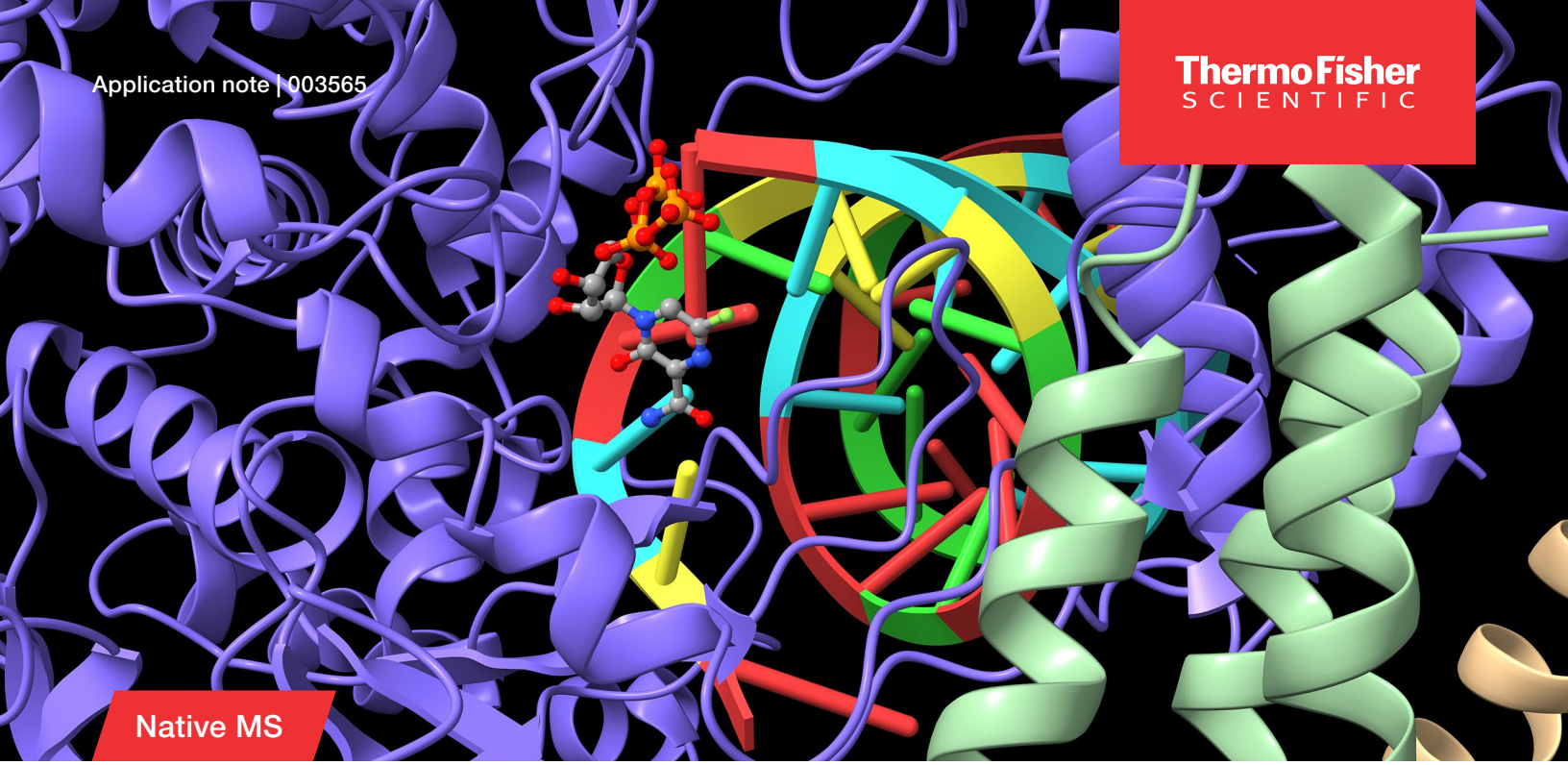


Gas phase affinity selection-native mass spectrometry for automated ligand screening

Authors

Weijing Liu[1], Wilson Phung[2], Christopher Mullen[1], Dennis Koehler[3], Lei Shi[4], Jacob Bills[1], Amanda Lee[1], Rosa Viner[1]

