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Design, Implementation, Simulation, and Visualization of a Highly Efficient RIM Microfluidic Mixer for Rapid Freeze-Quench of Biological Samples

Bryan Schmidt^{1,†,*}, Goher Mahmud[‡], Siowling Soh[‡], Sun Hee Kim^{2,†}, Taylor Page[†], Thomas V. O'Halloran[†], Bartosz A. Grzybowski^{†,‡,*}, and Brian M. Hoffman^{†,*}

[†]Department of Chemistry, Northwestern University, Evanston, IL 60208

[‡]Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL 60208

Abstract

Rapid freeze-quench (RFQ) trapping of short-lived reaction intermediates for spectroscopic study plays an important role in the characterization of biological reactions. Recently there has been considerable effort to achieve submillisecond reaction deadtimes. We present here a new, robust, high-velocity microfluidic mixer that enables such rapid freeze-quenching. It is based on the mixing method of two impinging jets commonly used in reaction injection molding (RIM) of plastics. This method achieves efficient mixing by inducing chaotic flow at relatively low Reynolds numbers ($Re = 140$). We present the first mathematical simulation and microscopic visualization of mixing in such RFQ micromixers, the results of which show that the impinging solutions efficiently mix within the mixing chamber. These tests, along with a practical demonstration in a RFQ setup that involves copper wheels, show this new mixer can in practice provide reaction deadtimes as low as 100 microseconds.

Introduction

Rapid freeze quenching (RFQ), the rapid mixing of two solutions, followed by rapid freezing of the mixture, has become a routine technique for trapping enzymatic reactive intermediates [1–5]. The frozen solution can be collected and analyzed by a variety of spectroscopic techniques, with electron paramagnetic resonance (EPR) spectroscopy being a particularly important method for the characterization of enzyme intermediates [6–8].

In general, the more reactive the intermediate to be studied, the more rapidly must the mixing and freezing be performed. However, for more than forty years after the technique was introduced, RFQ was limited to generating samples with reaction times greater than ten milliseconds. The classical freeze-quench design consists of a T-mixer, with a series of mesh grids that produce “split-and-recombine” mixing, that is connected to a jet nozzle by tubing of varying length (the aging hose), with the reaction being quenched in a liquid isopentane bath chilled to just above the freezing point ($\sim 130K$). The overall deadtime in this process is limited by the maximum flow rate, the minimum aging hose length, and the freezing process itself.

*Corresponding author: bmh@northwestern.edu, phone: 847-491-3104, fax: 847-491-7713.

¹Current address: Division of Science, Minot State University, Minot, ND 58707

²Current address: Department of Bioinspired Science, Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-750, Korea

In the last decade, great progress has been made in shortening the RFQ deadtimes to the sub-millisecond range by speeding both the mixing and the freezing processes [9,10]. However, current mixers still have limitations, and this has led us to design, implement, and test a robust new rapid freeze-quench micromixer based on an approach not previously applied to this problem: the mixing method of two impinging jets, commonly used in reaction injection molding (RIM) of plastics, which achieves efficient mixing by inducing chaotic flow at relatively low Reynolds numbers ($Re = 140$) [11–14]. We begin by summarizing the current state of the art, then describe the new design. We then describe finite-volume flow simulations that predict that it can achieve mixing under flow conditions appropriate to the RFQ experiment, and finally present direct microscopic observations that confirm that these predictions are realized in practice.

Background

The first breakthrough in freeze-quench design was the replacement of the chilled isopentane bath with rotating copper wheels temperature-equilibrated in liquid nitrogen [15]. This approach takes advantage both of the high thermal conductivity of copper and freezing on the solid wheels; when an isopentane bath is used, air entrainment of the impinging jet [16] slows the freezing time. Although the overall freezing time is dependent on the size of the jet stream, use of the wheels led to a 1000-fold reduction in freezing time, from approximately 5 milliseconds in an isopentane bath [6] to a duration approaching 5 microseconds for a stream width of 10 microns [17].

