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Letter

Collision-Induced Affinity Selection Mass Spectrometry for Identification of Ligands

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Cite This: *ACS Bio Med Chem Au* 2022, 2, 450−455

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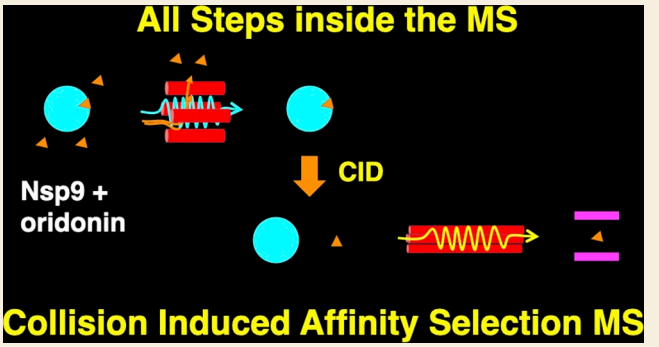
ABSTRACT: Hyphenated mass spectrometry has been used to identify ligands binding to proteins. It involves mixing protein and compounds, separation of protein−ligand complexes from unbound compounds, dissociation of the protein−ligand complex, separation to remove protein, and injection of the supernatant into a mass spectrometer to observe the ligand. Here we report collision-induced affinity selection mass spectrometry (CIAS-MS), which allows separation and dissociation inside the instrument. The quadrupole was used to select the ligand−protein complex and allow unbound molecules to be exhausted to vacuum. Collision-induced dissociation (CID) dissociated the protein−ligand complex, and the ion guide and resonance frequency were used to selectively detect the ligand. A known SARS-CoV-2 Nsp9 ligand, oridonin, was successfully detected when it was mixed with Nsp9. We provide proof-of-concept data that the CIAS-MS method can be used to identify binding ligands for any purified protein.



KEYWORDS: *affinity selection mass spectrometry, protein−ligand complex, SARS-CoV-2 Nsp9, CIAS-MS, oridonin, CID, Rabdosia rubescens*

INTRODUCTION

Electrospray ionization is a soft ionization technique that can be used to retain proteins in their native state. Native MS using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS) allows direct observation of a protein−ligand complex between an individual protein and a pure compound.[1−6] High-quality native MS protein spectra are required to distinguish the protein−ligand complex from unbound protein and to allow accurate calculation of the mass of the binding ligands. We have previously reported systematic optimization of MS instrument conditions for investigations of protein and ligand interactions.[1,7,8]

An alternative method that uses mass spectrometry to identify a ligand is hyphenated mass spectrometry, which can identify a bound ligand after affinity capture followed by dissociation (Figure 1). There are multiple methods to perform hyphenated MS, such as pulsed ultrafiltration mass spectrometry (PUF-MS), affinity selection mass spectrometry (AS-MS), size exclusion (SEC) AS-MS, and magnetic microbeads affinity selection screening (MagMASS).[9] These methods share the same principle, which involves mixing of protein and compounds, capture of the protein−ligand complex (e.g., SEC, ultrafiltration), dissociation of the protein−ligand complex, removal of the protein, and injection of the supernatant into a mass spectrometer for ligand observation.[10−13]

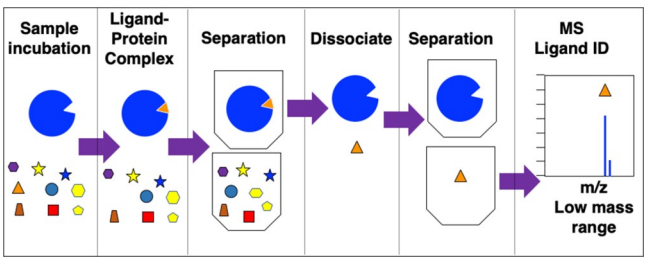


Figure 1. Basic strategy of AS-MS. Following incubation to allow formation of the ligand−protein complex, separation is needed to capture the high-molecular-weight components that are dissociated, requiring a second separation step to capture the low-MW ligand. The ligand mixture is finally injected into a mass spectrometer.

