Affinity Capture-Mass Spectrometry

Summary

One of the technical challenges in measuring and understanding the kinetics of multi component systems in complex biological and clinical samples has been the lack of instrumentation that simultaneously allow the quantification of kinetic rate constants and the identification of interacting partners. We here report the development of affinity-capture mass spectrometry (AC-MS) that enables the simultaneous measurement of kinetic parameters of protein-small molecule interactions with nanopore optical interferometry (NPOI), and the in-line identification of the interacting molecules by mass spectrometry (MS). This method has applications in drug-screening, affinity ranking of small molecules and proteins and the development of diagnostic reagents and assay conditions.

Introduction

A critical step in the development of drugs and biologics is the characterization of protein interactions. While binary interaction systems with purified compounds are widely used to determine kinetic properties of these interactions, the trend is to move to assays to test these interactions in more complex environments. For example, screening pooled drug libraries has the advantage to be able rapidly screen more compounds, however, the deconvolution of which compound(s) in this pool is the stronger binder is difficult and requires the design of secondary screens with pure compounds. Diagnostic assays are usually performed on complex biological specimens, like plasma or serum. When selecting reagents, such as antibodies that detect and quantify a specific biomarker, it is not always known how much these proteins cross react to non-specific plasma constituents and what these components are. Yet these cross reacting components significantly affect the fidelity of biomarker assays.

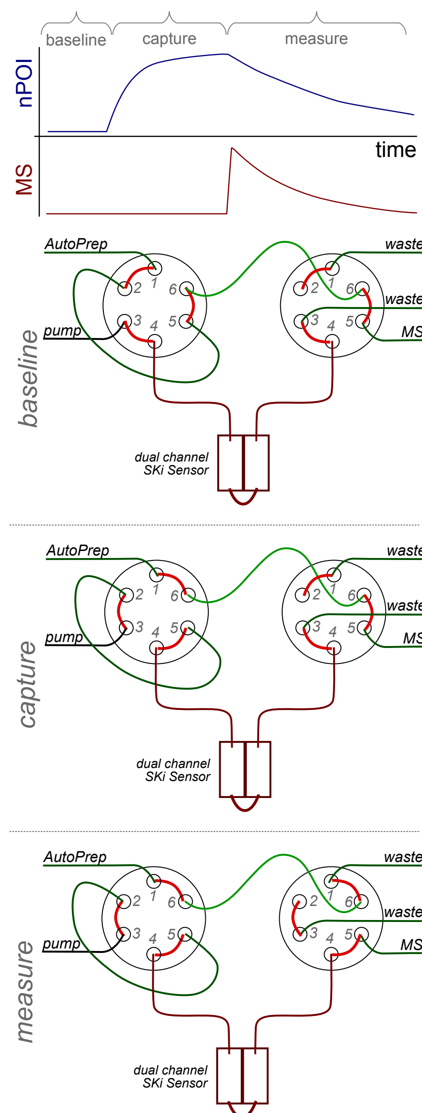

Figure 1: AC-MS using direct elution. Sample mixtures are applied to the surface, and association and dissociation is monitored in real time via optical interferometry measured as optical path difference (OPD). Eluent from the flow cell is injected directly in-line into the MS using two high pressure injection valves that are integrated within SKi Pro. Using a combination of SKi Report, as well as contact closure from the MS, a fully automated scheme for repetitive AC-MS analysis may be achieved.

To address these important limitations in today's screening applications, we have developed an AC-MS workflow that enables us to simultaneously measure on- and off- kinetics of ligands to their receptors and identify eluting ligands in-line or off line in a mass spectrometer.

