Sub-millisecond integrated mix-and-inject microfluidic sample delivery devices

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Abstract: Microfluidic devices which integrate both rapid mixing and liquid jetting for sample delivery are an emerging solution for studying molecular dynamics via X-ray diffraction. Here we use finite element modelling to investigate the efficiency and time-resolution achievable using microfluidic mixers within the parameter range required for producing stable liquid jets. Three-dimensional simulations, validated by experimental data, are used to determine the velocity and concentration distribution within these devices. The results show that by adopting a serpentine geometry, it is possible to induce chaotic mixing, which effectively reduces the time required to achieve a homogeneous mixture for sample delivery. Further, we investigate the effect of flow rate and the mixer microchannel size on the mixing efficiency and minimum time required for complete mixing of the two solutions whilst maintaining a stable jet. In general, we find that the smaller the cross-sectional area of the mixer microchannel, the shorter the time needed to achieve homogeneous mixing for a given flow rate. The results of these simulations will form the basis for optimised designs enabling the study of molecular dynamics occurring on millisecond timescales using integrated mix-and-inject microfluidic devices.

Keywords: Microfluidics, Micro-Jet, Sub-millisecond mixing, Simulation, Sample delivery for XFEL

1. Introduction

The availability of synchrotron and X-ray free-electron laser (XFEL) radiation sources enables time-resolved studies of structural changes in biomolecules to be carried out with atomic resolution[1]. A key challenge with performing these types of experiments is how to efficiently deliver molecules to the X-ray beam and then to rapidly trigger dynamic changes on the relevant molecular timescales. In order to address the first problem, devices capable of forming liquid micro-jets have been developed which offer precise control over the size of the interaction region and produce minimal background signal[2-6]. For the second problem of triggering a reaction, microfluidics offers a number of advantages in terms of efficiently and homogeneously mixing reactants such that the subsequent molecular dynamics can be probed by illuminating different points on the liquid jet with the X-ray beam. Benefits of using microfluidic devices for studying molecular dynamics include the ability to combine multiple fluidic components on a single chip, the flexibility of the geometries that can be produced, and the reproducibility in terms of fabrication. The use of microfluidics also enables a high surface-to-volume ratio to be used, enhancing heat and mass transfer whilst ensuring a low Reynolds number. This behaviour is characteristic of the dominance of viscous forces over inertial forces within the flow, i.e., the laminar flow regime[7]. However, in the laminar flow regime, there is a requirement for comparatively long mixing channels as mixing predominantly occurs via low-efficiency diffusion, which limits the temporal resolution than can be achieved using diffusion-based micromixers.
Passive mixing using microfluidics has been widely implemented in order to investigate the kinetics of biochemical reactions owing to the fast (milli- and micro-second) mixing times achievable, comparatively low sample consumption rates, and ease of integration with different experimental setups[27]. Employing droplet microfluidics in combination with passive micro-mixing further decreases sample consumption whilst allowing for a wide range of possible mixing times. For example, Jiang et al.[28] used two-photon fluorescence lifetime imaging microscopy combined with FEM simulations (using COMSOL) to generate 2D maps of chaotic mixing patterns inside of microdroplets travelling through a serpentine mixer. Their experimental and simulation results showed that their micro-mixer could achieve mixing efficiencies of up to 80% after just 18 ms. Of particular relevance to the current work is the fact that they demonstrated that by forcing the droplets through sharp turns within a serpentine mixing channel, they were able to enhance the degree of mixing. Another study of chaotic mixing in serpentine channels was carried out by Song and Ismagilov[29] who examined the millisecond kinetics of an enzymatic reaction by measuring the fluorescence intensities using nanolitre droplets. Micromixers have also been used for crystallisation studies, for example, Chen et al.[30] utilised a passive micromixer consisting of multiple 3D crossing channels in order to optimise the production of silver azide (SA) crystals. In addition to achieving high mixing efficiencies and low reagent consumption, the micro-mixer allowed for the optimisation of the size and shape of crystals which was not possible using macroscale crystallisation approaches.

By employing micromixers researchers have the means to trigger dynamic changes in proteins either in solution or as nanocrystals on sub-millisecond time scales. These time-scales are suitable for studying large-scale conformal changes in proteins such as folding and unfolding[31] which can be probed using a range of laboratory and synchrotron-based techniques including small-angle and wide-angle X-ray scattering (SAXS/WAXS). Examples of systems that could benefit from the availability of sub-millisecond sample mixing and delivery are human ubiquitin, a key protein found in almost all human cellular tissue whose conformational dynamics are still not well understood [32]. The structural dynamics of the four-helix bundle within the FF Domain from Human HYPA/FBP11 is also a suitable target and one which has previously been studied using...
Nuclear Magnetic Resonance (NMR) spectroscopy[33]. A particularly promising avenue for studying molecular dynamics at the atomic scale is via either serial femtosecond nanocrystallography (SFX)[34] or SAXS/WAXS. Liu et al.[35] and Kang et al.[36] previously used conventional capillary injectors to deliver protein solution to the X-ray beam in the form of a micro-jet for time-resolved SFX studies of G protein-coupled protein receptors.