The advantage of hyphenated methods is that they do not require the protein to be visible under MS conditions.

Received: March 25, 2022
Revised: May 15, 2022
Accepted: May 16, 2022
Published: May 18, 2022

ACS Publications

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https://doi.org/10.1021/acsbiomedchemau.2c00021
ACS Bio Med Chem Au 2022, 2, 450−455

Downsides of this screening method are the requirement for multiple washings of the sample to ensure complete removal of unbound chemical,[14,15] loss of low-affinity ligands,[16] and the possibility that fast-dissociating protein−ligand complexes could be dissociated during the filtering process, leading to false-negative results.

To overcome the necessity for hyphenated methods and to provide a complementary method to native MS, we sought to develop a simple, universally applicable affinity selection approach that can identify a ligand binding to proteins. In our method, termed collision-induced affinity selection mass spectrometry (CIAS-MS), electrospray ionization is used to preserve ligand−protein complexes in the native state, to capture the protein−ligand complexes with simultaneous removal of unbound molecules, and to dissociate protein−ligand complexes inside the mass spectrometer. The method removes the need for any hyphenated approach.

Collision-induced dissociation (CID) has been widely used to study biological systems. Ionic protein−ligand interactions have been shown to be extremely stable in the gas phase, as exemplified by adenylate kinase in complex with ATP. CID resulted in the formation of sequence ions that retained the diphosphate group non-covalently bound to the protein. This feature was exploited to yield positional information on the site of ATP binding on adenylate kinase.[17] CID has been used to study the stoichiometry of tryptophan RNA-binding attenuated protein (TRAP) with tryptophan with up to 46 protein and ligand molecules.[18] Hemoglobin is a tetramer structure that includes two α and two β chains that bind non-covalently to a heme group. CID dissociated the $(\alpha H)_2(\beta H)_2$ tetramer complex to observe the dissociated subunit mass of the α monomer, the $\alpha\beta_2$ trimers, and the heme group.[19] CID was used to examine dissociation products from different states of unfolding of tetrameric transthyretin (TTR) in complex with the natural ligand thyroxine.[20] An analytically useful application of CID is its use to induce unfolding in association with ion mobility to discriminate Type I and Type II kinase inhibitors.[21]

For biologically relevant small molecules as drug leads or probe molecules, the interest lies in identification of ligands. Many methods are based on MS identification of the ligand following affinity-based capture and/or release. The advantages of MS include rapidity, high sensitivity, and specificity. In particular, it does not require labeling on either proteins or ligands. Applications of MS to study the interactions between small molecules and biological macromolecules have been reviewed previously.[22−24] Other examples are AS-MS and PUF-MS.[9,25,26] They have been developed to conduct high-throughput screening.[10,27]

Native ESI-MS allows the possibility to directly investigate protein−ligand interactions under nondenaturing conditions. Both non-covalent and covalent protein−ligand complexes can be detected by native mass spectrometry.[8,28] Ligands are identified by observation of mass-to-charge ratio shifts, and the molecular weight (MW) of the ligand can be determined by calculation of the mass difference between the unbound protein and the protein−ligand complex.[1−4,29]

The isolation and collisional activation of a protein−micelle complex formed between the *Escherichia coli* multidrug-resistant protein EmrE and *n*-dodecyl-β-D-maltopyranoside (DDM) in the presence of the known ligand tetraphenylphosphonium (TPP^+) yielded a low-MW product ion corresponding to TPP^+ upon CID.[30] The use of CID to dissociate protein−ligand complexes for potential screening applications has been demonstrated downstream from a gel permeation step to remove unbound molecules. The protein−ligand complex was isolated in the linear ion trap and subsequently activated by the use of CID to dissociate the ligand.[31]

Here we report the use of a quadrupole to trap the protein−ligand complex and remove unbound compounds. This technique removes the requirement for prior removal of unbound compounds via a preliminary separation step. Subsequent dissociation of the complex by CID allows detection of the ligand. This mix-and-measure protocol, whereby direct injection of protein with mixtures of potential ligands and all operations of affinity capture, separation, and dissociation are achieved in the mass spectrometer, results in significant reduction in timelines.

