Native Mass Spectrometry

Automated online protein-ligand binding and its detection using native mass spectrometry

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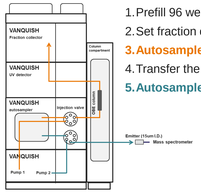
Abstract

Many proteins involved in diseases are considered "undruggable" using traditional small-molecule inhibitors due to their lack of well-defined binding pockets. Molecular glue (MG), by strengthening weak intrinsic interaction between target and E3 ligase, enables the targeted protein degradation via the ubiquitin-proteasome system. Native mass spectrometry (nMS) preserving the noncovalent interactions allows the identification of E3-MG-target ternary complex. However, offline manual sample preparation and direct infusion limits the throughput. A newly designed integrated LC system coupled to native MS streamlines the rapid online buffer exchange, parallel compound binding, and direct nMS analysis of complexes. The novel workflow has been applied for MG screening to assess their efficacy by directly detecting the ternary complexes.

Materials and methods

Methods

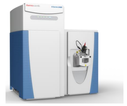


1. Prefill 96 well plate with one or more binders in each well
2. Set fraction collector temperature at desired binding temp
3. **Autosampler → Injection Valve 1 → OBE → UV → FC**
4. Transfer the sample plate from fraction collector to autosampler
5. **Autosampler → Injection Valve 2 → Easy-spray**

Instruments

Thermo Scientific™ Vanquish™ LC: UV detector, fraction collector, dual-injection autosampler, and dual Flex pumps

Thermo Scientific™ Q-Exactive™ UHMR



- m/z 350-80,000
- Quad up to m/z 25,000
- Native MS
- Native top-down
- Direct Mass Technology

Thermo Scientific™ Orbitrap™ Ascend™ Structural Biology edition

- m/z 50-16,000
- Quad up to m/z 8,000
- Native MS
- Native top-down
- PTCR/HCD/CID/ETD/UVPD

Data Analysis

Data analysis was performed with Thermo Fisher™ BioPharma Finder™ 5.0

Results

1. Carbonic anhydrase - Ligand binding

- We initially utilized Carbonic Anhydrase (P) as the target to evaluate ligand binding efficacy of the proposed workflow. Initial tests employed 4 ligands with varying K_d values, ranging from <100nM to >10uM.

Table 1. Carbonic Anhydrase ligands

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

Figure 1. Comparison of pre & post-column binding

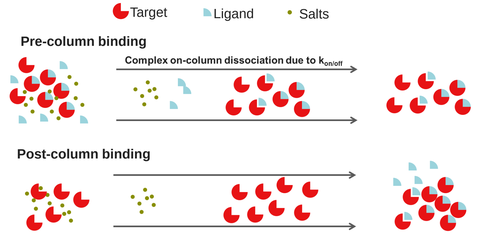
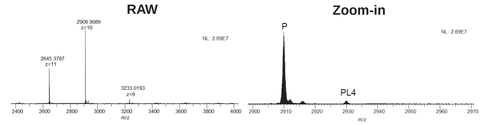
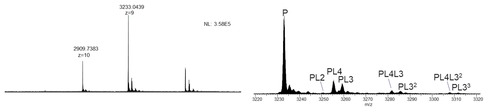


Figure 2. MS spectra of Carbonic binding to 4-ligand mix pre & post-column

- **Pre-column binding**



- **Post-column binding**



- ✓ Pre-column binding shows on-column dissociation
- ✓ Apparent K_d: L1>L2>L3>L4, aligning with published K_d
- ✓ Strongest ligand L4 shows most abundant primary binding

- Fraction collection of online desalted Carbonic Anhydrase prior to ligand binding

