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Microsecond Microfluidic Mixing for Investigation of Protein Folding Kinetics

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KEYWORDS: focusing, mixing, folding, protein

ABSTRACT
We have developed and characterized a mixer to study the reaction kinetics of protein folding on a microsecond timescale. The mixer uses hydrodynamic focusing of pressure-driven flow in a microfluidic channel to reduce diffusion times as first demonstrated by Knight et al.[1]. Features of the mixer include 1 μs mixing times, sample consumptions of order 1 nl/s, loading sample volumes on the order of microliters, and the ability to manufacture in fused silica for compatibility with most spectroscopic methods.

INTRODUCTION
Although important structural events in protein folding are known to occur on the sub-millisecond time scale, the investigation of fast protein folding kinetics has been limited by the time required to mix protein and denaturant solutions. Experiments have demonstrated protein folding with mixing times of ~50 μs but with high sample consumption [2]. Hydrodynamic focusing has been used in combination with small SAXS [3] and FTIR spectroscopy [4] to measure protein folding, both with mixing times of a few hundreds of microseconds. The mixer that we describe here reduces those time scales to a few microseconds and allows measurements both in the visible and the UV.

Our mixer creates a sub-micron jet using a 1 um nozzle and uses 1 to 10 m/s advection velocities to minimize mixing time. A protein-denaturant solution is hydrodynamically focused to a sub-micron wide stream and denaturant diffusion out of the stream induces protein folding. As shown in Figure 1, once protein folding is initiated, the process is monitored by observing spectroscopic changes at downstream locations.

The mixer was manufactured using silicon micromachining technology. A deep reactive ion (DRIE) process was used to etch a silicon wafer after photolithography. Figure 2 is an SEM image of the mixer before a glass wafer is anodically bonded to the top. We

characterized the mixers with micro-particle image velocimetry (microPIV), confocal scanning fluorescence microscopy, and fluorescence quenching measurements.

NUMERICAL MODEL

The mixer flow was analyzed using a commercial fluid dynamics solver, CFD Research Corporation's ACE code (CFD Research Corporation, City, State). The model geometry is a 2D non-uniform rectilinear grid. Non-dimensionalizing the Navier-Stokes equations gives 4 four non-dimensional parameters to fully describe the flow:

$$\begin{aligned}
&\frac{\partial u^*}{\partial x^*} + (\alpha\beta)\frac{\partial v^*}{\partial y^*} = 0 \\
&\mathrm{Re}(u^*\frac{\partial u^*}{\partial x^*} + \alpha\beta v^*\frac{\partial u^*}{\partial y^*}) = -\frac{\partial P^*}{\partial x^*} + (\frac{\partial^2 u^*}{\partial x^{*2}} + \alpha^2\frac{\partial^2 u^*}{\partial y^{*2}}) \\
&\mathrm{Re}(u^*\frac{\partial v^*}{\partial x^*} + \alpha\beta v^*\frac{\partial v^*}{\partial y^*}) = -\frac{\alpha}{\beta}\frac{\partial P^*}{\partial y^*} + (\frac{\partial^2 v^*}{\partial x^{*2}} + \alpha^2\frac{\partial^2 v^*}{\partial y^{*2}}) \\
&u^*\frac{\partial c^*}{\partial x^*} + \alpha\beta v^*\frac{\partial c^*}{\partial y^*} = \frac{1}{Pe_D}(\frac{\partial^2 c^*}{\partial x^{*2}} + \alpha^2\frac{\partial^2 c^*}{\partial y^{*2}})
\end{aligned} \tag{1}$$

with the following non-dimensional parameters; $\alpha=w_s/w_c$, $\gamma=w_e/w_c$ $\beta=U_s/U_c$, $\mathrm{Re}=U_c w_s/\nu$, and $Pe_D=U_c w_s/D$. The subscripts *s*,*c* and *e* refer to the side, center and exit channels, U is the maximum velocity, ν is the kinematic viscosity, and D is the diffusion coefficient. We optimized the geometry and flow conditions by parametrically varying these five parameters in the flow model to reduce mixing time. Since the flow in our mixer is laminar (Reynolds number based on nozzle diameter is 15), we can accurately calculate denaturant concentration histories and probability distributions of protein location in a given detection window.

Figure 3 shows the concentration of guanidine, ($D_{guanadine}$=1000 $\mu m^2/s$) for a 50 nm wide stream and focused stream velocity of 5.0 m/s from a CFDRC simulation. By integrating the stream-wise velocity along the vertical coordinate, we can plot local concentration versus time as is shown in Figure 4. Assuming the mixing time is that required for the concentration to drop to 50% of its initial value, a one microsecond mixing time is obtained with these conditions.

