Ultrafast Microfluidic Mixer and Freeze-Quenching Device

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The freeze-quenching technique is extremely useful for trapping meta-stable intermediates populated during fast chemical or biochemical reactions. The application of this technique, however, is limited by the long mixing time of conventional solution mixers and the slow freezing time of cryogenic fluids. To overcome these problems, we have designed and tested a novel microfluidic silicon mixer equipped with a new freeze-quenching device, with which reactions can be followed down to 50-ms. In the microfluidic silicon mixer, seven 10-μm-diameter vertical pillars are arranged perpendicular to the flow direction and in a staggered fashion in the 450-pL mixing chamber to enhance turbulent mixing. The mixed-solution jet, with a cross section of 10 μm × 100 μm, exits from the microfluidic silicon mixer with a linear flow velocity of 20 m/s. It instantaneously freezes on one of two rotating copper wheels maintained at 77 K and is subsequently ground into an ultrafine powder. The ultrafine frozen powder exhibits excellent spectral quality and high packing factor and can be readily transferred between spectroscopic observation cells. The microfluidic mixer was tested by the reaction between azide and myoglobin at pH 5.0. It was found that complete mixing was achieved within the mixing dead time of the mixer (20 μs), and the first observable point for this coupled device was determined to be 50 μs, which is ~2 orders of magnitude faster than commercially available instruments.

To achieve full understanding of a chemical or biochemical reaction, it is important to determine the molecular properties of the intermediates populated along the reaction coordinate. For spectroscopic characterization of metastable reaction intermediates, the reaction must be initiated in a way such that all of the molecules are in phase at the start of the measurement.1 This is commonly achieved through turbulent mixing in a stopped-flow or continuous-flow apparatus. The progression of the reaction is detected spectroscopically in real time or by chemical- or freeze-quenching methods for subsequent analysis. The earliest events that can be monitored with conventional instrumentation are limited by the mixing dead time of a few milliseconds. To overcome this restriction, many innovative protocols have been implemented over the years. One useful approach is to slow reactions to a feasible time window by lowering reaction temperatures.2,3 Another widely utilized methodology is to initiate reactions photochemically with a short pulse of light and probe them with ultrafast spectroscopic tools.4,5 These techniques, although powerful, apply to only a limited number of systems.

To obtain a more general tool, significant efforts have been placed on the development of faster solution mixers.6–10 Considerable success has been achieved in reducing the mixing dead time to the submillisecond time regime by reducing the dimensions of the mixing chamber. In the miniaturized mixing chambers, diffusive mixing can occur over a shorter distance; as a result, mixing efficiency is significantly improved. Unfortunately, this advantage is compromised by a concurrent decrease in the Reynolds number (Re), a dimensionless parameter describing the dynamics of a simple flow inside a tube with a circular cross section. The Reynolds number is defined as Re = \( \frac{vD}{\eta} \), where \( v \) and \( D \) are the average flow speed and the smallest cross section in the flow channel, respectively; and \( \rho \) and \( \eta \) are the density and the viscosity of the liquid, respectively. Under conditions of low Reynolds number, the flow dynamics are dominated by viscous drag rather than by inertia, resulting in laminar flow instead of the turbulent flow required for efficient mixing. To prevent laminar flow, the miniaturization of the mixer is typically accompanied by a fast flow speed. As an example, Regenfuss and co-workers designed a mixer in which two solutions to be mixed flowed

separately through two coaxial glass capillaries with converging tips and were mixed in a ~50-µm-wide space around a 50–100-µm sphere positioned between the tips of the inner and outer capillaries.21 With a fast flow rate of 300 µL/s, the first observable time point of this mixer was determined to be less than 100 µs. Building from this success, several derivatives of this mixer have been designed and constructed for studying enzymatic and protein folding reactions that exhibited dead times of 100 µs or less.16–19 Although the dead times are significantly reduced, the applications of these mixers are limited because of their high sample consumption rate and because the material used to fabricate these mixers (either glass or stainless steel) are either too fragile or too tough to fabricate reproducibly; furthermore, it is a nontrivial task to assemble these mixers due to their complicated designs.

