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Probing specific ligand-protein interactions by native-denatured exchange mass spectrometry

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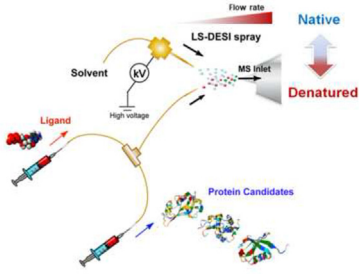
HIGHLIGHTS

- A native-denatured exchange (NDX) ionization approach is established.
- A native to denatured detection of ligand-protein complexes is enabled.
- The NDX approach readily recognizes nonspecific ligand-protein interactions.
- This approach holds promise for drug screening or target identification.

GRAPHICAL ABSTRACT



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ABSTRACT

Probing ligand-target protein interactions provides essential information for deep understanding of biochemical machinery and design of drug screening assays. Native electrospray ionization-mass spectrometry (ESI-MS) is promising for direct analysis of ligand-protein complexes. However, it lacks the ability to distinguish between specific and non-specific ligand-protein interactions, and to further recognize the specifically bound proteins as drug target candidates, which remains as a major challenge in the field of drug developments by far. Herein we report a native-denatured exchange (NDX) mass spectrometry (MS) acquisition approach using a liquid sample-desorption electrospray ionization (LS-DESI) setup, and demonstrate its capability in enabling a change from native detection of noncovalent ligand-protein complexes to denatured analysis using three model ligand-protein complexes including myoglobin, CDP-ribonuclease and N,N′,N″-triacetylchitotriose (NAG3)-lysozyme. Notably, we found the NDX-MS approach can readily discriminate specific ligand-protein interactions from nonspecific ones, as revealed by their distinct dynamic profiles of K_d as a function of the DESI spraying flow rate. Consequently, this NDX-MS approach holds promise for future applications to discovering specific protein

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targets for ligands of interest, and to screening compounds with high specificity to drug targets and thus eliminates off-target effects.

1. Introduction

Knowledge of ligand-protein interactions is essential for drug design in pharmaceutical research, and for deep understanding of molecular machinery involved in various biochemical processes. Consequently, the capability to probe specific ligands for target proteins serves as a fundamental basis for understanding their biological interactions, and holds promise for applications to drug screening and drug target discovery/validation [1].

Current methods, including NMR [2] and X-ray crystallographic analysis [3], reveal structural information of protein-ligand interactions at high spatial resolution. However, samples of high purity and large quantity are often required, together with the arduous and delicate process of sample preparation, making these approaches unsuitable for high-throughput analysis of ligand-protein complexes.

To this regard, mass spectrometry (MS), a routine tool for protein analysis due to its high sensitivity and speed, has been coupled to electrospray ionization (ESI) and set up under a native environment (native ESI-MS) for measuring ligand-protein complexes and their binding stoichiometry [4–10]. Besides native MS, liquid sample (LS)-DESI [11–14], microdroplet fusion MS [15] and extractive ESI (EESI) [16,17], have also been demonstrated to be capable of ionizing proteins and ligand-protein complexes in a native manner. These three methods all work by spraying charged solvents (MeOH/water, ACN/water) to plume with the assistance of nebulizing gas and concomitantly infuse protein samples from a separated sample transferring capillary. Since the proteins are neither directly exposed to high voltages nor dissolved in denaturing solvents during the ionization process, native conformations are retained for proteins infused from the sample introduction probe [13,18,19].

Although the above-mentioned methods manage to measure binding affinities of ligands to target proteins, they generally do not differentiate between specific and nonspecific ligand-protein target interactions since nonspecific interactions are frequently formed during the electrospray ionization process [20,21]. Such limitation thus remains as a hindrance to narrow down the ligand-binding protein candidates to actual targets that exert downstream pharmacological functions during drug screening.

Nonspecific ligand-protein interactions often involve weak binding to many sites at the solvent-exposed surface of proteins and are structure-independent, whereas specific interactions refer to the attractive interactions that occur at a well-defined pocket and are tertiary structure-dependent [20–26]. Based on such differences, we hypothesized that a change from native to denatured environment would affect specific ligand-protein binding due to denaturation-induced loss of protein's tertiary structures. Therefore, ligand-protein interactions that are preserved or even strengthened due to the increased solvent accessible area through unfolding events caused by denaturation can be assigned as nonspecific binding [20–23]. Monitoring the dynamic profiles of ligand-protein interactions as the change from native to denaturing environment thus holds promise for the recognition of specific ligand-protein complexes from nonspecific ones. Since MS can readily detect ligand-protein interactions based on the signal intensities of ligand-bound/free proteins, herein, we coupled a liquid sample-desorption electrospray ionization (LS-DESI) setup [11–14] that enables native-denatured exchange (NDX) ionization with a mass spectrometer to accommodate such purpose.