EXPERIMENTAL

The silicon microfluidic chips are mounted to an acrylic holder which includes reservoirs for samples and pressure interconnects. Pressure is applied to the reservoirs with computer controlled regulators and the applied pressure is measured with silicon-on-sapphire pressure gauges. The plastic holder allows fast switching of working fluids (<30 sec) and the large sample reservoirs (250μL) allow operation for hours before refilling. Sample consumption of the protein solution is on the order of 1 nL/s. Characterization of the mixers required correlating applied inlet pressures to fluid velocities and focused stream widths.

Micro-particle image velocimetry [5] measurements allowed us to accurately measure velocity fields in the mixer at velocities up to 1.0 m/s. A solution of water and 0.1% Triton-X100 surfactant was seeded with 300 nm polystyrene fluorescent beads (Interfacial Dynamics, Portland, OR) at 0.05% solids. Two pulsed, Q-switched Nd:YAG lasers (New Wave Research, Sunnyvale, CA) and a 5MHz interline CCD camera (Roper Scientific, Trenton, NJ) were used to acquire image pairs down to 10 μs apart. The image pair intensities were then cross-correlated to determine particle displacements.

We also verified the diffusive mixing process by measuring fluorescent dye quenching using a scanning confocal microscope. A solution of 2 megadalton dextran conjugated fluorescein ($D_{dextran}$=7 $\mu m^2/s$) at 10 μM was quenched with 2 M KI (D_{I^-}=2045 $\mu m^2/s$) . The confocal microscope used a 10 μm pinhole to spatially filter out of focus light and an avalanche photodiode for detection.

RESULTS AND DISCUSSION

Figure 5 shows a typical averaged velocity vector field in a 21 μm wide by 8.6 μm deep channel obtained from microPIV. Velocity vector resolutions of 1 vector/μm were achieved, and correlations were averaged over 100 image pairs for each vector. Figure 6 plots the external pressure applied to the center inlet channel versus the flow rate calculated from measured velocity profiles, along with the analytical solution. Variations in measured velocities between mixers are typically less than 10%.

The analytical solution for laminar flow in a rectangular duct is,

$$u(y,z) = \frac{16a^2}{\mu\pi^3}\left(-\frac{dp}{dx}\right)\sum_{i=1,3,5,\ldots}^{\infty}(-1)^{\frac{i-1}{2}}\left[1-\frac{\cosh(i\pi z/2a)}{\cosh(i\pi b/2a)}\right]\frac{\cos(i\pi y/2a)}{i^3} \qquad (2)$$

$$Q = \frac{4ba^3}{3\mu}\left(-\frac{dp}{dx}\right)\left[1-\frac{192a}{\pi^5 b}\sum_{i=1,3,5,\ldots}^{\infty}\frac{\tanh(i\pi b/2a)}{i^5}\right]$$

where a is the half-width and b is the half-height of the channel. This solution is used to calculate flow rate from PIV velocity data, and can be used to calculate the focused stream width based on mass flow. A simple model analogous to an electrical circuit [1] was also used to predict the stream width, w_s, at various conditions. This model gives,

$$w_s = \frac{2\lambda(\phi-1)+\phi\sigma}{\Gamma h(2+\phi\sigma)} \qquad (3)$$

where $\lambda=R_e/R_c$, (exit channel to center inlet channel impedance ratio), $\sigma=R_s/R_c$, $\varphi=P_c/P_s$, Γ=centerline velocity/ flow rate for exit channel, and h=depth of the exit channel. Figure 7 shows the stream width based on PIV velocity measurements and mass flow, as well as the analytical solution based on the circuit model (eq. 3).

Fluorescence quenching experiments were performed at a pressure ratio of φ=20 (w_s=1.6 μm) to show the diffusion of the iodide ions into the fluorescein stream. The fluorescein intensity decreases by 36% 10 μm downstream from the nozzle in the presence of iodide, verifying diffusional mixing.

Ongoing work includes the use of FRET to investigate the folding kinetics of a simple two-state folding protein, and the development of ultraviolet-absorption based system for detection of protein conformation using naturally occurring tryptophan. Furthermore, we plan to refine existing measurements of protein folding on a single molecule level (*6*).

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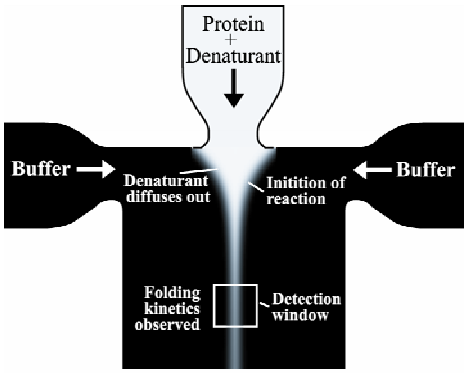

Figure 1

Schematic of diffusive mixing process. A solution of protein and denaturant enters from the top middle channel and is hydrodynamically focused into a thin stream. The

denaturant quickly diffuses from the focused, sub-micron-wide stream and induces folding of the protein.