Recently, microfluidic silicon mixers with predefined flow dynamics have been implemented, taking advantage of the robust mechanical strength of silicon and the ease of fabrication with modern photolithographic techniques.7–10,12,15 In the mixers designed by Mayo and co-workers, the mixing chamber was constructed with a simple “T” geometry in which the two solutions to be mixed, laterally enter a center channel with a 200-µm width.7 With an ultrahigh flow velocity of 500 µL/s, a Reynolds number of > 1000 was achieved and a dead time of 110 µs was observed through turbulent mixing. Although it is a nontrivial task to mix fluids at low Reynolds number, in the mixer designed by Whitesides and co-workers, staggered herringbone ridges were arranged on the floor of the inner surface of the flow channel (77 × 200 µm) to introduce chaotic flow.13 As a result, good mixing was achieved under conditions of very low Reynolds number (0 < Re < 100). In contrast, instead of avoiding laminar flow, Austin and co-workers took advantage of it and designed an ingenious mixer based on the hydrodynamic focusing principle, in which a reagent to be mixed enters the middle channel and is compressed by a second reagent (which enters from two side channels) into a 50-nm size stream such that mixing can occur rapidly through a diffusion-controlled process within 20 µs.10

Most of these novel microfluidic mixers were designed for continuous-flow applications. The progression of the reaction can be probed as a function of the distance along the solution stream. Unlike the stopped-flow applications that require fast real-time spectroscopic detection, the spectroscopic signal can be integrated for a long time at any given reaction time to obtain a satisfactory signal-to-noise ratio.17 However, the amount of sample consumption may not be economical, especially for precious biological samples. The freeze-quenching technique, introduced by Bray in 1961, provides a convenient means for overcoming this problem.20

The conventional strategy for freeze-quenching applications is to inject the mixed solution from a continuous-flow mixer through a small nozzle into an isopentane bath (−130 °C). The frozen samples thus collected contain trapped reaction intermediates that can be studied subsequently with various spectroscopic tools, including those that are difficult to directly couple to a continuous-flow or a stopped-flow apparatus (such as EPR and NM R).21 The time resolution of conventional freeze-quenching instrumentation is typically in the several milliseconds range, due to the limitations set by the dead time of the mixer and the freezing time of the solution jet by the cryogenic medium. In addition to the time resolution limitations, it is often problematic to physically pack the frozen powder into sample tubes and to obtain a satisfactory quality of the frozen sample for subsequent spectral measurements.22

Here we present the design of a novel microfabricated fluidic silicon mixer with small sample consumption (20 µL/s) and high mixing efficiency (~20-µs mixing time). The silicon mixer was interfaced with a freeze-quenching device, similar to that reported by Tanaka et al.,16 that rapidly freeze-quenches the reaction mixture at any given reaction time. The fine and stable solution jet from the silicon mixer, with a 10 × 100 µm cross section and a 20 m/s linear flow rate, is freeze-quenched within 20 µs and an ultrafine frozen powder with excellent spectral quality and high packing factor is obtained. The new coupled device was tested and characterized by the binding reaction of sodium azide to myoglobin with resonance Raman and EPR spectroscopies.

**EXPERIMENTAL METHODS**

Horse heart myoglobin and sodium azide were purchased from Sigma. Both myoglobin and sodium azide were buffered with 100 mM sodium phosphate buffer at pH 5.0. The stopped-flow measurements were carried out in a a*180 system from Applied Photophysics Inc. The concentration of myoglobin was 12.5 mM, and the concentration of potassium azide was varied from 0.14 to 1.9 mM. The reaction was probed in the optical absorption mode with a photodiode array detector. The reaction kinetics were analyzed based on the absorption at 425 nm.

For the resonance Raman measurements, the excitation laser beam at 406.7 nm (~9 mW) from a krypton ion laser (Spectra Physics) was focused onto an EPR sample tube containing the frozen sample with a cylindrical lens to avoid photodamage. The EPR sample tube was kept in a liquid nitrogen coldfinger to maintain the temperature at 77 K. A band-pass filter was used to eliminate plasma lines from the laser before the laser light reached the sample. The scattered light was collected in a backscattering geometry, filtered by a notch filter, and focused onto the entrance slit of a 1.25-m polychromator (Spex) where it was dispersed and then detected by a charge-coupled device (Princeton Instruments). The concentration of myoglobin used for the resonance Raman experiments was 50 µM. The acquisition time was 30 min for each spectrum. All the Raman spectra were calibrated with indene (Sigma).