Despite the dramatically improved freezing time, sample aging time was still limited to ~5 milliseconds with the commercially available Wiskind mixer. Although this mixer is capable of rapidly mixing two solutions, it suffers from the large mixing volume (~1.6 μL) and the need for a jet nozzle to accelerate the solution to decrease flight time of the solution to the freezing wheels. These two features set the lower limit of the reaction time when a reasonable flow speed is used. To reach sample aging times of less than 1 millisecond, new mixing designs were needed.

The first new mixer design, presented by Lin *et al.* [9], was a miniaturized T-mixer etched in silicon. The design goal of this mixer was to achieve a Reynolds number of $Re = 2000$ or greater in the flow, thereby crossing the threshold into turbulent flow to produce mixing. To help achieve this goal, staggered posts were placed in the mixing chamber, the aim being to alter flow patterns into a pseudo “split-and-recombine” flow [18,19], and thereby increase flow velocity. The small size of this mixer allows for high sample linear velocities (~20 m/s) which decrease flight time to the freezing wheels and removes the necessity for a separate jet nozzle, again decreasing reaction time.

Although this design was a breakthrough that successfully moved freeze-quench technology into the sub-millisecond regime, it nonetheless suffers from deficiencies in flow theory and in practical use. The notion that a Reynolds number in excess of 2000 is sufficient for efficient mixing is not strictly correct. It is based on the fact that water flowing in a large bore tube at standard temperature ceases to exhibit strictly laminar flow and begins to become turbulent at a Reynolds number of approximately 2300. However, truly turbulent flow is not fully achieved until a Reynolds number of $\sim 4 \times 10^3$ is achieved [20]. Even when turbulent flow has been established, further increase in Re causes even more efficient (faster) mixing [21]. Thus, generating a flow with $Re = 2000$ is not sufficient to ensure rapid and complete mixing. On the other hand, such a description of turbulence mixing in the RFQ mixer is based on observations of flow in large bore tubes. As the dimensions of the tube are scaled down to the micron scale, the onset of transitional and turbulent flows change, with the Reynolds number required to reach this state generally lower than standard theory [22].

This might suggest that the mixer of Lin *et. al.* could develop fully turbulent flow, and hence fast, efficient mixing, with the Reynolds numbers predicted. However, the calculation of $Re = 2000$ for this system is an overestimate, and a more precise calculation gives a Reynolds number of $Re \sim 270$ in the mixing chamber and $Re \sim 365$ for the outlet channel. Based solely on these numbers and without a clearly defined threshold for onset of turbulent flow on the scale of the mixer, one cannot predict that the flow will in fact be turbulent [23].

Clearly, it is difficult to achieve rapid mixing from fully turbulent flow that has been created by a flow at high Reynolds numbers at the low flow volumes required when using biological samples of limited supply. Fortunately, this is not necessary: rapid mixing also can be achieved through a stretch of a laminar flow caused by chaotic advection. This method is popular in “lab-on-a-chip” designs [24], and uses structural elements to create a chaotic flow, similar to turbulence, that diminishes the distance between layers of the two solutions, allowing for rapid molecular diffusion across the layers and resulting in rapid mixing. The efficient mixing by the commercial Wiskind mixers also relies on this principle, using the “split-and-recombine” flow pattern to induce chaotic flow. The obstruction posts in the Lin *et. al.* mixer could be considered as introducing such chaotic mixing elements, and a similar design element had been tested before [18,25]. However, even were this design to allow sub-millisecond freeze-quenching (but see below), it still suffers from a fatal fault in practice. Because of the high pressures in the mixing chamber required to reach a flow velocity of 20 m/s, these posts are quite prone to break, clogging the outlet channel and rendering the mixer inoperable. In our hands, using flow velocities of 25 m/s, these mixers had lifetimes from one to five ‘mixes’ before they became unusable due to clogging.