[1]Thermo Fisher Scientific, San Jose, CA, USA

[2]Genentech, South San Francisco, CA, USA

[3]Thermo Fisher Scientific, Germering, Germany

[4]Thermo Fisher Scientific, Shanghai, China

Keywords

Orbitrap Ascend Structural Biology Tribrid mass spectrometer, structural biology, ligand binding, Orbitrap Ascend MS, Vanquish Neo UHPLC, BioPharma Finder software

Introduction

Ligand screening is a critical process in drug discovery and structural biology, aimed at identifying compounds that can selectively bind to target biomolecules. This interaction can modulate the biological function of the target, leading to potential therapeutic effects. Ligand screening evaluates potential ligands by assessing their binding affinity, specificity, and stability. By optimizing these interactions, ligand screening aids in the development of effective and targeted drugs, ultimately reducing time and costs in the drug discovery pipeline. Advanced technologies like high-throughput screening, computational docking, and biophysical techniques such as surface plasmon resonance have improved the efficiency and precision of ligand screening,[1,2,3] enabling researchers to make more informed decisions about advancing candidates in preclinical testing.

Mass spectrometry (MS) has become a valuable tool in ligand screening, offering high sensitivity, accuracy, and specificity in detecting and characterizing ligand-target interactions.[3] In conventional MS-based screening, affinity selection MS (AS-MS)[1,2] involves incubating a target protein with a mixture of compounds. Compounds with affinity bind to the protein, while non-binding molecules remain in solution. Size-exclusion chromatography (SEC) is often used to separate bound protein-ligand complexes from unbound compounds. Following separation, binders are dissociated, for further analysis and identification by reverse-phase liquid chromatography (RPLC)-MS.

AS-MS is recognized as a high-throughput platform for ligand screening; however, it does not directly interrogate binding complexes, and on-column dissociation may result in the loss of weak binders.

Native mass spectrometry (Native MS) allows for the analysis of intact, non-denatured protein-ligand complexes, preserving native protein conformations and providing insights into stoichiometry, binding strength, and ligand interaction dynamics.[3] Conventional native MS is limited in throughput due to labor-intensive manual sample preparation. This study introduces an integrated liquid chromatography (LC) system coupled to native MS, streamlining rapid online buffer exchange, parallel compound binding, and direct native MS analysis of complexes. Additionally, a post-column binding approach is proposed to enhance weak binder detection. For unknown binders, the MS^3 capability of the mass spectrometer can provide structural insights of bound ligands. We utilize carbonic anhydrase (CA) as a model system due to its well-studied ligand library. This novel workflow holds promise for screening targeted protein degraders by directly detecting ternary complexes to assess efficacy.

Experimental

Consumables

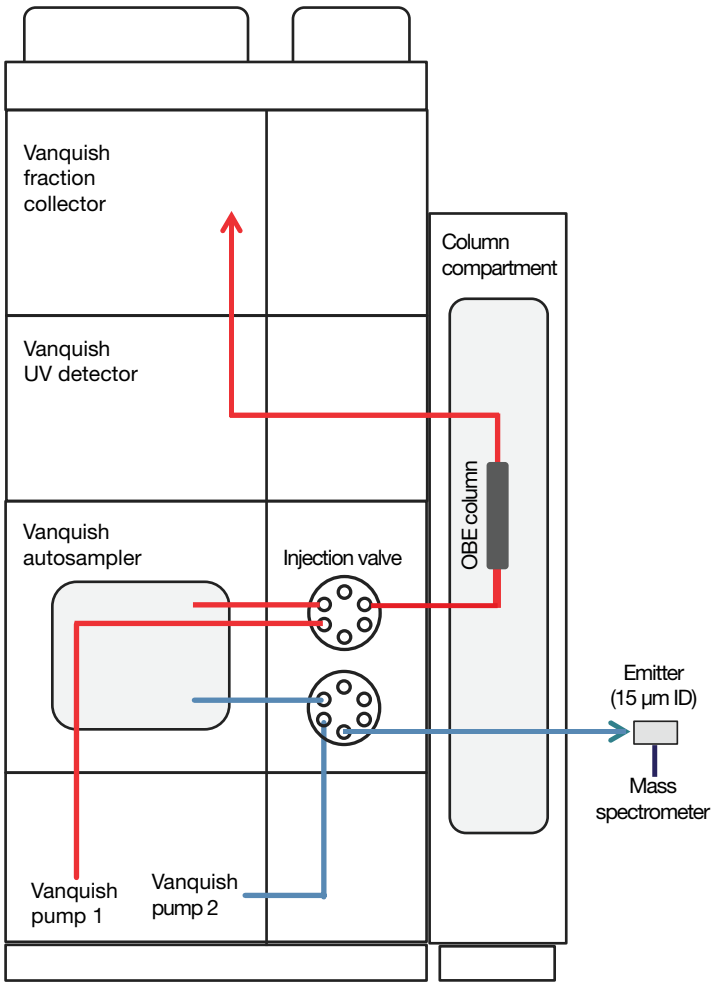
- Ammonium acetate (MilliporeSigma, P/N 372331-10G)
- Fisher Scientific™ Optima™ LC-MS grade water (P/N 10505904)
- Carbonic anhydrase (MilliporeSigma, P/N C2522-5MG)
- Thermo Scientific™ SureSTART™ TPX screw top microvial with glass insert (P/N 60180-1655)
- Eppendorf™ Protein LoBind™ 384-well plate (P/N 951040589)
- Thermo Scientific™ NativePac OBE-1 SEC column (P/N 43803-052130)
- Thermo Scientific™ EASY-Spray™ capillary emitter, bullet type (P/N ES994)