As shown in Fig. 1, the physical separation of the two probes for sample infusion and subsequent desorption/ionization process allows us to freely tune the ionization spray. By simply increasing the flow rate of the LS-DESI spray and consequently the denaturant contained in the ionization plume, a native to denatured detection of proteins and ligand-protein complexes is accomplished as demonstrated by myoglobin, NAG3, CDP and their binding proteins. The K_d-flow rate curves of ligand-protein complexes are plotted as the proteins experience a change from native to denatured states, which are found to be useful to differentiate specific interactions from nonspecific ones. We thus expect this NDX-MS acquisition approach to be widely applicable to discover novel compounds for drug targets with high specificity to eliminate off-target effect and reveal target proteins for ligands of interest with reduced false positive rates.

2. Materials and methods

2.1. Chemicals and reagents

Ubiquitin (bovine), lysozyme (chicken egg white), myoglobin, ribonuclease A (RNase A, from bovine pancreas), cytidine 5′-diphosphate disodium salt (CDP), N,N′,N″-triacetylchitotriose (NAG3), formic acid (FA), acetonitrile (ACN), NH_4OAc were all purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Native/denatured exchange ionization sources

All NDX-MS experiments were carried out on a home-built LS-DESI source. A nanoESI source is used to generate charged microdroplets for the ionization of protein or ligand-protein complex ions. The nanoESI probe is fabricated by a piece of silica capillary with an i. d. of 25 μm and an o. d. of 380 μm. The spray solvent consists of ACN/water/formic acid (50:50:0.1, v/v). The sample introduction capillary (i.d. 100 μm, o. d. 190 μm) was positioned between the nanoESI probe and the MS inlet (Fig. 1A). For native NDX experiments, the model proteins (ubiquitin and lysozyme) were dissolved in 20 mM NH_4OAc. The protein solutions were delivered through the sample introduction capillary, while the DESI spraying solvent was infused through the nanoESI emitter. The flow rates were both set at 3 μL/min.

For NDX experiments to probe specific ligand-protein complexes, the CDP solution was prepared at 500 μM and the NAG3 solution was prepared at 200 μM, and their potential binding partners, including RNase A, ubiquitin and lysozyme, were all prepared at 100 μM. The protein and ligand solutions were infused separately and mixed via a Tee mixer (Fig. 1B). The flow rates of both protein and ligand solutions were set as 5 μL/min (Fig. 1B) After the Tee mixer, a piece of silica capillary (i.d. 100 μm, o. d. 190 μm) was employed as a micro-reactor, where protein-ligand complexes were formed upon mixing prior to MS analysis. The DESI spraying solvent was infused at stepwisely increased flow rates of 5, 10, 15 and 25 μL/min as a cycle for each NDX experiment, whereas the flow rate of the ligand-protein complexes was kept

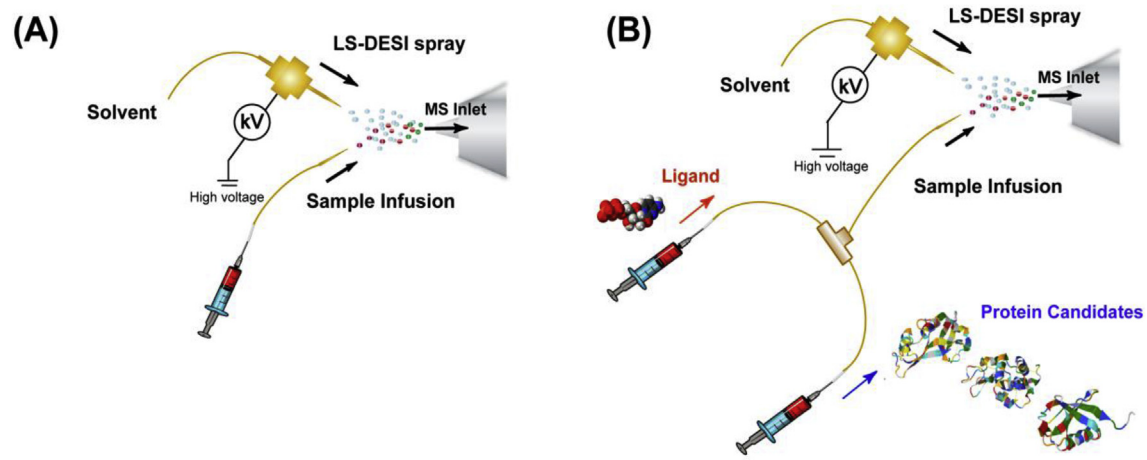


Fig. 1. Illustration of the LS-DESI ionization source for native denatured exchange (NDX)-MS acquisition. This approach enables (A) direct analysis of ligand-protein interactions, and (B) allows for probing specific ligand-protein complexes held by noncovalent interactions by infusing ligands and potential binding proteins through a micro-reactor before being subjected to NDX-MS analysis.

constant during the cycle. The protein candidates were infused in the order of RNase A, ubiquitin and lysozyme.