Shortly after the report of Lin *et. al.*, Cherepanov and de Vries [10], described another mixer based on the principle of achieving a sufficiently high Reynolds number to ensure turbulent flow. Despite some calculation errors in determining the pressures required to achieve desired flow rates, direct tests showed this device to mix efficiently, but only at the highest flow speed/Reynolds number was used shown to mix efficiently, leaving mixing efficiency at the slower speeds still in question. Because the mixer employs continuous solvent flow at high flow rates, very precise timing is required to switch the flow from the solvent collection vessel to the freeze-quenching cold plate. This causes operational difficulties, and failure to time the event properly will result either in loss of sample (into the solvent collection vessel or on the ground between it and the cold plate), or dilution of samples with excess solvent. Recently, a design designed for lab-on-a-chip applications has been described as potentially being useful in RFQ applications [26].

We here report the design, implementation, and testing of a new rapid freeze-quench micromixer based on the mixing method of two impinging jets, commonly used in reaction injection molding of plastics. This design has the advantages of requiring lower Reynolds numbers ($Re = 140$) for the onset of chaotic flow without the intricate designs that are required in most chaotic mixers. Mixing efficiency was confirmed by simulation and by microscopic visualization; the practical use of the instrument for mixing biological samples was demonstrated by trapping intermediates of the reaction of myoglobin with azide.

Experimental

The design of the mixing chamber is discussed below. The chamber and inlet channels were arranged to fit within a 1×2 cm silicon chip as in the design by Lin *et. al.*, so that their mixer and the one discussed here could be interchangeably inserted into the same mounting plate and mated to an Update Instruments Rapid Freeze Quench device (Update Instrument Inc, Madison, WI) and rotating copper freezing wheels based on the design of Tanaka *et. al.* [15], as discussed in the text. One mm thick Pyrex coverslips with 0.8 mm drilled inlet holes

were produced by Bullen, Inc (Eaton, OH). Mixers were etched in silicon and bonded to the coverslips by Integrated Sensing Systems, Inc (Ypsilanti, MI) using chrome-plated soda-lime photomasks made with a 0.5 micron tolerance by Advance Reproductions Corporation (North Andover, MA). The mounting plate mates the mixer to PEEK tubing (Upchurch Scientific, Oak Harbor, WA) from the drive syringes. Horse-heart myoglobin, fluorescein and sodium azide were used as obtained from Sigma.

The flow in the microfluidic mixer was simulated with a commercial software package, “Fluent” by ANSYS, Inc. The Navier-Stokes equation and a standard k - ϵ model [27] were used to solve for the turbulent flow together with a transport equation for the mixing of fluorescein dye. The chaotic flow found in the mixer can be simulated with the standard k - ϵ turbulence model [11].

In these simulations, the two inlets were each set with an inlet volumetric flow rate of 12.5 $\mu\text{L/s}$. One of the streams contained $c = c_0$ (where c_0 is the concentration of the injected dye), while the other contained $c = 0$. The outlet was set at constant, atmospheric pressure.

Reynolds number for the inlet was calculated as follows. The geometry of the inlet is rectangular with cross-sectional dimensions of $10\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$, with an inlet velocity, $v = 25\text{ m/s}$, and the hydraulic diameter of a rectangular duct can be expressed as [20] $D_H = 2HL/(H+L)$, where H and L are the cross-sectional dimensions of the inlet. This gives $D_H = 16.7\text{ }\mu\text{m}$. The Reynolds number can then be expressed as,

$$\text{Re} = \rho v D_H / \mu$$

where ρ is the density of the fluid and μ is the viscosity. The fluid is essentially water in our case, $\rho = 1000\text{ kg/m}^3$ and $\mu = 0.001\text{ Pa}\cdot\text{s}$; for this mixer and the assumed value of v , $\text{Re} = 420$.