Sample preparation

CA was received in powder and directly dissolved in 200 mM ammonium acetate (AmAc) to prepare the 100 µM stock solution. Ligands were solubilized in DMSO to prepare either the 10 mM or 100 mM stock solution.

Test methods

Protein buffer exchange, protein-ligand mixing, and protein-ligand detection procedures are listed in Figure 1. The Thermo Scientific™ Vanquish™ LC system equipped with a UV detector, fraction collector, dual-injection autosampler, and dual Vanquish Flex pumps was used.



1. Prefill 96 well plate with one or more ligands/well
2. Set fraction collector temperature at desired incubation temp.
3. Place protein vial into autosampler
4. Autosampler → Injection valve 1 → OBE → column → UV → FC
5. Transfer the sample plate from FC to autosampler
6. Autosampler → Injection valve 2 → EASY-Spray emitter

Figure 1. Workflow schematic. Red lines show protein-ligand binding and blue lines, detection workflow.

Table 1. Orbitrap Ascend Structural Biology MS parameters for MS^1 scans of CA-ligand complex, MS^2 for ligand release, and MS^3 for ligand fragmentation

Source parameters			
Spray voltage (+V)	1,200–1,400		
Capillary temperature (°C)	275		
Orbitrap scan parameters			
Method type	**Full MS**	**MS^2**	**MS^3**
Application mode	Intact		
Pressure mode	Standard		
RF lens (%)	60		
Source fragmentation (V)	0		
Source CID compensation scaling	0		
Resolution	240,000 at *m/z* 200		
Scan range (*m/z*)	1,500–6,000	100–4,000	100–500
AGC target value	200	400	400
Max injection time (ms)	100	100	100
Isolation mode	-	Quadrupole	Ion trap
Isolation window (MS^2)	-	60 Th	20 Th
Microscans	3	3	3
Fragmentation	-	HCD	CID
Fragmentation energy	-	15–50	10–25

The mobile phase was 200 mM AmAc and the flow rate was 0.3 mL/min. Proteins were online buffer exchanged into 200 mM AmAc using a NativePac OBE-1 column and fractionally collected into a multi-well plate at the rate of 1.5 min/sample (Figure 2). Each well of the 96-well plate was preloaded with one or more ligands. The fraction collector chamber was set at the desired binding temperature. Upon mixing proteins with ligands, incubation began while the remaining wells awaited desalted proteins. The final molarity ratio between CA and ligand was between 0.5 to 10. After all the wells were filled with desalted proteins, samples from each well were directly infused into either the Thermo Scientific™ Orbitrap™ Ascend Structural Biology Tribrid™ mass spectrometer or the Thermo Scientific™ Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap™ mass spectrometer using a 15 µm capillary EASY-Spray bullet type emitter and the EASY-Spray source. With a throughput of 1 ligand per well, this setup enables processing of over 300 samples per day.

Data analysis

The data were analyzed using Thermo Scientific™ BioPharma Finder™ 5.0 software. Dissociation constant K_d was calculated using GraphPad Prism™ (GraphPad Software, LLC).

Results and discussion

Pre-column binding vs. post-column binding

Using CA as a model system, we evaluated ligand binding efficacy across a range of dissociation constants (from K_d < 100 nM to K_d > 10 µM, Table 2) using online buffer exchange, fraction collection, and flow injection-native MS analysis.