EPR spectra were measured using an ESP 300 spectrometer (Bruker Instruments) equipped with an ESR 10 continuous-flow cryostat (Oxford Instruments). All spectra were acquired with the following parameters: temperature 10.0 ± 0.3 K; microwave frequency 9.47 GHz; microwave power 2 mW; modulation amplitude 8.0 G; time constant 327 ms. The reaction was followed by monitoring the integrated intensity of the high-spin ferric g′ = 6 peak (after baseline subtraction) as the iron was converted from high-spin (azide-free) to low-spin (azide-bound) forms.

**RESULTS AND DISCUSSION**

Previously, we reported the design and application of a continuous-flow solution mixer with a mixing dead time of 100 µs. In that mixer, a “T”-shaped mixing chamber was created by

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sandwiching a rectangular quartz observation cell against a metal plate with a 100-μm-wide and 25-μm-deep flow channel. This continuous-flow mixer was robust, easy to assemble, and reliable. Although it has been successfully applied to study early intermediates of important biological reactions, there are two limitations for the future application of this mixing device: (1) a fast flow speed of 125 μL/s is required to achieve efficient mixing, which is not economical for precious samples; (2) the mixing chamber is mechanically cut into a metal plate; the design of the mixing chamber is thus restricted by the machinery used to fabricate the mixer. These drawbacks prompted us to develop a new generation of microfluidic mixer on silicon substrates, taking advantage of the excellent mechanical strength of silicon and the well-developed photolithographic technology for fabricating silicon devices. Several silicon mixers with a variety of mixing chambers with predefined flow dynamics have been designed and tested based on a trial-and-error basis. Here we present the most reliable mixer that exhibited the best mixing efficiency.

**Design of the Microfluidic Silicon Mixer.** The microfluidic silicon mixer was fabricated by using standard photolithographic techniques in the Pennsylvania State Nanofabrication Facility. The top view of the mixing chamber is shown in Figure 1. The two solutions flow head to head through the 50-μm-wide channels prior to entering the 50 × 100-μm mixing chamber that is arranged in a T geometry. Within the mixing chamber, seven vertical pillars with a diameter of 10 μm are arranged perpendicular to the flow direction and in a staggered fashion. With this design, the flow velocity is modulated by the alternating 20-, 50-, 10-, and 50-μm passages as the mixed fluid travels down the mixing chamber (Figure 1, right). The associated changes in the Reynolds number, Re, ranges between ~200 and 2000 along the flow direction (the density and the viscosity of the solution are assumed to be 1 g/cm³ and 0.0089 P, respectively). The spatial and temporal modulation of the flow dynamics introduces turbulent eddies in the mixing chamber that ensure efficient fluid mixing.

![Micro-Fabricated Silicon Mixer](image)

![Mixing Chamber](image)

**Figure 1.** Photograph of the microfluidic silicon mixer taken under a bright-field confocal light microscope. A schematic illustration of the scaled mixing chamber is shown on the right. The detailed design of this mixer is described in the text. The two channels where the two solutions to be mixed are introduced are labeled (1) and (2). The alternating 10-, 20-, and 50-μm passages along the flow direction are indicated on the right by the solid, dashed, and dotted double arrows, respectively.

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The designed pattern was etched into the silicon substrate with an isotropic etching reagent that resulted in 100-μm depth with a rectangular cross section. The silicon substrate with the etched features was sealed with a 1-mm-thick glass window via anodic bonding with high voltage and high temperature based on previously reported procedures. The binding of the seven pillars in the mixing chamber to the cover glass was confirmed by visual inspection under an optical microscope. The resulting silicon–glass assembly was placed in a plastic holder, in which the two solutions to be mixed are introduced through two HPLC fittings into the two through-holes drilled on the glass window, which matched the two inlet channels built on the silicon substrate as indicated by (1) and (2) in Figure 1. The two solutions to be mixed are loaded into two syringes and pumped into the mixer through the HPLC tubing by a syringe pump equipped with a gear-driven mechanical motor (Harvard Apparatus Inc.) to ensure a constant flow speed. The mixed solution exits the mixing chamber through a 50-μm-long flow channel as a free jet with a cross section of 10 μm × 100 μm and a linear velocity of 20 m/s as shown in the inset in Figure 2. The mixing dead volume of this mixer is calculated to be 450 pL, resulting in a dead time of 20 μs with a mass flow speed of 20 μL/s.