Such experiments have been performed in the past using planar sensors—biochips employing a solid surface, as opposed to a porous one—but suffered from too little surface capture. Here we use SKi Sensors,[1] which are 80% porous. The pores have on average a 40 nm radius (r), and are 1.5 μm deep (h). In the case of a planar sensor, any particular 80 nm circular region has area πr^2, or 5,000 nm^2. Now considering this area as a cylindrical pore with area $\pi r^2 + 2\pi rh$ the available surface area become 400,000 nm^2. When accounting for the fact that 80% of the surface area is porous, there ends up being an 60× enrichment of surface area than with a planar sensor of the same size. SKi Sensors therefore, have much more surface capacity for the affinity capture of analytes. Using the known conversion of 1 nm OPD is 50 pg/mm^2 of material on the surface[2], the flow cell area of 2.4 mm^2, and the 60× enrichment factor, one can calculate the amount of material captured based on ΔOPD, which is typically 3 ng of small molecule or 100 ng of protein.

The NPOI flow cell will measure the association and dissociation of one of more ligands, which serves as measure that ligands were able to successfully bind and dissociate to the surface immobilized receptor. Eluting ligands can either be directly routed into a mass spectrometer (in-line modality), or trapped, salt exchanged and then injected (binning modality).

The subsequent study exemplifies both commonly utilized AC-MS modalities, using a nanoparticle-drug combination and carbonic anhydrase II (CAII) with a set of seven common CAII inhibitors as affinity-ligands.

Method

All interactions were monitored using SKi Sensor, COOH chips (p/n SKI-SENSOR-FC-8PACK-C) using carboxyl coupling reagents (p/n SUPPLY-0309) from Silicon Kinetics (San Diego, CA). Carbonic Anhydrase II (CAII) from bovine erythrocytes and sulfonamide inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). All experiments were done on SKi-Pro X10 flow cell instrument.

[1]See Silicon Kinetics Application note 2
[2]Ibid.

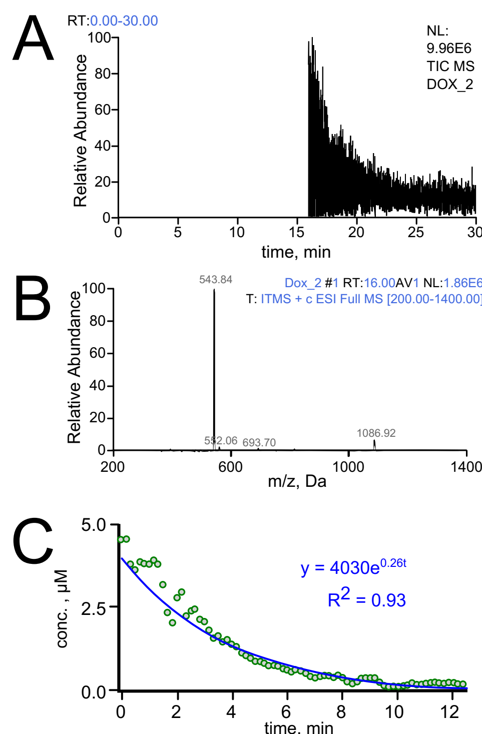

Figure 2: Real time elution measurement. **A** Total ion current spectrum from the eluent is shown starting during the dissociative phase.. **B** Mass spectrum at 16 minutes. **C** Dox ions quantified over time in XIC mode.

Label Free Binding Studies

CAII was immobilized onto the sample channel of the flow cartridges using standard amine coupling procedure. Enzyme was dissolved in water immediately prior to immobilization. Surface was activated with a 7-minute injection of EDC/sulfo-NHS mixture at 200/50 mM concentration. Protein was diluted to 200 μg/mL into 20 mM Acetate buffer pH 5.0 and injected for 10 minutes. Active sites were quenched with 1M Ethanolamine buffer pH 8.0 and washed with phosphate-buffered saline, pH 7.2 (PBS). Binding surface was stabilized with 3–5 blank injections of PBS. PBS was used as a running and sample dilution buffer in all experiments The tested sulfonamide CAII inhibitors were Furosemide, Acetazolamide, Benzenesulfonamide, 1,3-Benzenedisulfonamide, 4-Carboxybenzenesulfonamide, Dansyl amide, Sulfanilamide, Sulpiride and Methanesulfonamide. All inhibitors were first prepared as 100 mM stock solutions in Dimethyl Sulfoxide (DMSO) and further diluted into PBS immediately prior to the

experiments. Each analyte binding was analyzed in automated runs of 10 two-fold dilution series plus 2 "zero concentration" blank injections. No regeneration steps were necessary since all bound compounds spontaneously dissociated to baseline.