Table 2. CA ligands

Ligand	Compound	MW	Reported K_d/µM[4-6]
L1	Sulfanilamide	172.20	13.2
L2	Benzenesulfonamide	157.19	1.44
L3	1,3-Benzenedisulfonamide	236.27	≈1.1
L4	4-Sulfamoylbenzoic acid	201.20	0.27

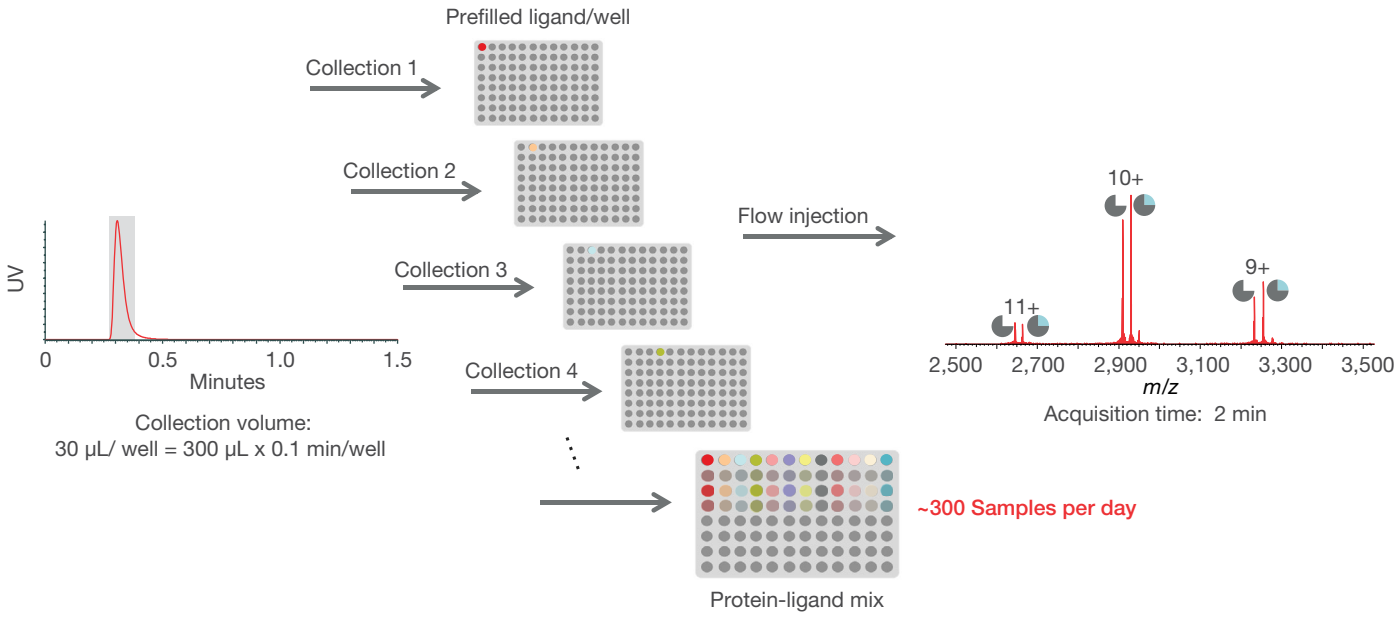


Figure 2. Fraction collection of online desalted protein prior to post-column ligand binding

Conventional pre-column binding often suffers from on-column dissociation due to dilution effects or high off-rates (Figure 3A). This issue limits the detection and analysis of weak binders, as these interactions are prone to dissociation during chromatography. However, by adding ligands to the protein solution post-column, researchers can bypass dissociation issues inherent in on-column binding (Figure 3B). This approach allows for more accurate assessment of binding efficacy across a broader range of affinities.

When CA was mixed with a set of four ligands, pre-column binding resulted in on-column dissociation, with only the strongest binder remaining bound after passing through the SEC column (Figure 3C). In contrast, the post-column binding approach preserved binding complexes with relatively weaker, including L2 and L3. The apparent dissociation constants (K_d), derived from the fractional abundance of CA-L complexes, ranked as L1 > L2 > L3 > L4, consistent with published K_d values.[4,5,6] This alignment highlights the merits of post-column binding in characterizing a wider range of binding interactions.

