are in the SI.

**CONCLUSIONS**

We investigated the effects of nanoscale ESI emitter tips on the binding affinity of protein–ligand interactions in native MS. For three functionally different classes of proteins, the use of nanoscale ion emitters with inner tip diameters as narrow as 250 nm can be used to measure the binding affinities of small ligands to proteins with significantly higher resolution than by use of conventional tips (2000 nm and larger). For example, the binding of low-molecular-weight anions (formed from sodium salts) to a 29 kDa protein can be directly probed using narrow-bore emitters, unlike for the conventional emitters under the same conditions. The use of nanoemitter tips can significantly reduce the saltaddition in ESI, and thus, the binding affinities of small molecules to proteins can be measured in the presence of high concentrations of nonvolatile salts and common biochemical buffers (e.g., 20 mM Tris-HCl and 50 mM NaCl). By increasing the spectral resolution owing to reduced salt addition using nanoscale ion emitters, the binding affinities of at least six ligands can be directly measured simultaneously in a single mass spectrum for protein–ligand complexes that differ in relative mass by as little as 0.06%, which is a factor of 7 lower than that reported previously for ESI-MS competition experiments. Although ligand–protein binding constants cannot be measured in solutions with NaCl concentrations that have ionic strengths near that of intracellular matrices (150 mM) owing to significant peak broadening, in the future it may be possible to use narrower bore emitters that are surface functionalized with antifouling monolayers to prevent clogging. Owing to the improved resolution resulting from the use of nanoemitters, it is now feasible that the cooperative effects of multiple different ligands binding to a single protein target that are challenging to investigate using traditional biochemical assays can be quantified by native MS. Overall, it is anticipated that nanoscale emitters in native MS will be beneficial in the rapid screening of small-molecule libraries to accurately identify ligands that bind potently to druggable protein targets with high sensitivity.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00787.

Additional scheme, experimental details, equations, tables, and figures including ligand structures, results from a ligand-binding assay, and SEM images (PDF)

Code for PLbinding that was used to calculate K_d values (PDF)

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**Notes**

The authors declare no competing financial interest. Safety statement: no unexpected or unusually high safety hazards were encountered during the course of our studies.

**ACKNOWLEDGMENTS**

We acknowledge the financial support from the Australian Research Council (DP160102681). All the experiments were carried out in Bioanalytical Mass Spectrometry Facility (BMSF) and Electron Microscope Unit (EMU) of Mark Wainwright Analytical Centre (MWAC).

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