**Coupling to the Freeze-Quenching Device.** The microfluidic silicon mixer was interfaced with a freeze-quenching device. The freeze-quenching device was constructed of two oxygen-free copper wheels with a diameter of 4 cm, which are arranged in a side-by-side fashion as illustrated in Figure 2. The drive wheel, attached to a motor, drives the slave wheel through frictional...
contact. As a result, the two cooper wheels rotate in opposite directions at the same speeds. The bottom halves of the two wheels are immersed in a liquid nitrogen bath to maintain the temperature of the wheels at 77 K. The ultrathin sheet of the mixed-solution jet (10 μm x 100 μm) impinges on the drive wheel at a position that is offset from the center of the interface between the two wheels to allow a close contact between the microfluidic silicon mixer and the drive wheel. It is also offset from the center of the drive wheel to avoid back-splash. The mixed solution instantaneously freezes on the surface of the wheel and is ground into a fine powder as it is carried down through the interface of the wheels. The fine powder is subsequently collected into an EPR tube through a collecting funnel situated in the liquid nitrogen directly below the interface of the wheels. On average, it requires 100 μL of solution to pack into a 1-cm-high sample in an EPR tube (o.d. = 5.00 mm; i.d. = 4.62 mm). The packing factor of the frozen sample was estimated to be 0.6 based on the ratio of the volume of the frozen powder and that of the melted counterpart.

The microfluidic silicon mixer is attached to a three-dimensional translation stage that was driven by electronic actuators with ~1-μm precision (Figure 2). The vertical actuator controls the distance between the microfluidic silicon mixer and the drive wheel, which in turn determines the reaction time. Based on a linear flow velocity of 20 m/s and a closest manageable distance between the Si mixer and the copper wheel of 0.5 mm, the earliest collectable time point is 50 μs.

Characterization of the Microfluidic Mixer and the Freeze-Quenching Device. The performance of the coupled microfluidic silicon mixer and the freeze-quenching device was evaluated by the binding reaction of azide to myoglobin. The reaction was first tested in a stopped-flow system with 12.5 μM myoglobin and 0.1–1.9 mM sodium azide in pH 5 buffer at room temperature.28 The kinetic traces measured by optical absorption spectroscopy at 425 nm were fitted with single-exponential functions. The observed pseudo-first-order rate constants (k_{obs}) were plotted as a function of the azide concentration (Figure 3, left panel) and fitted with a linear line that exhibited a bimolecular rate constant of 1.1 \times 10^5 M^{-1} s^{-1}. The same reaction was carried out as a function of temperature from 1 to 17 °C in the presence of 0.6 mM sodium azide. Figure 3 (right panel) shows the plot of ln(k_{obs}) versus 1/T, where T is the temperature. The data were fitted with a linear line with a slope of 2500. Based on the Arrehenius equation (k_{obs} = A \exp(-E_a/RT)), the activation energy for the reaction was 5 kcal/mol.

The microfluidic silicon mixer was tested with 50 μM myoglobin and 28.4 mM sodium azide in pH 5 buffer at 5 °C. The reaction mixture was freeze-quenched as a function of time from t = 50 to 3300 μs. The sample tube filled with the collected frozen powder was transferred into a liquid nitrogen coldfinger to maintain the temperature at 77 K and characterized with reso-

nance Raman spectroscopy. The typical time-resolved resonance Raman spectra thus collected are shown in Figure 4. The changes in the spectral patterns in the 1350–1600 cm\(^{-1}\) region reflect the changes in the heme ligation states of myoglobin following the binding of the azide ion to the heme iron atom. To obtain quantitative information, the resonance Raman spectra were deconvoluted into a linear combination of the azide-free and the azide-bound spectra, shown as the top and the bottom traces in Figure 4, respectively. A typical example of the deconvolution result is shown in Figure 5, which was obtained at 50 µs following the reaction. It was deconvoluted into an 82% azide-free spectrum and an 18% azide-bound spectrum. The residuals from the deconvolution process, shown in the bottom of Figure 5, are negligible, confirming the reliability of the deconvolution process. The population of the azide-free species extracted from the deconvolution process was plotted as a function of the reaction time in Figure 6. It was fitted with a single-exponential function with a lifetime of 630 µs, corresponding to a rate constant of 1600 s\(^{-1}\). This rate constant is very close to the theoretically predicted pseudo-first-order value based on the concentration and temperature-dependent plots shown in Figure 3, suggesting that full mixing was achieved within the 20 µs mixing dead time of the microfluidic mixer and the freezing time of the freeze-quenching device is equally fast.