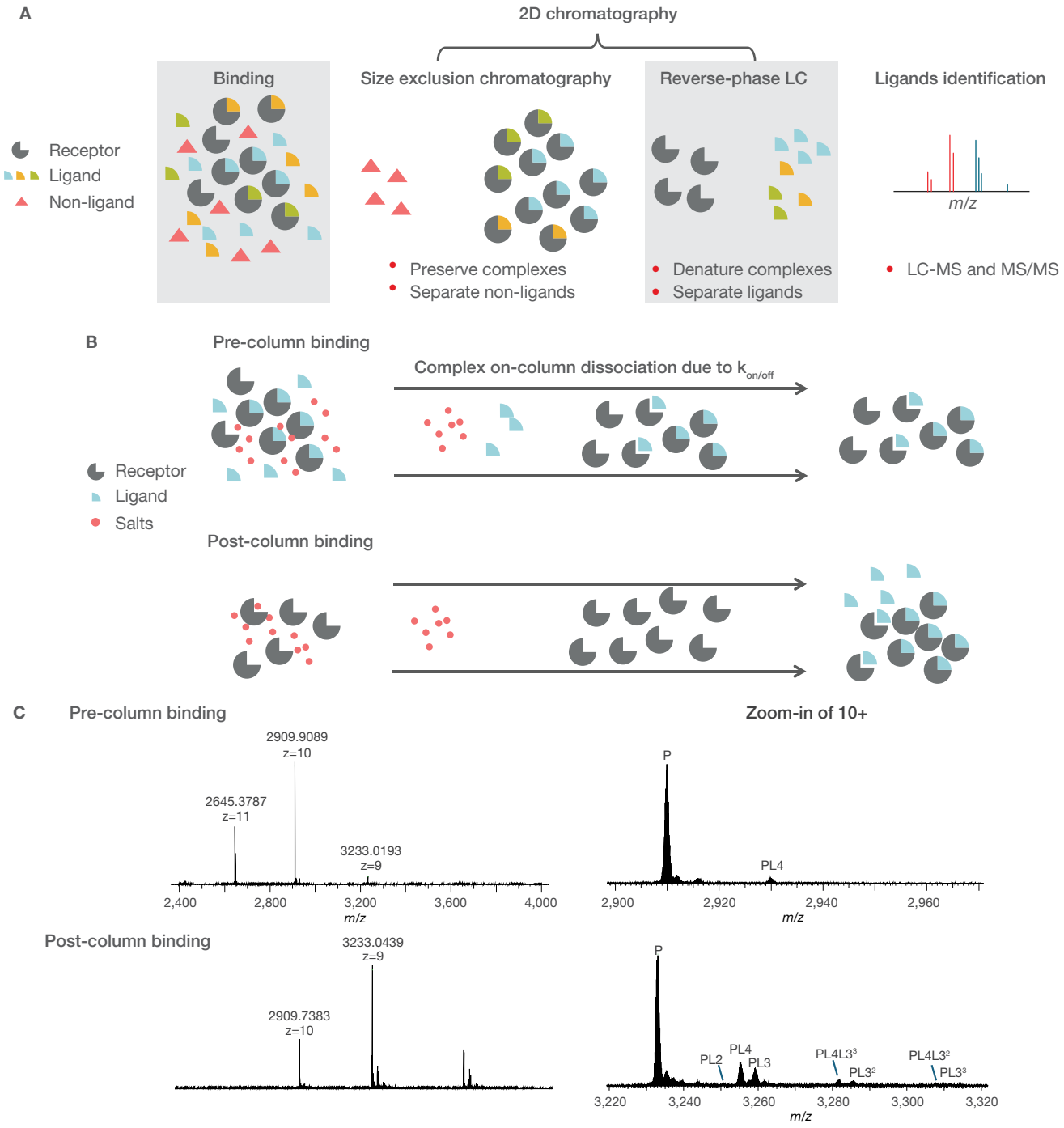


Figure 3. (A) AS-MS workflow; (B) Comparison of pre & post-column binding; (C) MS spectra of CA binding to 4-ligand mix pre & post-column

Automated ligand screening and K_d measurement

Inspired by preliminary studies comparing pre-column and post-column binding, we used the proposed LC set-up to mix CA with each of the four ligands separately for assessing automation and calculating apparent K_d. Online buffer exchanged CA was collected into a multi-well plate containing ligands L1 to L4 at varying concentrations. The final molarity ratios of CA to ligand were 1:0.5, 1:2, 1:4, 1:8, and 1:10. Figure 4A displays the raw spectra and zoom-in on the 10+ charge state from the 1:1 binding event. As expected, the fractional abundance of CA-ligand complex increases as the K_d decreases. Apparent K_d measurements in Figure 4B provide quantitative insights, demonstrating consistency with K_d values previously measured by other biophysical assays (Table 2).[4,5,6] The results demonstrate the capabilities of this workflow in automation and precise K_d measurement.