All regular ESI experiments, both denatured and native ESI-MS acquisitions, were carried out on the very nanoESI source used for the NDX experiments by simply removing the sample introduction capillary and infusing protein solutions of the same concentration as those used for the NDX experiments. The flow rates of native and denatured ESI spray were both set at 3 μL/min. For denaturing ESI, the model proteins were dissolved in ACN/water/formic acid (50:50:0.1, v/v) before being infused into MS, whereas they were dissolved in 100 mM NH_4OAc for native ESI-MS acquisitions.

2.3. Mass spectrometry

An AB SCIEX TripleTOF 5600 mass spectrometer (Framingham, MA, USA SCIEX, Milford, MA) was used for data acquisition. The source conditions were set as follows: Ion Source Gas 1 (GS1) as 60–90 psi (optimized for different flow rate), Ion Source Gas 2 (GS2) as 0 psi, Curtain gas (CUR) as 30 psi, source temperature 80 °C, Ion Spray Voltage Floating (ISVF) 3500–4000 V in the positive ion mode. Mass spectrometer conditions were as follows: Collision gas N_2, Q1 vacuum gauge $2.4*10^{-5}$ torr, TOF vacuum $0.28*10^{-6}$ torr. In the TOF-MS acquisition mode, the instrument was set to acquire over the *m/z* range of 300–3000 Da for TOF-MS scan. Declustering potential (DP) was set as 100 V. The accumulation time for TOF-MS scan were set at 1 s. The acquired spectra were deconvoluted by MagTran 1.0.3 (Amgen, Thousand Oaks, CA) and manually validated.

A Waters Synapt G2 Si Q-TOF was employed to cross-validate the general applicability of the NDX approach. The instrumental conditions were as follows: mass range 50–3000 Da, capillary voltage +4 kV, source temperature 100 °C, desolvation temperature 300 °C, cone gas flow 25 L/h, nanoflow gas pressure 0.3 bar, desolvation gas flow 600 L/h, scan time 1 s, interscan time 0.014 s, data format continuum, analyzer operated in the resolution mode.