MATERIALS AND METHODS

Nsp9 Protein

Nsp9 was cloned, expressed, and purified as previously reported.[32] Nsp9 was buffer-exchanged with 150 mM ammonium acetate (Sigma-Aldrich, St. Louis, MO, USA) (pH 6.8) using Nalgene NAP-5 size G25 from GE Healthcare (Parramatta, NSW, Australia).

Oridonin

Oridonin was sourced from Nanjing NutriHerb BioTech (Nanjing, Jiangsu, China).

Plant Extract

Rabdosia rubescens extract powder (Nanjing Nutri-HerbBio Tech Co., Nanjing, China) (110 g) was mixed with boiling methanol (80 mL), and the mixture was cooled, filtered, and washed with cold MeOH. The soluble solution was then dried (44 g dry weight).

Each injection sample contained 49 μL of Nsp9 (10 μM) mixed with 1 μL of *R. rubescens* extract dissolved in methanol (1 mg/mL). The screening concentration of the extract was 20 μg/mL.

Pooled Compounds

Nine compounds were dissolved individually in DMSO and then mixed, freeze-dried, and dissolved in methanol as stock solutions. Each injection sample was 49 μL of Nsp9 (10 μM) mixed with 1 μL of compound dissolved in methanol. The screening concentration of each compound was between 45 and 166.6 μM.

Instrument Settings

All of the MS experiments were performed on a Bruker SolariX XR 12 T ICR-FTMS.

Native MS Experiment. The ESI source was operated in the direct injection configuration with the use of a 500 μL Hamilton syringe on the built-in syringe pump on the Bruker SolariX mass spectrometer at a flow rate of 120 μL/h. Nitrogen was used as the nebulizing gas at a pressure of 2 bar, and the capillary voltage was set at 4000 V. The end-plate voltage was set at −500 V. The dry gas flow rate was 4 L/min, and the temperature was 200 °C. The voltages of the source optics including capillary exit, deflector plate, funnel 1, and skimmer 1 were set at 200, 220, 150, and 30 V, respectively. Optic transfer was set at 2 MHz with a time of flight (TOF) of 1.5 ms.

CIAS-MS. The ESI source was operated in the direct injection configuration at a flow rate of 120 μL/h. Nitrogen was used as the nebulizing gas at a pressure of 2 bar, and the capillary voltage was set at 4000 V. The end-plate voltage was set at −500 V. The dry gas flow rate was 4 L/min, and the temperature was 200 °C. The voltages of the source optics including capillary exit, deflector plate, funnel 1, and skimmer 1 were set at 200, 220, 150, and 30 V, respectively. The quadrupole was set to capture ions between m/z 1800 and 2000. Argon was used as the collision gas in this experiment with 80% gas flow and 25 V ion acceleration to provide enough energy. Optical transfer was set to 4 MHz with a TOF of 0.65 ms.

https://doi.org/10.1021/acsbiomedchemau.2c00021
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RESULTS AND DISCUSSION

CIAS-MS Strategy and Proof of Concept

A mixture of protein and ligands was directly injected into the ESI source for ionization. The ionized sample was transferred to the quadrupole for mass selection, trapping the protein−ligand complexes and excluding any unbound small molecules. The protein−ligand complexes were then transferred to the collision cell for collision-induced dissociation. The dissociated protein and ligands were transferred to the ion guide with a low time-of-flight setting that allowed only the small ligand to reach the ICR cell for detection (Figure 2).