Fluorescent imaging of micromixer performance was achieved by mixing a solution of 1 mM fluorescein dye with water. Direct observation of fluid flow was performed on an inverted microscope (TMD, Nikon) equipped with phase-contrast optics (10X, 0.25 NA objective) and CCD camera (Sensys, Photometrics, Tucson, AZ) and driven by Metamorph imaging software (Universal Imaging Corp., Worchester, PA). Time-lapse videos of fluid movement of ~60 frames at 3-second intervals (for total time of 3 minutes in each experiment) were obtained.

EPR experiments were performed on a Bruker ER-220 X-band spectrometer with an Oxford liquid helium cryostat; spectra were recorded at 20 K. Samples were collected at various delay times after mixing, the shortest estimated to be 200 microseconds based on the flow rate set with the Update Instruments pumps, the set distance from the mixer outlet to the rotating wheels (see text), and the freezing time reported for the use of such wheel. The data were normalized and then fitted to an exponential decay to give the pseudo first-order rate constant for azide binding and the second order rate constant then calculated from the azide concentration.

Results and Discussion

Mixer design

The new micromixer is based on the principles employed in reaction injection molding (RIM) in the plastics industry. These mixers have several similarities with standard T-mixers, but with important variations. The mixer is constructed such that the high-velocity

inlet streams collide “head-to-head” in what is commonly referred to as “impingement mixing” [27]. In order for mixing to occur in such a case, there must be a third dimension for the fluid to flow, producing a region of high energy dissipation that cannot be bypassed [28]. To achieve this, as shown in Figure 1, the mixing chamber of the new mixer is twice the depth of the inlet arms and extends away from the outlet, thereby providing the necessary 3D space for the impingement film to form and to create efficient chaotic mixing.

As shown in Figure 1, the two impinging jets of the mixer are separated by 70 microns and the d/D (diameter of the inlet jets per the diameter of the mixing chamber) is approximately 5. The mixing chamber is 4.3 diameter lengths (300 microns), terminating in a 10 micron wide channel, etched to a depth of 100 microns, and 100 microns long. The injection channels are 10 microns wide, 50 microns deep and 100 microns long. The mixing chamber is etched 100 microns deep and the injection jets are placed 30 microns down from the top of the mixing chamber to expand the three-dimensional space beyond that provided by the extra depth of the mixer relative to the depth of the inlets, enhancing the turbulent collision of the impinging jets.

The mixer is etched into a 1×2 cm silicon chip bonded to a glass coverslip with provision for attachment to the sample lines of an Update Instruments Rapid Freeze Quench apparatus, so that it is interchangeable with the mixer described by Lin *et al* [9]. The mixer is mounted on two adjustable stages for alignment over the copper freezing wheels. The frozen solution is collected in a cup containing liquid nitrogen, then transferred to EPR tubes and packed using the pressure method described by Tsai *et al* [29].

Impingement mixing has been experimentally determined to be highly efficient due to the onset of chaotic flows beyond a critical Reynolds number of ~ 140 (inlet streams) [12,27]. When our mixer is driven by the Update Instruments apparatus, the inlet velocity ranges from 2300 cm/s to 7500 cm/s, resulting in Reynold’s numbers of 380 to 1250 for the injection jet. The linear velocity of the outlet ranges from 2300 cm/s to 7500 cm/s, with corresponding Reynolds numbers of 420 to 1360.

Given the volumetric flow rate of the inlet streams relative to the volume of the mixing chamber, the linear velocity of the outlet stream, the closest practical distance between the mixer and the freezing wheels of 5 mm, and the rate of freezing of the stream on the copper wheels, this setup yields a shortest achievable reaction time of approximately 100 microseconds. Given the longest distance achievable between the mixer and the wheels being 3.5 cm, the longest reaction time for this setup is approximately 1.5 milliseconds. (Reaction times were calculated by adding the time to fill the mixing chamber – 2.1 pL mixer volume divided by flow rate – to the flight time from the mixer to the freezing wheels – flow rate divided by 1 nm^2 area of the outlet times the flight distance – plus a freezing time of 5 μs).

