Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information for

Mass Spectrometry-Based Monitoring of Millisecond Protein-Ligand Binding Dynamics Using Automated Microfluidics

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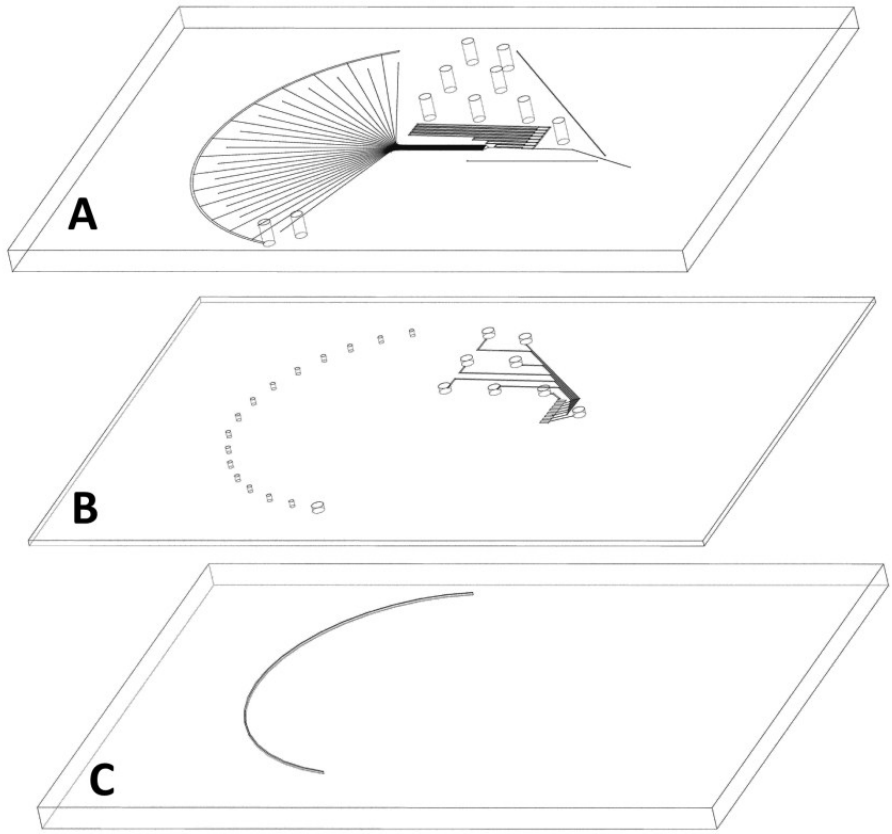

Figure S1. Microchip fabrication schematic showing PDMS chip assembly. (A) Plate with flow channels facing down. (B) Membrane with "push-up"-style[1] control channels facing down. (C) Cover plate with ligand introduction channel facing up. The flow layer incorporates the multi-lamellar flow mixer, an incubation chamber comprising eight distinct flow paths, a flow channel for electrospray, two mark channels to guide emitter cutting, and a protein introduction channel. The ligand introduction channel connects to the shorter co-flowing channels after bonding via holes punched through the membrane layer.