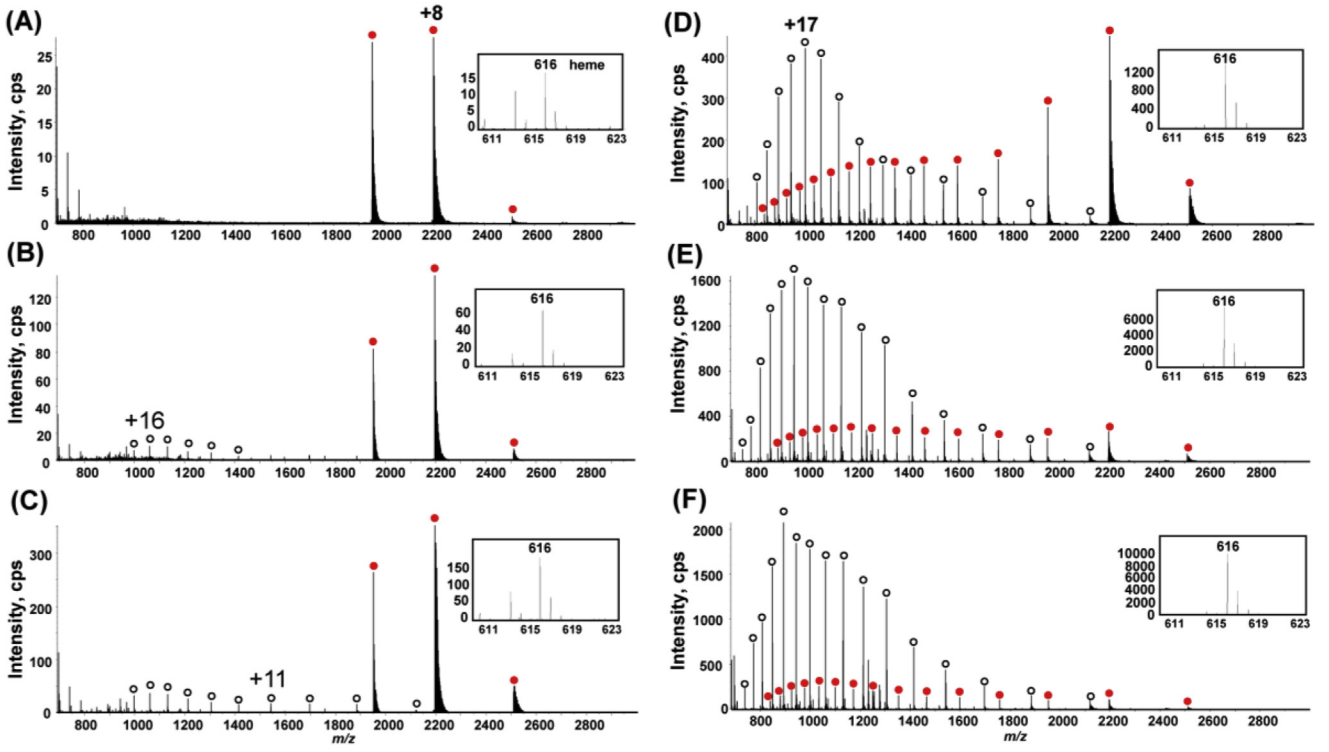
3. Results & discussions

We first selected ubiquitin as a model protein for proof-of-principle study of NDX ionization. With the denatured ESI-MS approach, a wide charge state distribution (CSD) between 7 + to 13 + was observed when ubiquitin was directly mixed with the DESI spraying solvent that contains denaturant ACN and formic acid (FA) (H_2O:ACN:FA, 50:50:0.1 by volume) followed by being infused through the nanoESI emitter into MS (Fig. S1A). In contrast, the native ESI mass spectrum exhibited a narrow CSD centered at 6 + corresponding to a folded structure (Fig. S1B). Interestingly, the NDX-MS produced a mass spectrum with CSD similar to that acquired by native ESI when the DESI spraying solvents was infused at a relatively low flow rate of 3 μL/min (Fig. S1C). Our observation indicated that the native conformation of ubiquitin can be maintained by LS-DESI ionization even when the DESI spraying solvent is capable of partially denaturing ubiquitin by in-solution mixing. This also agrees with previous literature that reports native detection of proteins by indirect ionization approaches[22–24], and can be explained by that charges are introduced to native proteins through rapid mixing within the ionization plume while the denaturant present in the microdroplets has evaporated to an appreciable extent and cannot suffice to denature analytes. Following direct protein analysis, we subsequently examined native lysozyme incubated with its specific binding ligand, N,N′,N″-triacetylchitotriose (NAG3), dissolved in NH_4OAc buffer (pH = 7.4) and infused through the sample introduction probe. The resulting NDX-MS spectrum shows a dominant ion corresponding to the NAG3-lysozyme complex (z = 8+) (Fig. S1F). Expectedly, the pattern of the mass spectrum with a low flow rate of DESI spraying solvent resembled that of the native ESI-MS spectrum (Fig. S1E) rather than the denatured ESI-MS spectrum (Fig. S1D). We further calculated the ratios of the ligand-bound protein (holo-form)/the free protein (apo-form), denoted by R (holo/apo ratio, relative intensities of the bound versus unbound protein ions as measured in the spectrum), for the NAG3-lysozyme complexes. The R values of the NAG3-lysozyme complexes acquired under the denatured ESI, native ESI and native NDX setup were calculated as 0.41, 3.49 and 2.95, respectively. Based on the holo/apo ratios, we reasoned that the NDX acquisition is able to provide native-like ionization at current settings.

Next, we exploited the characteristic of DESI source that the sample is separately infused from the DESI solvent, and tested whether tuning the DESI spraying solvent that forms the ionization plume manages a change from native to denatured ionization environment. We anticipate that, by increasing the flow rate of the DESI spraying solvent and thus enhancing the exposure of samples to denaturants contained in the ionization plume, the native conformations of ligand-protein complexes cannot be maintained and

a change from native to denatured detection is achieved.