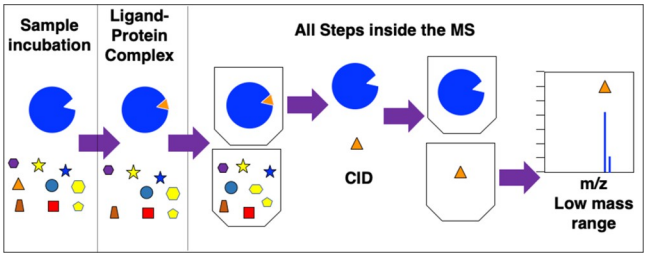

Figure 2. Basic strategy of CIAS-MS. The sample mixture is directly injected, and the ligand mass spectrum is observed directly. Inside the mass spectrometer, the quadrupole captures the protein−ligand complex, which is then dissociated by CID.

As a proof of principle, we examined the SARS-CoV-2 Nsp9 protein and its known ligand, oridonin, which was previously identified by native mass spectrometry.[33] The quadrupole was used to separate and remove unbound compounds so that only bound compounds (i.e., the high-MW components) remained in the mass spectrometer. Blank experiments were conducted by injecting Nsp9 or oridonin alone, and no ions were detected in the mass range of m/z 200−1000. Nsp9 (10 μM) and oridonin (10 μM) were incubated and infused into the mass spectrometer. No ion signals were detected without application of CID (Figure S1), indicating that the mass selection range in the quadrupole was capable of removing all unbound ligand. With CID on, three signals were detected, at m/z 387.17, 403.15, and 419.20 (Figure 3), corresponding to an oridonin sodium adduct, a potassium adduct, and a manganese adduct, respectively. Under positive-mode ESI conditions, oridonin appeared as the hydrogen adduct (base peak) with a 17% Na adduct (Figure S2). The oridonin $[M + H]^+$ species was not

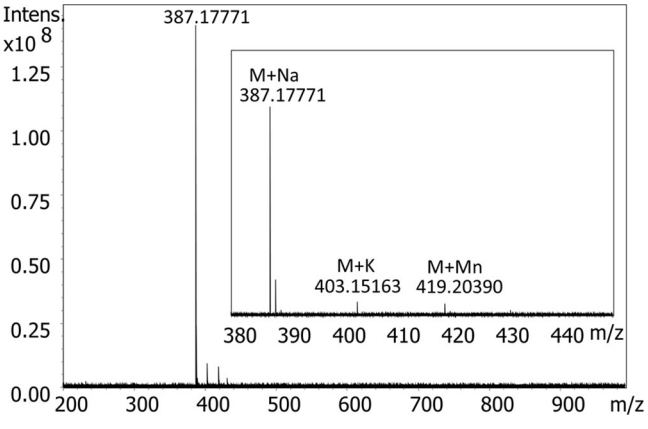

Figure 3. CIAS-MS of Nsp9 mixed with oridonin.

observed after CID from the oridonin−Nsp9 complex, indicating that adducted ions were generated within the CID cell. Our results indicate that CIAS-MS can capture a ligand−protein complex and remove unbound molecules using the quadrupole analyzer and that CID can dissociate the ligand−protein complex, allowing the dissociated ligand to be captured inside the mass spectrometer for detection.

CIAS-MS Using a Complex Compound Mixture

Next, we applied CIAS-MS to a pooled compound library containing oridonin (Figure 4). To demonstrate feasibility, we

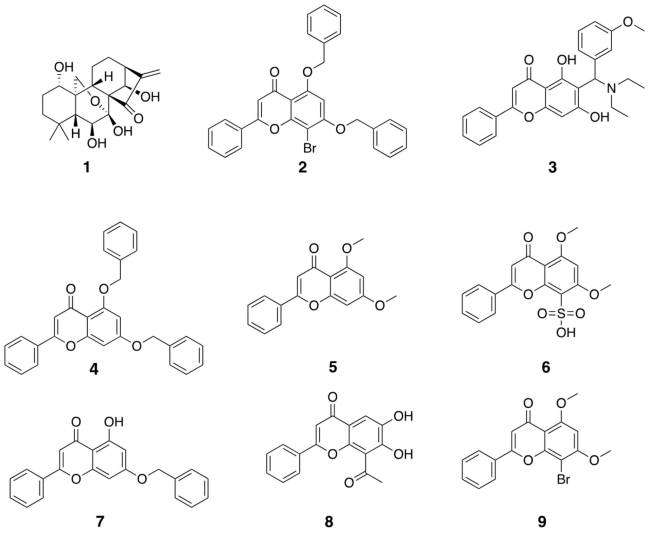

Figure 4. Structures of the nine compounds.