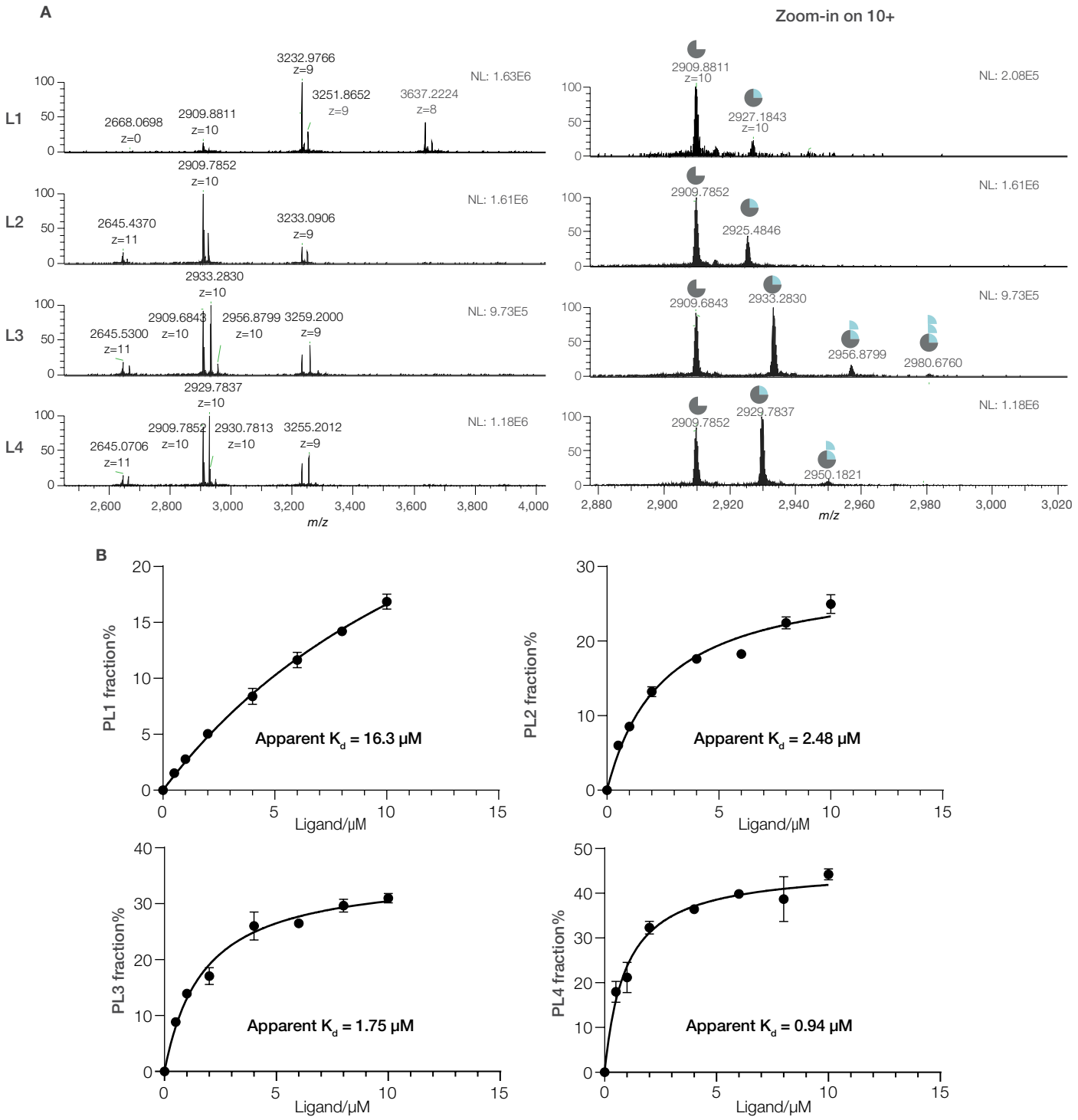


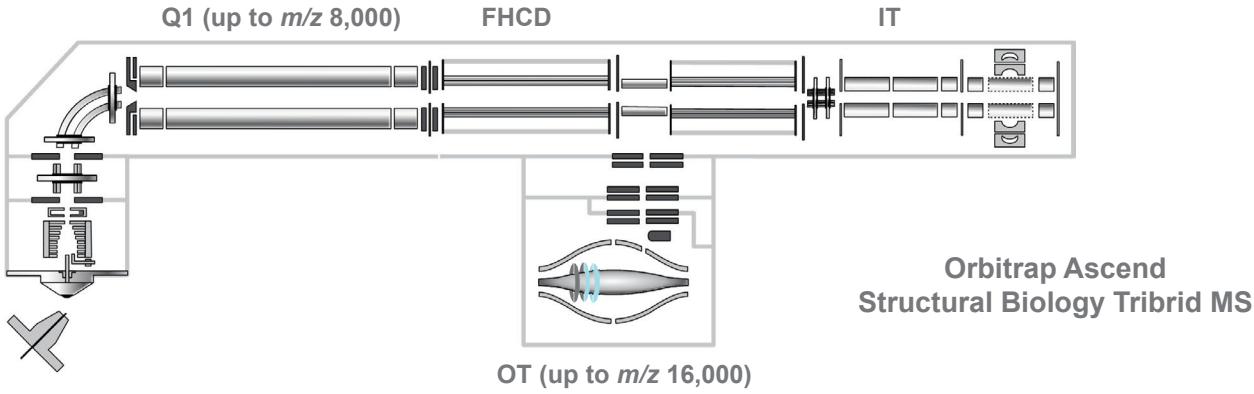
Figure 4. (A) CA binding to four ligands respectively; (B) L1 to L4 binding curve for K_d calculation to CA

Gas phase affinity selection MS for ligand identification and elucidation

To further enhance throughput, we increased the number of ligands per well. However, many ligands within the same class have very similar molecular weights, differing only by a functional group. Moreover, adducts and cofactors can be present in the complex binding. These factors may negatively affect or reduce the resolution of native MS. Consequently, increasing the number of ligands per well may introduce ambiguity in distinguishing binders from non-binders. To overcome this challenge, we developed a strategy called "Gas Phase Affinity Selection MS." This approach involves purifying the binding complex using a quadrupole, then releasing the bound ligands by applying collision energy before identifying them in the Orbitrap mass analyzer.

Gas phase affinity selection MS to increase throughput

1. **Full OTMS injection into OT**
2. **Target complex isolation in Q1**
3. **Target complex dissociation in FHCD**
4. **Dissociated complex and ligands detection in OT**
5. **Dissociated ligands fragmentation in IT for structure information**



View an animation of the gas phase affinity selection MS here.