We first chose heme-containing myoglobin as an exemplary ligand-protein complex, and gradually ramped up the flow rate of the DESI spraying solvent while keeping the sample infused at a constant rate to test our hypothesis. Firstly, we set the flow rate of DESI spraying solvent at 1 μL/min and that of the sample at 5 μL/min. The acquired mass spectrum (Fig. 2A) showed a narrow CSD with the dominant peak assigned to holo-myoglobin at z = 8+. Meanwhile, the intensity of the ion at *m/z* 616.18 corresponding to heme was almost negligible. This agrees to our previous observation that ligand-protein complexes, myoglobin in this case, can be maintained in native conformations. The possibility of unfolding-refolding of myoglobin has been excluded since the reincorporation of heme has a time constant on the order of hundreds of milliseconds to seconds [14] and is thus unlikely to occur within the short droplet lifetime of DESI spray (~submillisecond timescale) [15,27]. Subsequently, we increased the flow rate of DESI spraying solvent to 3–5 μL/min (Fig. 2B–C), and observed the presence of apo-myoglobin ions with a broad CSD although the holo-myoglobin peaks are still dominant. This indicates that myoglobin became partially denatured as the proportion of denaturant was increased in the DESI plume. The deconvoluted MS spectra are shown in Fig. S2. Concomitantly, the impairment of the native conformation resulted in enhanced signal intensity of heme. We then adjusted the flow rate of the DESI spray to two fold that of the infused sample (Fig. 2D), and noticed the intensity of the apo-



(G)

Holo form

Charge	NDX flow rate (μL/min)					
	1	3	5	10	15	20
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						

Apo form

Charge	NDX flow rate (μL/min)					
	1	3	5	10	15	20
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
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21						
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24						

(H)

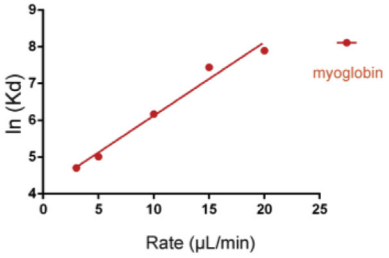

Fig. 2. Adjusting the flow rate of the DESI spraying solvent enables a change from native to denatured analysis of ligand-protein complexes using the heme-bound holo-myoglobin as a model via the NDX-MS approach. The myoglobin solution was constantly infused at a moderate flow rate of 5 μL/min, whereas the flow rate of DESI spray varied from 1, 3, 5, 10, 15–20 μL/min as shown in (A) to (F), respectively. (G) Heatmap showing charge state distributions of holo- and apo-forms of myoglobin when the DESI spraying solvent was infused at increasingly high flow rates and concomitantly induced protein denaturation. (H) Linear regression fit of ln (K_d) as a function of DESI flow rate. The data point corresponding to the DESI flow rate at 1 μL/min was not included due to the absence of apo-myoglobin peak in the spectrum.

myoglobin increased dramatically, and the highest charge was shifted to 21 + at *m/z* 808.22. The balance between the holo- and apo-forms of myoglobin was further interrupted by increasing the flow rate of DESI spray to three~four times that of the sample. Dominant peaks corresponding to both denatured apo-myoglobin and heme with negligible peaks of native holo-myoglobin were shown (Fig. 2E-F). These results combinatorially suggest that a change from native to denatured ionization is readily achieved by NDX. Such phenomena have been cross-validated by performing the NDX acquisitions on another mass spectrometer (Fig. S3, SI).

In addition to the CSD shift shown in Fig. 2G, the decreasing holo/apo ratios calculated from the deconvoluted spectra (Fig. S3 and Equation (1) [21–23,28]) also demonstrated a change from native to denatured ionizing environment as the increment of the DESI spray flow rate. The binding affinities of ligand-protein interactions under different DESI spray flow rates, suggested by K_d (dissociation constant), are further elucidated by a direct ESI-MS model via Equation (2) [21–23].

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

Note: The R value is obtained by dividing the summed intensities of the complex ions at all charge states, denoted by [PL], by the sum of the intensities of protein ions, denoted by [P], of all charge states.

$$K_a = \frac{1}{K_a} = \frac{R}{[L]_0 - \frac{R}{1+R}[P]_0} \quad (2)$$

Note: $[P]_0$ and $[L]_0$ refer to the initial concentration of protein and ligand in solution.

A titration curve is thus obtained by plotting the K_d against the flow rate of the DESI spraying solvent, whose increment indicates a change from native to denatured analysis of protein ions in Fig. 2H. As the flow rate increases, myoglobin becomes gradually denatured and an increase in K_d is concomitantly shown. Such change can be explained by that specific ligand-protein complexes is sensitive to conformational changes of proteins induced by increasingly high concentrations of denaturant within the DESI plume, which ultimately leads to impaired heme-myoglobin interactions and the loss of ligands. In contrast, ligand-protein interactions that exhibit no significant changes or even reduction of K_d as the increment of DESI flow rate are postulated to be nonspecific interactions.