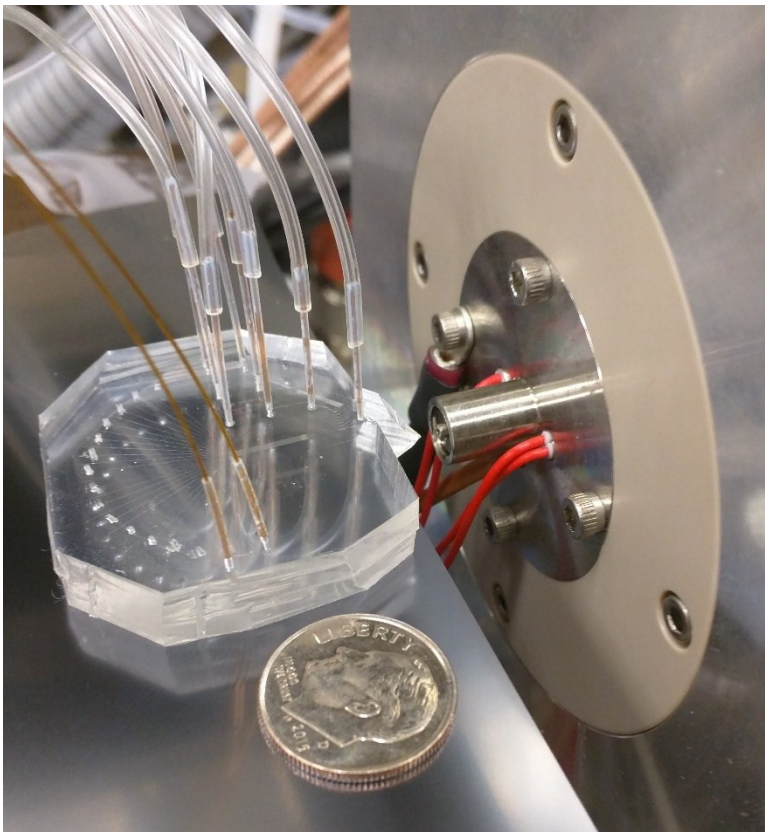

Figure S2. Microchip assembly interfaced with mass spectrometer inlet. A US dime is included for scale.

Materials and sample preparation.

Carbonic anhydrase (hCAI), furosemide, ammonium acetate and HPLC grade methanol were purchase from Sigma-Aldrich (St. Louis, MO). Colored dyes were purchased from ESCO Foods Inc. (San Francisco, CA). PDMS elastomer base and curing agent were purchased as Dow Corning Sylgard 184 from Ellsworth Adhesives (Germantown, WI). Water was purified using a Barnstead Nanopure Infinity system (Dubuque, IA). Carbonic anhydrase was desalted using an Amicon ultra-0.5 mL centrifugal filter (Millipore, Billerica, MA) and stored at a concentration of 120 µM in a 60 mM ammonium acetate buffer. For protein-ligand binding kinetics, the protein and furosemide were dissolved in 10 mM ammonium acetate buffer with 10% methanol added to enhance electrospray stability.

Microchip design and fabrication.

The device was fabricated using multilayer soft lithography[1] and created from three layers: a substrate containing flow channels (Figure S1A); a membrane (~50 µm thick) containing control channels (Figure S1B); and a cover containing a ligand introduction channel (Figure S1C). The flow layer incorporates a multi-lamellar flow mixer with 28 co-flowing channels (10 µm wide, 10 µm deep) that merge into a single narrow channel (20 µm wide, 10 µm deep), the incubation chamber contains eight flow paths, a flow channel (25 µm wide, 10 µm deep) for electrospray, two marker channels to guide emitter cutting with a razor blade[2, 3], and a protein introduction channel (200 µm wide, 10 µm deep) which connects to 14 long co-flowing channels of the mixer distributed alternately with the other 14 short co-flowing channels. The control layer had eight dead-end microchannels that were 100 µm wide and 25 µm deep, aligned at corresponding flow paths after bonding. The PDMS membrane between flow and control channels served as pneumatic microvalves, which were actuated by applying pressure in the control channels. The ligand introduction channel (200 µm wide, 10 µm deep) is on the cover layer and connects to the 14 shorter co-flowing channels after bonding via holes punched on the membrane layers (Figure S1B) using a 20-gauge catheter punch (Syneo, West Palm Beach, FL). Figure 1C shows the side view of the connection between the protein/ligand introduction channels and the co-flowing channels of the micromixer. In this design, the protein and ligand can be introduced and distributed to the co-flowing channels of alternating composition.

Three templates were produced on silicon substrates by photolithographic patterning from a single photomask as described previously[4]. The photomask was designed using AutoCAD software (Autodesk, San Rafael, CA) and printed onto a photoresist-

coated chrome-on-glass photomask blank (Telic, Valencia, CA) using an Intelligent Micro Patterning SF-100 Express direct-write lithography system (Saint Petersburg, FL). There are two steps to create the flow layer mold. The first step is to create the mold for the rounded channels[1] on which the control channels sit as microvalves in the incubation chamber. A silicon wafer was spin-coated with AZ P4620 (Capitol Scientific, Austin, TX) at 1600 rpm for 30 s. After soft-baking for 2 min at 70 °C and 3 min at 110 °C and then allowing the resist to rehydrate at room temperature for ~20 min, the wafer was loaded into the photomask aligner (Neutronix-Quintel 4006, Morgan Hill, CA) and exposed for 30 s. The features were then developed in a 1:3 solution (v/v) of AZ 400 K Developer (Capitol Scientific, Austin, TX) in water and the wafer was hard-baked at 180 °C for 25 min. The second step to finish the flow layer mold is to pattern the channels having rectangular cross-section over the rounded channels. The AZ P4620-patterned wafer was spin coated with SU-8 25 (MicroChem, Newton, MA) at 2000 rpm for 30 s and soft-baked at 65 °C for 3 min and then at 95 °C for 7 min, after which the wafer was again loaded into the photomask aligner and exposed for 20 s. The SU-8 was post-exposure baked at 65 °C for 1 min and 95 °C for 4 min, developed in SU-8 Developer (MicroChem), rinsed with SU-8 developer, blown dry with N_2 and then hard baked at 180 °C for 20 min. The control layer and cover layer molds were created on separate silicon wafers using SU-8 25 that was spin-coated to the same thickness as that on the flow layer and processed using the same conditions.