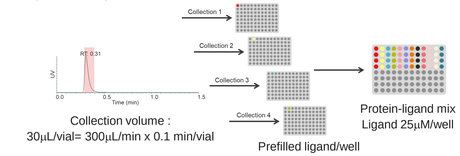
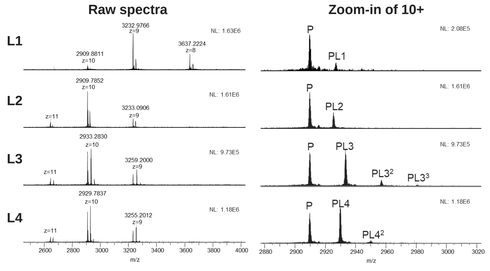


Figure 3. Carbonic Anhydrase binding to 4 ligands respectively



- ✓ PL fractional abundance%: L1<L2<L3<L4
- ✓ Apparent K_d: L1>L2>L3>L4
- ✓ Rank of K_d aligns with published results

2. Molecular glues screening

- Molecular glues stabilize interactions between E3 ligases and therapeutically relevant substrates to initiate substrate degradation via the ubiquitin-proteasome system.

Figure 4. Molecular glue (MG) strategy premise

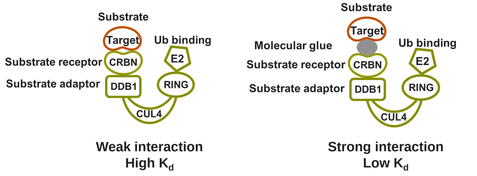


Table 2. Molecular glues of CRBN-DDB1 and target

Glue	MW	Ternary complex K_d (μM)	Comments
A1	483.46	0.0941	Control, strong K_d
B1	584.02	0.003	Top Glue with low K_d
C1	577.00	0.035	Top Glue with low K_d
D1	456.51	0.913	High K_d
E1 (-Ctrl)	441.86	>1	Negative control binds CRBN

Figure 4. MS spectra of CRBN-DDB1 and target binding to 5 MGs

CRBN-DDB1:Target:MG = 2.3μM:2.3μM:23μM

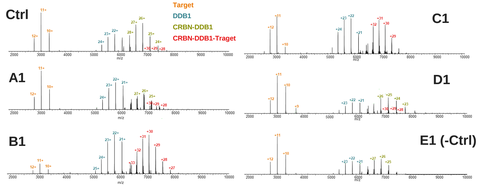
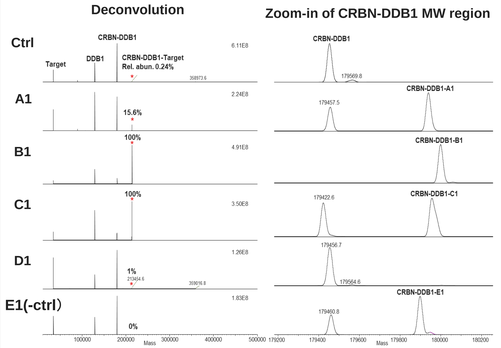


Figure 5. Deconvoluted spectra of CRBN-DDB1 and target binding to 5 MGs



- ✓ Apparent K_d: C1<B1<A1<D1<Ctrl<E1 (-Ctrl) aligns with results from biophysical assays
- ✓ Strong MGs show more binding on CRBN-DDB1

Table 4. Stability of CRBN-DDB1-Target complex

Glue	K_d	Frac. Abun. @ RT 10min	Frac. Abun. @4°C overnight
B1	Low	100%	97%
C1	Low	100%	22%
D1	High	1%	1.5%
E1 (-Ctrl)	Negative control	0%	0%

- ✓ Relative abundance of CRBN-DDB1 and CRBN-DDB1-Target dropped overnight
- ✓ Samples are unstable in 200mM AmAc, reflecting the importance of rapid screening after buffer exchange

Conclusions

1. On-column dilution and dissociation prevent the detection of weak binders.
2. Post-column binding facilitates the observation of weak binding event K_d>10uM.
3. Apparent K_d aligns well with published results or measurements from other assays.
4. Fraction collection coupled to direct infusion enables high-throughput ligand screening.
5. This workflow has been applied for MG screening to assess their efficacy by directly detecting the ternary complexes.

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