We thus tested our postulations by exploring whether the NDX-MS acquisitions can differentiate specific ligand interactions from nonspecific ones by plotting their K_d as a function of the DESI flow rate. The ligand CDP and its specific binding protein, RNase A, were selected as a model of specific ligand-protein interactions, while ubiquitin and lysozyme served as exemplary nonspecific binding proteins. These three models of specific and nonspecific interactions have been validated by microscale thermophoresis (MST) as shown in Fig. S4. The protein and ligand solutions were infused and mixed via a Tee mixer as illustrated by Fig. 1B (detailed in SI) [29,30]. After the Tee mixer, a piece of silica capillary was employed as a micro-reactor, where protein-ligand complexes were formed upon mixing prior to MS analysis. The DESI spraying solvent still contained a certain fraction of denaturant (ACN:-H_2O:FA, 50:50:0.1), while the proteins and ligands were both maintained in native conditions (20 mM NH_4OAc). The dynamic profiles of K_d of CDP with RNase A and with the other two non-target proteins were recorded as a function of the DESI solvent flow rate, when the flow rate of the infused sample is kept constant as shown in Fig. 3. The enlarged (Fig. 3A) and deconvoluted mass spectra (Fig. S5) both showed that the dominant ion corresponds to the RNase A-CDP complex, indicating that RNase A maintained its native conformation and tended to specifically interact with its ligand CDP when the DESI spraying solvent was infused at a moderate flow rate of 5 μL/min. Nevertheless, when the flow rate of DESI spray was stepwisely increased to 10, 15 and 25 μL/min, the increased concentration of denaturant present in the DESI plume resulted in gradually denatured RNase A and subsequent dissociation of CDP-RNase A complex (Fig. 3A) as revealed by the inversely decreased holo/apo ratio of CDP-RNase A (Fig. 3D–E). Correspondingly, the K_d values were gradually increased as the protein ions were changed from native to denatured states as shown in Fig. 3F. Such trend agrees well with previous knowledge that a compact conformation of RNase is a prerequisite for its binding to the ligand, whereas the denaturation-induced unfolding would result in ligand-target dissociation [20,31]. The K_d values determined by the direct ESI-MS model are higher than those fitted by MST, which are probably attributed to the propensity of the ionized protein complexes to dissociate in the gas phase as previously reported [28].

In contrast to the CDP-RNase A complexes, the non-target proteins, ubiquitin and lysozyme, and CDP showed distinct trends in the enlarged and deconvoluted mass spectra (Fig. 3B–C, Fig. S5). The spectral trends were then translated to titration curves of holo/apo ratio (R) in Fig. 3D–E and K_d in Fig. 3F. Intriguingly, the holo/apo ratios held almost constant even when the flow rate of DESI spray was increased (values summarized in Table S1). The titration curves of K_d against DESI flow rate can thus be used to differentiate specific ligand-protein interactions from nonspecific ones. Moreover, the advent of such non-specific binding can be explained by the highly concentrated ligands present in the droplets of DESI plume, which is prone to result in nonspecific binding to co-existent protein species [20–22,28].

To verify the general applicability of the DESI approach, we further investigated the interactions of NAG3 with its specific binding partner lysozyme and two nonspecifically bound proteins, ubiquitin and RNase A. As shown in Fig. 4, the NAG3-lysozyme complexes were also sensitively influenced by the conformation of lysozyme when specific interactions hold the ligand NAG3 and lysozyme together (Fig. 4A), whereas the non-specific interactions between both ubiquitin and RNase A with NAG3 were not affected by the denaturing-induced conformational changes of proteins (Fig. 4B–C). Such finding highlights the general applicability of the LS-DESI approach to rapidly and efficiently distinguishing specific binding partners from nonspecific ones. The deconvoluted spectra were summarized in Fig. S6. The curves of R were further plotted against the DESI flow rate in Fig. 4D, illustrating the distinctions between specific and nonspecific interactions. Accordingly, the increased K_d values as the ligand-protein complexes shifted from native to denatured conditions reiterate the influence of protein conformation on specific binding interactions (NAG3-lysozyme), whereas no pronounced changes of K_d were observed for the nonspecifically bound ligand-protein complexes, ubiquitin- or RNase A-NAG3, in Fig. 4E and Table S2. Such phenomena also revealed the binding sites of non-specific interactions in this case are located at the surface of the target protein, since the denaturation-induced unfolding of target proteins and subsequent exposure of buried hydrophobic residues failed to increase the holo/apo ratio.