To implement this strategy, we incubated CA with a 4-ligand mixture. Figure 5A shows the isolation of a single charge state of the binding complexes in either positive or negative mode. Upon applying collision energy to the isolated complexes, we observed that the negative mode revealed all the ligands in the low *m/z* region, while the positive mode did not. This suggests that when ligands are released in the gas phase, they may not always carry a positive charge, making *m/z* measurement in positive mode ineffective. Switching to negative mode allows successful identification of the ligands. A zoom-in of the low *m/z* region in the MS^2 spectrum collected in negative mode shows the presence of L1 to L4, along with an unknown ligand observed at *m/z* 270.9007 (Figure 5B). We further isolated this unknown ligand in MS^3 mode and fragmented it to determine its structure. Unsurprisingly, the most abundant fragments at *m/z* 234.9638 and *m/z* 171.0221 correspond to the molecular weights of L3 and L1. It indicates this unknown ligand belonging to the same "sulfanilamide" class. The mass shift between the precursor ion at *m/z* 270.9007 and the fragmented ion at *m/z* 234.9638 is 35.977 Da. It exactly matches with the monoisotopic mass of HCl. Therefore, we elucidated the unknown ligand as Di-benzenedisulfonamide plus a -Cl functional group. It is likely an impurity from one of the ligand products.