Data was analyzed with SKi Report control software for the SKi Pro X10-FC instrument. Referenced and blanked binding-dissociation curves were fit globally with two-state binding kinetics model. Sigmoidal dose-response fit was used for affinity analysis. For statistical analysis of data fidelity two populations were selected. One included all data points during last 10 seconds before inhibitor injection and represented background. The second included all data points during last 10 seconds of inhibitor injection and represented signal.

AC-MS

2.5 μg of a proprietary nanogel matrix was coupled to a COOH activated NPOI surface, washed extensively with water, and doxyrubicin (Dox, Selleck Chemicals, Houston, TX) from a 10 μM aqueous solution was captured onto the sample surface, followed by elution at a flow rate of 3 μL/min. Upon release of ligand, the flow is directed into the mass spectrometer, where the eluting sample is mixed with a 1% formic acid 80% methanol solution, which is t-ed into the flow-path at a flow rate of 2 μL/min. Dox ions were monitored and quantified in extracted ion mode in a ThermoFinnigan LTQ mass spectrometer.

For the real time work, 25 μL of a 0.1 mg/mL CAII solution was immobilized on a COOH activated nanoporous silicon surface and extensively washed with PBS. The immobilized OPD of CAII was typically 35 nm, which corresponds to ca. 1.8 ng/mm^2. Various concentrations of different sulfonylamides ranging from 0.3 to 30 μM were captured and monitored by NPOI. Eluting sulfonylamides were trapped on a 2.5 μL aqueous C18 trap column and exchanged into water (Silicon Kinetics). Contact closure from the SKi Pro triggered the elution of trapped sulfonylamides in 0.05% formic acid, 60% acetonitrile solution via an externally attached LC Packings HPLC and their subsequent identification and quantification in a QStar XL mass spectrometer (Applied Biosystems, Foster City, CA).

Results

As shown in Figure 2, we were able to measure, in real time, the elution of bound Dox from the nanogel matrix. We observed the characteristic parent ion of Dox, but also a number of ions derived from buffer contaminants and likely breakdown products from the nanogel matrix. After monitoring the extracted ion current (XIC) for Dox over time, it was now possible to determine the specific elution kinetics of Dox from the nanogel matrix. Using calibration standards that are directly injected into the mass spectrometer under identical injection conditions, it is now possible to determine the concentration of Dox that is eluting over time, thus establishing the binding capacity and stoichiometry of the nanogel matrix for Dox in addition to its elution kinetics.

We used an equimolar mixture consisting of seven sulfonylamides at 33 μM each and applied it to a 35 OPD carbonic anhydrase surface. After capture, we collected two 40 second bins—the first from 130–170 s the second from 185–225 s (Figure 3). After buffer exchange of each bin content into formic acid:acetonitrile