The frozen samples were further examined by EPR spectroscopy. EPR is a convenient probe for the reaction, because the high-spin heme iron associated with the azide-free myoglobin exhibits a characteristic EPR peak at 1130 G (\(g' = 6\)), while the low-spin heme iron associated with the azide-bound form is EPR silent. The EPR spectra obtained at various time points are shown in Figure 3. Left: Pseudo-first-order rate constants of the bimolecular binding reaction of azide to myoglobin at pH 5.0 as a function of the concentration of sodium azide obtained in the stopped-flow apparatus. The second-order rate constant obtained from the slope of the linear line is 1.1 \(\times 10^5\) M\(^{-1}\) s\(^{-1}\). Right: Arrhenius plot of the pseudo-first-order rate constants of the binding reaction of azide to myoglobin. Based on the slope, the activation energy is 5 kcal/mol. 

![Figure 4](image-url) Time-dependent resonance Raman spectra obtained during the binding reaction of azide to myoglobin at t = 50–3300 µs as indicated. The excitation wavelength was 406.7 nm, and the laser power was 9 mW. The acquisition time for each spectrum was 30 min. 

![Figure 5](image-url) Deconvolution of the resonance Raman spectrum obtained at t = 50 µs during the binding reaction of azide to myoglobin as shown in Figure 4. The raw data trace shown in blue is deconvoluted into an 82% azide-free form and an 18% azide-bound form. The red trace is the simulation. The bottom trace shows the residuals between the raw data and the simulated trace from the convolution process. 

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in Figure 7. The relative integrated intensity of the 1130 G peak is plotted on top of the resonance Raman data shown in Figure 6. The excellent agreement between the EPR and Raman data demonstrated the reliability of the frozen powder for spectroscopic measurements.

Figure 6. Relative concentration of the unreacited azide-free protein as a function of reaction time, obtained by deconvoluting the time-dependent spectra shown in Figure 4 into the contributions from the azide-free myoglobin and azide-bound myoglobin. The solid line shows a single-exponential fit to the data with a rate constant of $1.6 \times 10^3$ s$^{-1}$. The EPR data, from Figure 7, are included on the same plots demonstrating the consistency of the two spectroscopic measurements.

CONCLUSIONS

The novel microfabricated silicon mixer described here achieves a dead time of 20 $\mu$s with a relatively low sample consumption rate (20 $\mu$L/s). The complete mixing is accomplished by placing seven staggered pillars in the mixing chamber, thereby inducing the turbulent flow necessary for efficient mixing at moderate Reynolds number ($Re \sim 200-2000$). Although it is unclear as to whether all seven pillars are required for the complete mixing, a similar mixer with a simple T mixing chamber and a smallest cross section of 10 $\mu$m in the flow channel, but without any barrier placed in the mixing chamber, has been tested and failed to reach a similar level of mixing efficiency.

By coupling this mixer with a copper wheel-based freeze-quenching device, the ultrathin solution jet with a cross section of $10 \times 100$ $\mu$m can be rapidly frozen and a dead time of 50 $\mu$s was achieved. The ultrafine frozen powder thus produced exhibits a high packing factor and outstanding spectroscopic quality for resonance Raman and EPR measurements. Furthermore, due to its ultrafine quality, the frozen sample can be easily transferred between observation cells for additional spectroscopic measurements, such as NMR, FT-IR, fluorescence, or optical absorption. Two additional advantages of the new rapid freeze-quenching method are as follows: (1) it provides a useful means for preventing aggregation of solute in frozen solution without the need to add a cryoprotectant such as glycerol; and (2) it can preserve the room-temperature phases of biological systems in the frozen solution, which is especially important for a system containing lipids that undergo a phase transition during a slow freezing process.

The successful design of the microfluidic silicon mixer opens a new window for future applications. In a new generation of the microfluidic silicon mixers, we have extended the length of the exit channel for continuous-flow applications. By varying the flow speed, a comprehensive time window can be covered. Various optical detection systems can be employed to probe the progression of a reaction in real time in a back-reflection mode by point-by-point scanning along the flow channel. Furthermore, to study more complicated reactions, multiple mixers can be coupled together through aging channels built on a single silicon chip. In summary, silicon mixing devices were demonstrated to be very useful in trapping reaction intermediates with microsecond lifetimes and they can be readily implemented with various existing schemes and spectroscopic detection systems for continuous flow applications.

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