4. Conclusions

In summary, we reported a NDX-MS acquisition approach in this study by coupling a tunable DESI spraying probe to a sample introduction capillary, and demonstrated such approach can change the ionization environment from a native to denatured

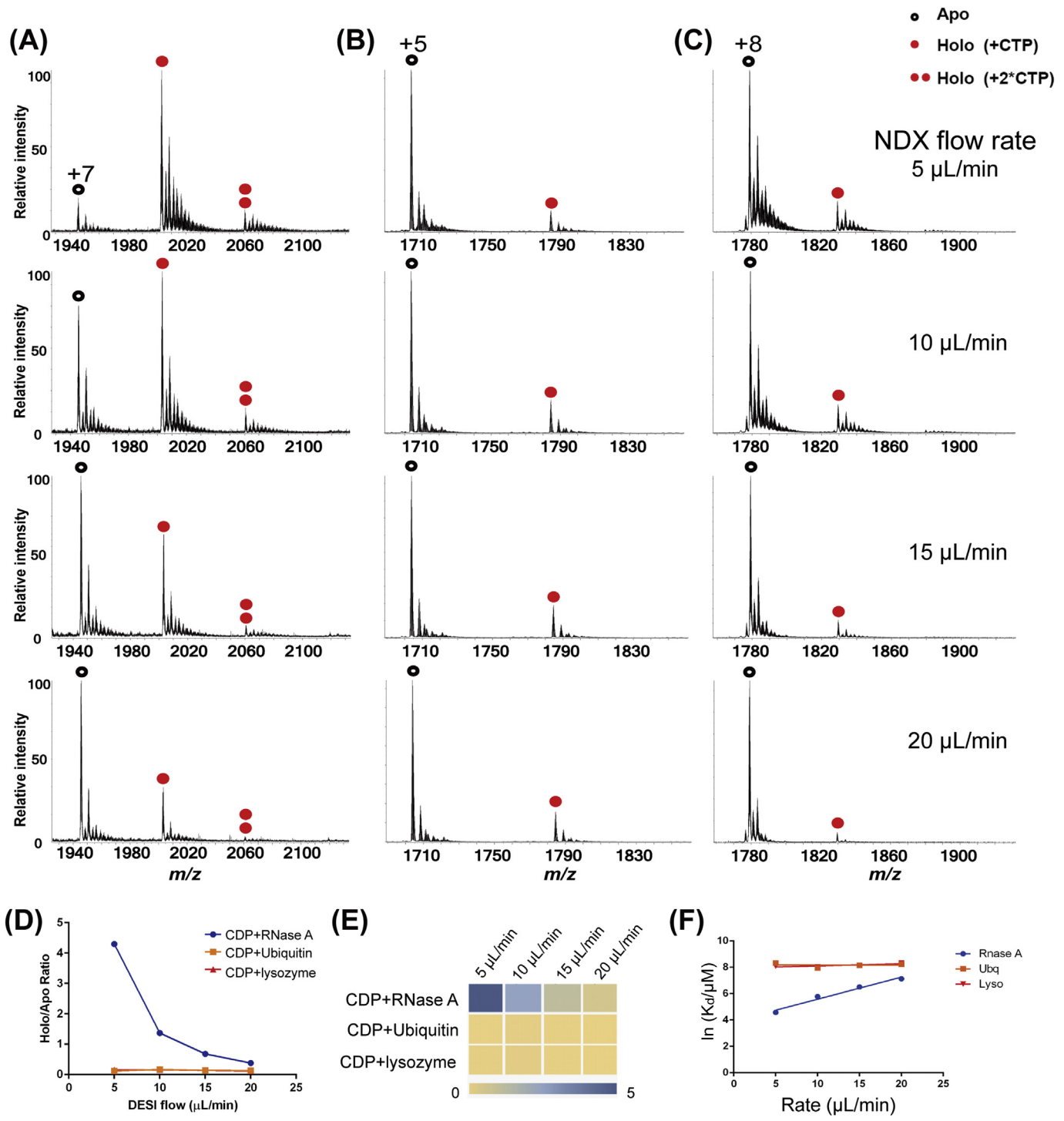

Fig. 3. NDX-MS spectra of ligand-protein complexes collected at different flow rate of DESI spray. CDP was used as a model ligand, whereas proteins including a specific binding protein (A) Rnase A and its nonspecific binding partners including (B) ubiquitin and (C) lysozyme were used. The corresponding flow rates of DESI spray are as labeled. (D) Plots and (E) heatmap of holo/apo ratios of ligand-protein complexes as a function of the flow rate of DESI spraying solvent. (F) Linear regression fit of ln (K_d) as a function of the flow rate of DESI spray.