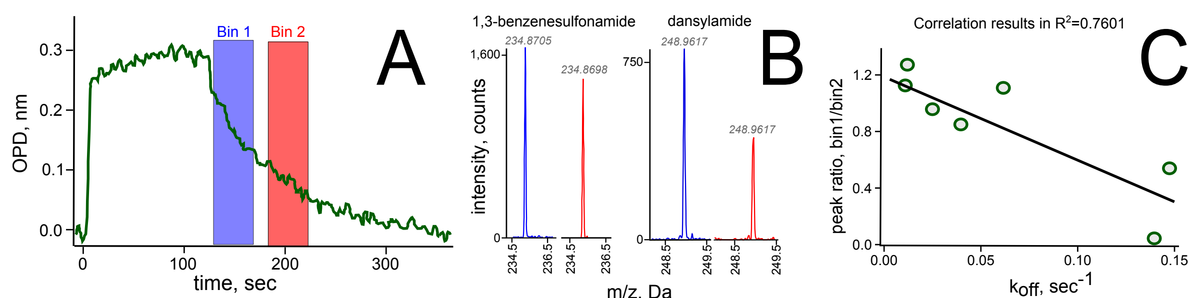

Figure 3: Results of binned AC-MS In **A** the total, label-free NPOI signal is shown for the mixture of sulfonylamides interacting with an immobilized CAII surface. The shaded areas shown indicate the 'bins' of eluent which were collected. **B** shows an example mass spectra segments comparing the peaks between 1,3-benzenesulfonamide and dansylamide. While **C** shows the inverse linear relationship between peak ratio between bin 1 to bin 2 and k_{off}.

on an aqueous C18 trap column, each bin was analyzed by mass spectrometry and each sulfonylamide was quantified in comparison to a concentration standard matrix that was established just prior to the sample acquisition run. It is easily apparent that 1,3-benzenesulfonamide (slower k_{off}) shows a minor reduction in concentration between first and second bin, while dansylamide (faster k_{off}) is reduced by half between the first and second bin (**B**). When comparing peak area rations between sulfonylamides simultaneously captured to carbonic anhydrase and their measured k_{off}rates (in singleplex), we are able to detect an inversely linear correlation between their peak ratio and k_{off}rate (**C**).

Discussion

The above studies demonstrates the feasibility of using the SKi Pro X-10FC system for affinity capture mass spectrometry and affinity ranking of ligand-analyte interactions in complex mixtures. Specifically, it combines the label-free kinetic characterization of molecular interactions with the analytical powers of the mass spectrometer to identify and orthogonally quantify interacting partners.

In the case of studying the release properties of a novel nanoparticle polymer designed to chelate Dox, AC-MS could establish the release kinetics of bound Dox by specifically quantifying Dox ions as they were eluting from the NPOI flow-cell surface in water. In addition to independently verifying the release kinetics measured by NPOI, AC-MS now enables us to study mixtures of molecules embedded in these nanoparticles by being able to independently quantify each component in the mixture, as long as they can be ionized by electrospray or CI and FAB ionization methods. In these instances NPOI serves to verify that the ligand was able to bind analyte from solution and to determine the release kinetics of the entire mixture. The in-line MS then serves to deconvolute the ions in the mixture and enables us to quantify each molecule as a function of time. In this particular example, we added a mixture of organic and formic acid to the flow path after the flow-cell to facilitate efficient ionization of the eluting Dox.

The second case study focuses on the enzyme CAII and seven inhibitors belonging to the sulfonamilamide family. While the binding parameters of each compound to CAII is known, we wanted to establish proof of concept to rank all seven inhibitors in terms of binding strength. We were indeed able to experimentally establish a direct relationship between off rate of each molecule and the elution behavior observed by AC-MS. This finding established the utility of AC-MS in determining the release kinetics and affinity ranking of small molecule inhibitors in complex mixtures.

Since the intramolecular interactions in the latter study require PBS as buffer system, which is incompatible with mass spectrometry, we collected fractions (bins) early and late during the elution process. Each bin was in-line buffer exchanged into an organic formic acid buffer to facilitate ionization in an electrospray source. While the binning strategy looses the real-time resolution power of direct in-line injection, it still can rank the inhibitors according to interaction strength.

Overall, AC-MS is a versatile and highly reproducible method that allows us to understand and quantify the simultaneous interactions of many molecules with an immobilized ligand, or to determine possible analytes binding to a ligand from a complex mixture. Its applications are in drug-target interactions, mixed library screening, affinity ranking of complex intramolecular interactions, nanoparticle formulation development, pharmacokinetics, biomarker discovery and biomarker assay development. AC-MS is enabled by the significantly augmented surface area of nanoporous silicon, whose binding capacity is compatible with the analytical performance of modern mass spectrometers, thus setting it apart from capture on planar surfaces.

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