performed CIAS-MS using Nsp9 (10 μM) incubated with a pool of nine compounds (including oridonin) with concentrations between 45 and 166.6 μM. With CID on, two ion signals were detected. The major signal was m/z 387.18 (Figure 5), which was consistent with an oridonin sodium adduct. Interestingly, a minor signal at m/z 363.05 was also observed, and it was subsequently identified as compound **6** (MW = 362.05 Da). A native MS experiment was performed to validate binding of compound **6** to Nsp9. Protein−ligand complex signals were directly detected at m/z 1851 (7+) and 1620 m/z (8+), which correspond to the mass of Nsp9 (13

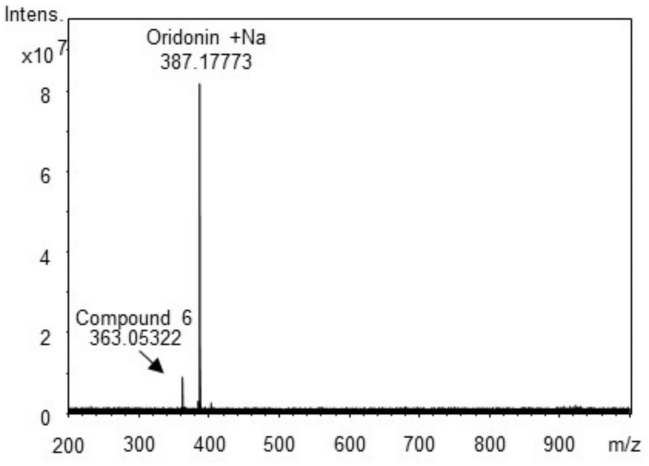

Figure 5. CIAS-MS of Nsp9 mixed with the mixture of nine compounds.

https://doi.org/10.1021/acsbiomedchemau.2c00021
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kDa) plus compound **6** (362.05 Da) (Figure S3). This result indicates that CIAS-MS can detect binding ligands from pooled compound libraries. Furthermore, CIAS-MS can also detect multiple binding in one experiment.

CIAS-MS Using Natural Product Extract

Next, we applied CIAS-MS to identify binding ligands of Nsp9 from extract of *Rabdosia rubescens*, a plant that is known to produce oridonin and its analogues. With CID on, a larger number of MS signals were detected, with the most intense signals being those of oridonin: m/z 387.17 ($[M + Na]^+$), 403.15 ($[M + K]^+$), and 419.20 ($[M + Mn]^+$) (Figure 6). A

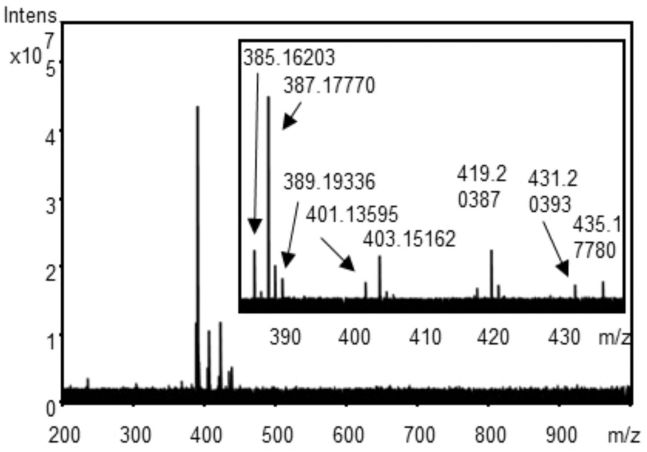

Figure 6. CIAS-MS of Nsp9 mixed with *Rabdosia rubescens* extract.

minor species with m/z 385.16 ($[M + Na]^+$) and 401.14 m/z ($[M + K]^+$) was also observed, and its MW was calculated as 362 Da. Apart from this newly detected ligand, we also detected several other ions, such as m/z 389.19, 431.20, and 435.18, which are likely to be analogues of oridonin produced by *R. rubescens*.