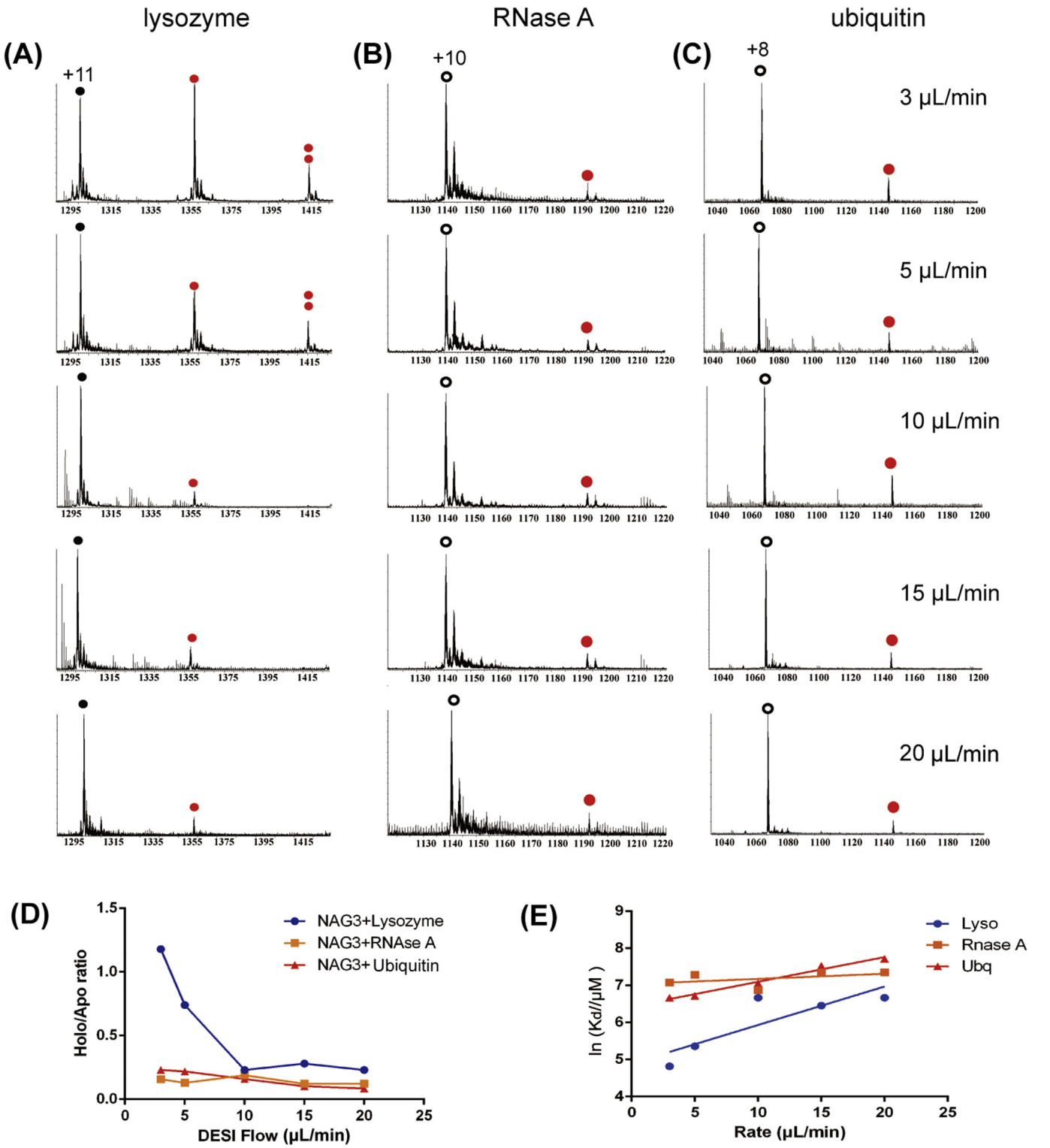

Fig. 4. NDX-MS spectra of ligand-protein complexes collected with DESI spraying solvent infused at increasingly high flow rate and with ligand-protein mixture infused at a constant flow rate. Noncovalent interactions between NAG3 and its specific binding protein, (A) lysozyme, and its nonspecific binding partners including (B) RNase A and (C) ubiquitin were characterized by NDX-MS. (D) Plots of holo/apo ratios of ligand-protein complexes and (E) Ln (K_d) as a function of the flow rate of DESI spraying solvent.

state by flexibly adjusting the flow rate of the DESI spraying solvents that contains denaturant at appropriate proportions. The K_d curves of ligand-protein complexes as the conformations of proteins shifted from native to denatured states can thus be used to readily differentiate specific interactions from nonspecific ones. The NDX-MS approach is anticipated to shed light in discovering new compounds for selected targets with high specificity to eliminate the off-target effect and discovery of target proteins for ligands of interest with reduced false positive rates.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.07.072.

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