PDMS was prepared by thoroughly mixing Sylgard 184 base and curing agent at a 10:1 (w/w) ratio. The PDMS was spin-coated on the control layer mold at 1500 rpm for 30 s. PDMS was also poured over the flow layer mold and the cover plate mold to a thickness of 3–4 mm and degassed under vacuum. All substrates were cured at 70 °C for 2 h. The flow layer substrate was then peeled from its template and holes were punched at the end of the protein introduction channel. The holes were cleaned with water to wash out PDMS debris and then dried with compressed nitrogen. The surfaces of the flow layer and control layer substrates were activated in an oxygen plasma system (PX-250, March Plasma Systems, Westlake, OH) at 50 W power and 200 mTorr pressure for 30 s. After activation, the flow layer and control layer were aligned, bonded and placed in an oven at 70 °C for 1 h to improve bond strength. The bonded flow and control substrates were then cut and removed from the control layer template. A punch was used to penetrate the bonded layers at the end of the short co-flowing channels to create the holes that connect the short co-flowing channels with the ligand introduction channel. These bonded layers were then cleaned, oxygen plasma activated, aligned and bonded to the cover layer that contained the ligand introduction. The assembly was then placed in an oven at 70 °C for 1 h to improve bond strength.

Protein-ligand binding dynamics measurement.

A Nexus 3000 syringe pump (Chemyx Inc, Stafford, TX) was used to deliver protein and ligand solutions using two 500 µL glass Hamilton syringes. Two 50-cm-long, 360-µm-o.d., 150-µm-i.d. capillaries (Polymicro Technologies, Phoenix, AZ) were used to connect syringes to the protein and ligand introduction channels. One end of each capillary was connected to the syringe via the assembly of Upchurch union and fittings (IDEX Health & Science LLC, Oak Harbor, WA), while the other end was inserted into a short section 762 µm o.d., 304 µm i.d. polytetrafluoroethylene (PTFE) tubing (Cole-Parmer, Vernon Hills, IL). The PTFE tubing was in turn inserted into the 20-gauge punched holes at the ends of the protein and ligand introduction channels. To monitor protein-ligand binding kinetics, the introduced protein and ligand solutions were initially split into 14 long and 14 short co-flowing channels, respectively, and then mixed rapidly. The mixing is triggered at the merging triangle and completed in the single narrow microchannel. The mixture was incubated in the incubation chamber with the reaction time determined by the volume of the selected flow path. The selection of the flow path was controlled by the actuation state of the microvalves. The microvalves in the PDMS device were actuated by computer-controlled solenoid valves (Festo, NY). A home-written program was used to control the valve operation and a pressure of 40 psi was applied to the control channels to actuate the valves[5]. After incubation, the mixture was electrosprayed for determination using an ion funnel-modified[6] IMS/MS platform. The ion mobility separation was not employed for protein-ligand binding kinetics study, but the ~1 m drift tube ensured gentle desolvation of the noncovalent complexes. 10% methanol was added into the solution to improve electrospray stability. The microchip emitters were positioned 2–3 mm from the MS inlet capillary, which was heated to 200 °C. A potential of 5 kV was applied to the syringe needle of ligand solution for electrospray.

The raw MS files were processed and the resulting mass spectra were exported to Microsoft Excel. For kinetic rate constant determination, complex and unbound protein concentrations were obtained for each time point based on the known starting concentrations and the ratio of complex to unbound protein. The concentrations of protein and protein-ligand complex after incubation were calculated by the relative peak intensity ratio of the native protein and protein-ligand complex after baseline subtraction. This approach assumes that ionization efficiency is the same for protein and complex[7]. The ratio was determined based on the intensity of the 10^+ charge state of hCAI (*m/z* 2885.5) and hCAI+furosemide (*m/z* 2918.6) after baseline subtraction. The baseline was taken as the average intensity from *m/z* 2870–2880 for hCAI and *m/z* 2903–2913 for hCAI+furosemide. The bound and unbound concentrations were determined for starting concentrations of 5 µM hCAI and both 25 and 50 µM furosemide at each time point. The concentrations at each time point and their standard deviations based on

three replicate measurements were entered into Graphpad Prism 6 (La Jolla, CA), and the rate constants were determined using the Association Kinetics function based on pseudo first order kinetics.

Note and reference:

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