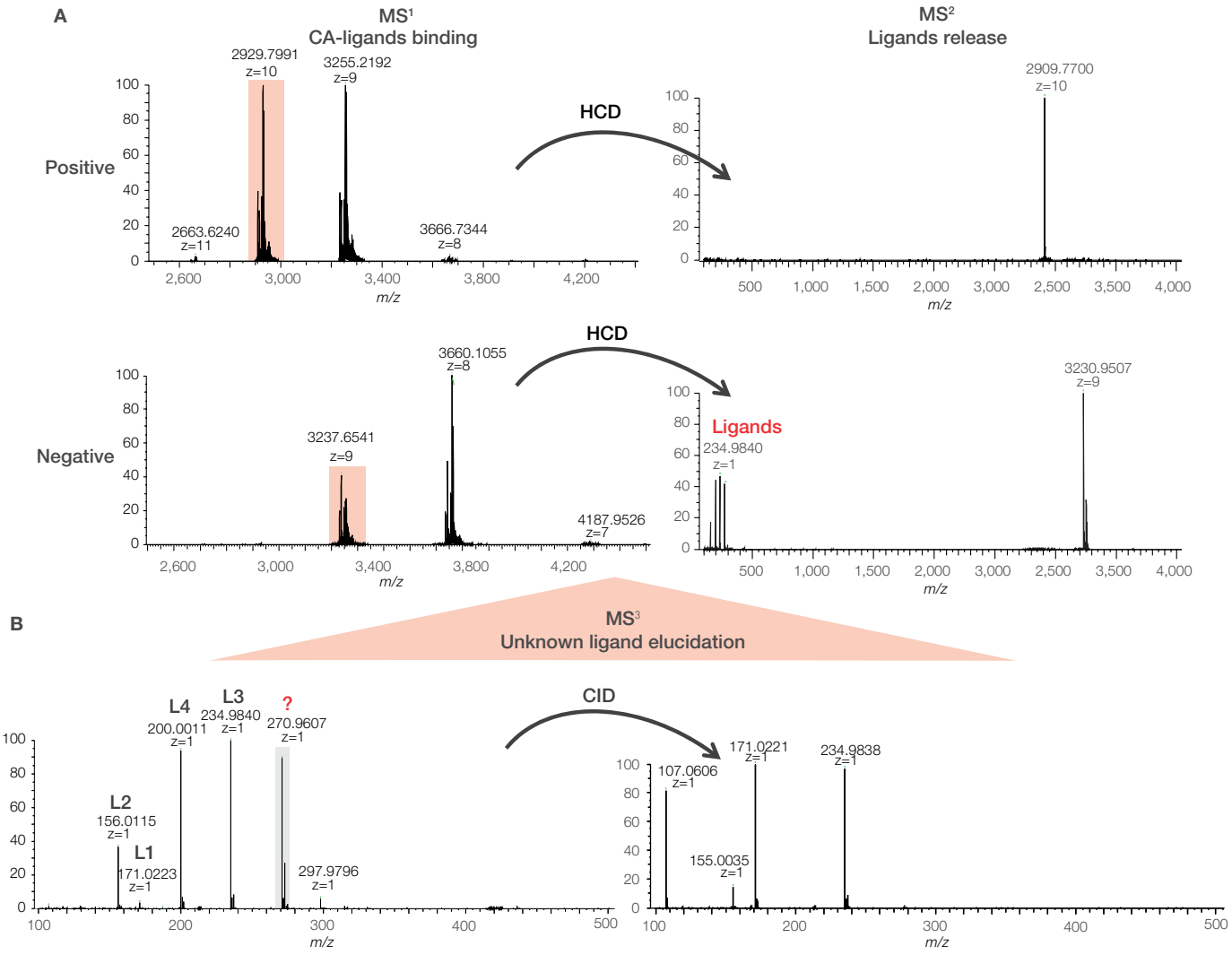


Figure 5. (A) Full scan of CA-ligands binding (left) and MS^2 scan for ligands release using HCD (right); (B) Isolation of unknown ligand at *m/z* 270 (top) followed by CID fragmentation for elucidation (bottom)

Conclusions

- An integrated LC-nMS system streamlines rapid online buffer exchange, multiplexed compound binding, and direct native MS analysis of complexes.
- This workflow has been applied for ligand screening to assess binding affinity, with a particular focus on preserving weak binders.
- The apparent K_d ranking correlates well with reported K_d values.
- "Gas Phase Affinity Selection MS" increases screening throughput, and MS^n ligand identification enables the identification of bound ligands and their structural elucidation.

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