In order to investigate the dynamics of proteins, a reaction must be initiated prior to measurement. To achieve the necessary sub-millisecond time-resolution, rapid, homogeneous mixing of the reactants is required immediately prior to measurement. Microfluidic devices incorporating micromixers and injector nozzles into one integrated structure (i.e. 'mix-and-inject') serve as the ideal platform for initiating and measuring molecular dynamics for time-resolved structural biology. Recently Knoska et al.[37] have reported the development of an ultra-compact 3D microfluidic mix-and-inject device for performing time-resolved serial femtosecond crystallography (SFX) measurements. For their device, they used a two-photon additive manufacturing technique to produce an integrated 3D helical mixer. Kim et al.[38] have also previously produced a 3D serpentine micro-mixer in order to examine the dynamics of carbamates. The serpentine geometry they employed was again found to significantly enhance the mixing efficiency enabling them to achieve a high synthesis throughput of 5.3 g/hour with continuous flow. Maeots et al.[39] also used the same mixer design incorporating a nozzle for mixing-and-spraying a protein solution onto a copper grid for electron microscopy measurements. Using this approach, they were able to capture different conformations of RecA-ssDNA filaments using cryo-electron microscopy. Their mixer was able to achieve high mixing efficiencies (up to 90%) on millisecond mixing time scales (2-5 ms) using nanolitre volumes of sample.

Whilst the influence of passive mixing on micromixers in isolation has been reported before in the literature, here we are interested in investigating the range of parameters and mixing times that can be achieved using integrated mix-and-inject devices. Understanding and optimising these characteristics is critical to achieving the maximum time-resolution for molecular dynamics experiments. Such devices are currently used on both synchrotron and XFEL sources to study dynamic changes in proteins and also have the potential to be deployed in a range of different molecular sensing applications. The two key aims with the present work are: 1) To determine the influence of the device geometry on mixing performance under conditions of stable jetting and 2) To predict the minimum time required for homogeneous mixing which will determine the limit on the timescales for the dynamic processes which can be studied.

Previously, we experimentally demonstrated the application of a new approach to fabricating planar microfluidic mix-and-inject devices based on high-resolution photolithography using SU8 on glass[40]. These devices have the advantage of being relatively straightforward to fabricate whilst having very high mechanical rigidity and chemical inertness. One of the primary applications for these devices is the investigation of molecular dynamics using X-ray diffraction at XFEL sources. In our previous experimental work, we used fluorescence intensity analysis to demonstrate millisecond and sub-millisecond combined mixing and jetting. In the current simulation study we employ an FEM analysis conducted using COMSOL in order to investigate the mixing behaviour, efficiency, and minimum achievable mixing times possible using these mix-and-inject devices for a wide range of different flow rates. The simulation results presented here are validated against experimental data. We anticipate that this work will guide the development of future integrated mix-and-inject devices as well as help researchers to plan and design experiments aiming to capture sub-millisecond molecular dynamics.

2. Materials and Methods
2.1. Experimental work

The devices investigated here consist of an integrated serpentine micro-mixer channel and a gas dynamic virtual nozzle (GDVN), fabricated using our recently developed SU8 on glass technique[40]. The nominal width (W) and height (H) of the serpentine micro-channel is 25 µm and 45 µm, respectively. The mixing performance was experimentally characterised using fluorescence measurements at five different positions along the micromixer channel starting at just after the inlets. During the experimental measurements three different types of the liquid jet (cylindrical, ribbon, and planer) were observed; the schematic in Fig. 1 is an approximate representation of the regime (in terms of flow rate and gas pressure) over which combined homogeneous mixing and stable jetting can be achieved using our devices.

![Fig. 1. Schematic of the stable operating conditions for our mix-and inject microfluidic devices40 based on optical experiments. Uniform mixing was observed to occur over a flow rate range of 80-220 µL/min. The green area indicates the optimal conditions where both uniform mixing and stable jetting were observed.](image)

For the simulation study, the mixing behaviour of the microfluidic devices was investigated over a range of conditions for which both homogeneous mixing and stable jetting was observed experimentally shown in Fig. 1. One of the key goals of the simulations was the characterisation of the mixer in terms of the minimum time required to achieve homogeneous mixing as a function of the flow rate and length of the serpentine mixer. This, in turn, determines the maximum time-resolution possible using these devices.