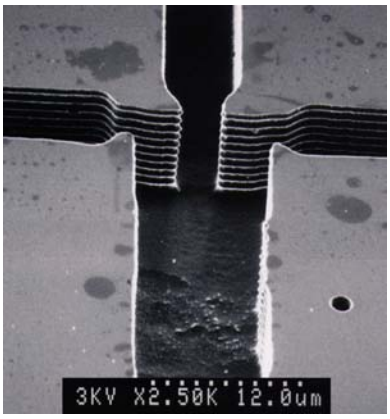

Figure 2

SEM image of the nozzle area before the glass cover-slip is bonded on top. Channels are 8.6 μm deep and the nozzles are 2.5 μm wide. Scalloped walls are a result of the deep reactive ion etching process.

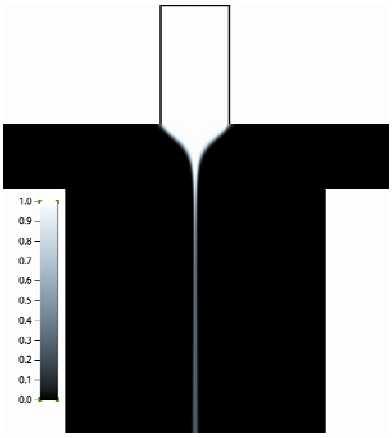

Figure 3.

2D Simulation of guanidine diffusing in water using CFDRC's ACE^{TM} code. Stream width is 50 nm, and stream velocity is 5 m/s. Intensity indicates normalized guanidine concentration.

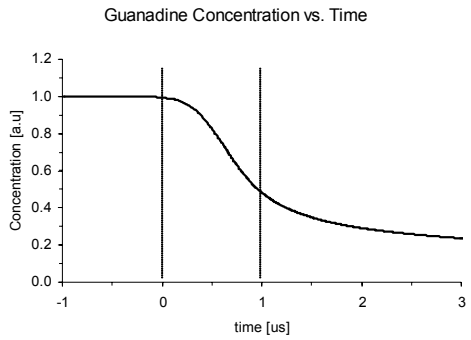

Figure 4.
Concentration profile along centerline of guanidine stream as a function of time. The velocity profile along the centerline was integrated to get time as a function of stream-wise position. These conditions give a 1 μs mixing time (for a 50% concentration drop).

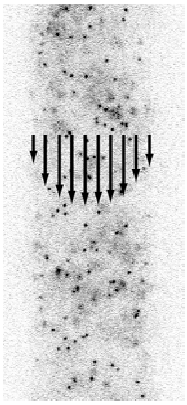

Figure 5
MicroPIV image and overlaid vectors of downstream mixing region showing expected velocity profile. Measurements obtained using images of 0.3 μm particles, 2 μm wide interrogation windows with 50% overlap, and using 100 image pairs. Inverted image and one in two vectors are shown for clarity of the presentation.

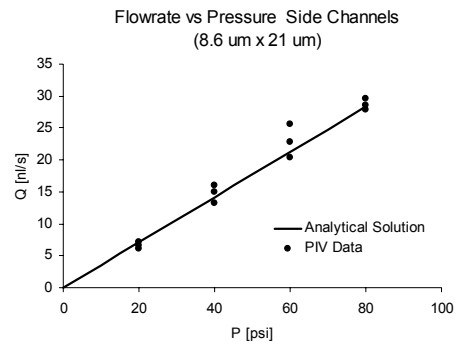

Figure 6

Plot of flow rate versus applied pressure for the side inlet channels. Flow rates calculated from velocity measurements. Dots represent measurements of various mixers, and the solid line is the analytical solution based on channel geometry and applied pressure.

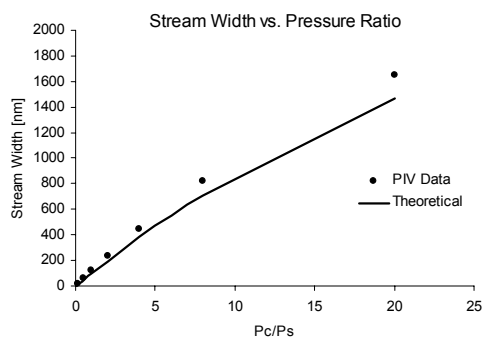

Figure 7

Width of the focused stream versus ratio of applied inlet pressures. Width calculated from PIV velocity data shown in dots, and circuit model as solid line. A pressure ratio of ~12 gives a 1 µm wide stream.

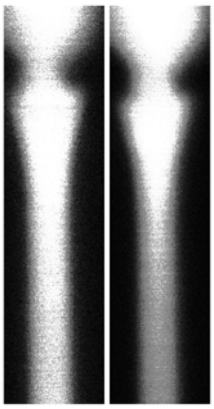

Figure 8

Confocal scanning intensity images of fluorescence quenching. Pressure ratio is φ=20 corresponding to a stream width of 1.6 μm. The left image is without iodide on the sides and the right has 2M KI on the sides. Diffusive mixing is verified by the decrease in intensity (quenching) of the fluorescein in the right image.