Simulation of flow in the mixer

The flow in the microfluidic mixer was studied for a model case of mixing of a solution of fluorescein with water. The flows were simulated using the commercial software package, “Fluent” by ANSYS, Inc, as described in Materials and Methods. The 2D and 3D steady-state model was solved for the slowest (25 $\mu\text{L/s}$) and fastest (75 $\mu\text{L/s}$) flow velocities, with the resulting flow fields shown in Figure 2.

Figure 2, shows the mixing of an inlet stream of fluorescein dye solution (represented by the red color) that enters the top right of the microfluidic mixer with pure water (blue) entering from the left. At the flow rate of 25 $\mu\text{L/s}$, Figure 2a, these streams mix completely (green) before exiting the mixing chamber; there is little visual difference in the mixing pattern upon

increasing the flow rate to 75 $\mu\text{L/s}$, Figure 2e. The velocity vector plot of Figure 2b gives a corresponding sense of the flow pattern. The ‘face-on’ view of Figure 2a shows that the three-dimensional volume above the inlets indeed contributes strong mixing, while the perspective view of Figure 2c shows the importance of the three-dimensional depth of the mixer. To investigate the variation of mixing with depth, a full 3D simulation was also carried out. Figure 2c shows that the streams are well-mixed even at the top of the mixing chamber near the inlet streams, as indicated by the white arrow. As flow velocity is increased to the maximum value achievable with our pump, 75 $\mu\text{L/s}$, mixing efficiency changes negligibly (Figure 2d).

For illustrative purposes, we also simulated the flow of the original “T-shaped” microfluidic mixer of Lin *et. al.*, both with the incorporated baffles and without. As seen in Figure 3, in both cases mixing is poor within the mixing chamber, with incompletely mixed dye leaving the chamber, but the simulations indicate that diffusion within the narrow outlet channel completes the mixing. This computation thus gives insight into the experimental finding of effective mixing. The computation further suggests that there would be no loss in efficiency, and a gain in robustness, by eliminating the posts.

Visualization of mixing

The predicted efficient mixing of our micromixer was confirmed experimentally by visualizing the mixing of fluorescein and water. To position the mixer, it was loaded with a fluorescein dye solution and placed on the stage of the microscope in the viewing frame. Once the mixer was aligned, the mixer and one inlet arm (left) were loaded with water; the other (right) inlet arm was loaded with a 1 mM fluorescein solution. Images were first recorded prior to injecting solutions into the mixer, then consecutive images were recorded every three seconds as the solutions were injected into the mixer at the lowest flow speed of 12.5 m/s until the syringe pumps stopped and the system returned to equilibrium. As seen in Figure 4, during active mixing the high intensity of fluorescein fluorescence from the inlet jet diminishes quickly down the mixer, revealing that the solution in the bottom 1/3 of the mixer has been fully mixed (Figure 4c). These visual results are in very good agreement with the simulation results, and completely verify the efficiency of this new design. Once the syringe pumps are stopped, this pattern is lost (Figure 4d), indicating mixing is no longer efficient. Once the system has equilibrated (approximately 10 seconds after mixing), a clear boundary layer separation can be seen (Figure 4e). This boundary layer separation is a result of the dissipation of back-pressure within the tubing, causing a slow flow through the mixer and a velocity that is too slow to induce impingement mixing.

RFQ EPR with the new mixers

As the first implementation in an experimental setup, the mixer was integrated with an apparatus that incorporated the Update Instruments syringe RAM drive, a holder for the mixer, and copper wheels as described by Lin *et. al.* Of particular interest was to verify the effects of the proximity of the mixer to the wheels on reaction times. It has previously been suggested that the temperature of the mixing stream may drop as much as 15 $^{\circ}\text{C}$ during flight time [10], dramatically decreasing kinetic rates. In this measurement, we examined the well-characterized binding of azide to myoglobin.