The Nsp 9−oridonin complex was confirmed by native MS, and the result showed no sign of nonspecific binding (Figure 7). Oridonin has been shown to have broad antiviral activity, reducing the viral load of SARS-CoV-2 in Calu3 cells.[33]

CID requiring a gel permeation step and three buffer exchange steps has recently been reported. This was rapid (10 min per sample) prior to MS analysis.[31] Online buffer exchange with an autosampler has been developed to provide

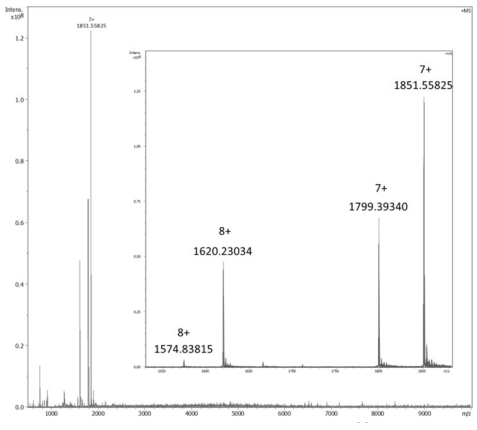

Figure 7. Native MS of the Nsp9−oridonin complex.

removal of nonvolatile buffer prior to MS injection.[34] The advantages of CIAS-MS is its direct nature, with all steps being completed within the MS, and an order of magnitude time savings. The CIAS-MS method uses a single buffer exchange of protein followed by incubation with compounds. This removes virtually all prehandling, allows automation for injection into a mass spectrometer, and circumvents the need for prior separation of bound and unbound ligand in the current hyphenated methods. By achieving the separation of bound and unbound ligand in the quadrupole, our method provides a protocol for single-step identification of ligands.

CONCLUSION

Collision-induced affinity selection mass spectrometry (CIAS-MS) is a new method that relies on the affinity between a protein and its ligand for identification of ligands. Previous hyphenated AS-MS methods for ligand identification have had substantial success, but many limitations remain. The key advantage of CIAS-MS is that it does not require externally performed washing or dissociation steps, as all steps are performed inside the mass spectrometer. It is much more time-efficient. Using CIAS-MS, we detected a ligand dissociated from a ligand−protein complex, a ligand in a pooled compound library, and a ligand in a natural product extract. Unbound ligand(s) were separated from ligand−protein complexes by quadrupole selection, and bound ligand(s) were dissociated by CID. CIAS-MS simplifies hyphenated mass spectrometry methods by providing a direct mix-and-measure method.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.2c00021.

CIAS-MS under current CIAS-MS conditions for Nsp9, oridonin, pooled compounds, and *R. rubescens* extract (Figure S1); mass spectrum of 10 μM oridonin in 150 mM ammonium acetate with 5% methanol (Figure S2); native MS of NSP9 mixed with **6** (Figure S3) (PDF)

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https://doi.org/10.1021/acsbiomedchemau.2c00021
ACS Bio Med Chem Au 2022, 2, 450−455

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Author Contributions

J.R. and D.R.L. cloned and purified the NSP9 protein. T.M., M.L., and R.J.Q. conceived and conducted the MS experiments. All of the authors contributed to the manuscript and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by the Australian Research Council Linkage Project (LE120100170). The authors thank Wendy Loa for maintenance of the mass spectrometry platform.

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https://doi.org/10.1021/acsbiomedchemau.2c00021
ACS Bio Med Chem Au 2022, 2, 450−455