In the test experiment, 100 μM ferric myoglobin, buffered at pH 5.0, was mixed with 300 mM sodium azide and allowed to react for various times as set by the distance of the mixer outlet to the copper wheels and fluid flow-rate. The samples were collected and the content of high-spin (unliganded) heme was analyzed by EPR. EPR intensities were normalized to that of the high-spin heme of the myoglobin prior to mixing with azide, and the time course was fitted to an exponential decay, yielding a pseudo-first-order rate constant of 1130 s^{-1} .

for the binding of azide (data not shown). This rate is fully consistent with the rate constant of azide binding calculated by Cherepanov and deVries [10]. Further comparison with the analysis of deVries suggests an average reaction temperature to be 13 °C for our setup.

Conclusion

We have designed and tested a new freeze-quench system using a micromixer employing RIM impinging jets to cause chaotic mixing. Simulations predicted that this design would give highly efficient mixing, and this was confirmed by visual microscopic inspection. In addition, a complete RFQ system was tested experimentally by reaction of ferric myoglobin with azide. The results of the simulations and visualization are in close agreement, both indicating efficient mixing within the mixing chamber at flow rates of 25–75 $\mu\text{L/s}$. Based on the flow rate, physical layout (distance to freezing wheels), and expected freezing times [17], we estimate that the deadtime of this instrument could be as low as 100 μs . Finally, the simplicity of the design (Figure 1) makes the mixer more robust than previous RFQ micromixers which required more intricate designs to help achieve mixing. This robustness allows a single mixer to be used for months, far longer than previous RFQ micromixers could be used.

The reaction of azide with myoglobin demonstrates the application of this system to biological samples and achieves reaction deadtimes as low as 200 μs . By varying the solution velocity as well as varying the distance from the mixer to the freezing wheels, this setup is capable of achieving samples with reaction times from 100 μs to 1.5 ms. Coupling this system with a commercially available mixer will allow for studying reaction times ranging from 100 μs to seconds. The simple design of a mixer with no moving parts and without fragile baffles allows for robust use even under the high pressures such a system generates, making it an advance on the ground-breaking micromixers that initiated the micromixer-based improvement of RFQ techniques.

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Fig. 1. Image of the micromixer chip (a), with a schematic enlargement of the mixing chamber (b).

Fig. 2.

Two-dimensional (a)–(b) and three-dimensional (c)–(d) views of fluid simulations of the micromixer at 25 $\mu\text{L/s}$ flow rates and two-dimensional views of 75 $\mu\text{L/s}$ flow rates (e)–(f). For (a), (c) and (e) fluid from one inlet is shown as blue with fluid from the other inlet shown as red. A perfect mix of both streams is shown in green. Complete mixing occurs in the top half of the mixing chamber and the extra depth of the mixing chamber allows for the rapid mixing. (b), (d) and (f) shows the velocity vector plot of the fluid in the chamber.

Fig. 3. Fluid simulations from a standard T-mixer (a) and a previously published micromixer that contains obstructions to aid in mixing (b). While posts do seem to aid slightly in mixing, neither mixer has completely mixed streams prior to exiting the mixing chamber.

Fig 4.

Fluorescent imaging of micromixer channels. A fluorescein dye (a) was filled in the channels and direct observation of fluid was performed on an inverted microscope (TMD, Nikon) equipped with phase-contrast optics (10X, 0.25 NA objective) and CCD camera (Sensys, Photometrics, Tucson, AZ) and driven by Metamorph imaging software (Universal Imaging Corp., Worchester, PA). Time-lapse videos of fluid movement of ~60 frames at 3-second intervals (for total time of 3 minutes in each experiment) were obtained. Initially, (b) the channels are empty of dye. Then, (c) the pump is turned on to deliver water from the left channel, and dye from the right. Note that when two fluids initially meet each other, there is no mixing, but later in the channel, the fluorescein dye completely fills the channel. Immediately after the pump stops moving the fluid, (d) the fluorescein “swirls” in the channel. At long times after the pump has stopped, (e) a clear boundary layer is formed between the water and the fluorescein, due to dissipation of back-pressure in the system.