2.2. Geometric structure of microfluidic devices

In order to characterise our devices, we considered the mixing of two solutions. For solution #1, we chose a 6.64 mM solution matching the concentration of the fluorescein sodium salt in DI-water that was measured experimentally using our devices. Solution #2 was taken to be pure water. The two solutions (solution #1 and solution #2) conjugate via a Y-shaped junction, followed by a serpentine-shaped mixer comprising 19 turns.
Within the COMSOL Multiphysics simulation package, the 'laminar flow' and the 'transport of diluted species' modules were employed. We defined 12 probe cross-sectional surfaces at which the device characteristics were probed (see Fig. 2). When comparing to the experimental data, the simulated cross-sectional surfaces were averaged along the y-axis resulting in line profiles which could be directly matched to the fluorescence measurements.

2.3. Governing equations

For the simulations, we used a single-phase laminar fluid flow interface to model the diffusive/convective transport properties of the solutions in the mixer microchannel. All fluids were considered to be incompressible, and steady-state conditions were applied. The model solves the continuity equation:

\[ \rho \nabla \cdot \mathbf{u} = 0 \]  

(1)

and the Navier–Stokes equation,

\[ \rho (\mathbf{u} \cdot \nabla) \mathbf{u} = \nabla \cdot (-p \mathbf{I} + \mathbf{K}) + \mathbf{F} \]  

(2)

where \( \rho \) is the solution density (kg/m³), \( \mathbf{u} \) is the velocity vector (m/s), \( \nabla \cdot \) is the gradient operator, \( p \) is the pressure inside the mixer microchannel (Pa), \( \mathbf{I} \) is the identity matrix, \( \mathbf{K} \) is the viscous stress tensor (Pa), and \( \mathbf{F} \) is the volume force vector (N/m³). The diffusive/convective transport of a dilute solute is defined by:

\[ -\nabla \cdot (D \nabla c + c \mathbf{u}) = 0 \]  

(3)

where \( c \) (mol/m³) is the concentration, and \( D \) (m²/s) is the diffusion coefficient of the solute. A diffusion coefficient of \( D = 4.9 \times 10^{-10} \text{ m}^2/\text{s} \), consistent with the molecular diffusion of fluorescein in water, was used in the simulations[41]. The flow and mass transport models were used in order to solve for the concentration distribution in the 3D domain.

2.4. Numerical simulations

At the walls of the micromixer, the no-slip condition was applied. The pressure was assumed to have no viscous stress at the outlet. The flow rate was varied over a range of 80 to 220 µL/min consistent with the stable jetting regime indicated in Fig. 1. For the numerical solution of the governing equations, finite element discretisation based on linear functions for the velocity was used, whilst the pressure and concentration fields were described by second-order functions. A mesh consisting of 3986106 domain elements
was used to represent the micromixer model. Fig. 3 shows representative results for the flow field and concentration field at a flow rate of 80 µL/min.

Fig. 3. Distribution plots in the x-y plane at a flow rate of 80 µL/min for (a) velocity, (b) Peclet number, and (c) concentration. The intensity of the Peclet number (Pe) distribution in (b) directly corresponds to the intensity of the chaotic advection effect (chaotic mixing is higher in darker areas). The chaotic mixing of the two solutions is also apparent from the concentration distribution shown in (c). (d) An experimental optical fluorescence image of mixing in the serpentine also at 80 µL/min shows the same chaotic effect as was observed in the serpentine microchannel during simulations. Note that the outline of the channel is indicated by the dotted white line in c) and d).

Fig. 3a shows the simulated velocity magnitude distribution within the serpentine micromixer. The serpentine geometry induces the maximum fluid velocity to vary from the microchannel centre towards the edges in the y-direction. This shift affects the concentration profile by disrupting the interface between solution #1 and solution #2, which otherwise would be dominated by diffusion. The physical quantity, which describes the convective and diffusion dominant mechanism of a mixing system is given by the Peclet number, which is expressed as 

\[ \text{Pe} = \frac{u l}{D} \]

where \( l \) is the characteristic length. For the present simulation, due to the low diffusivity of fluorescein in water and a relatively high velocity range of 1.89 - 4.72 m/s, the maximum values of Pe are around 65,000. A large Pe indicates a convective dominated mass transport, which in this case is highly dependent on the chaotic advection phenomenon in the mixer microchannel[42]. The micro-mixer areas, where the chaotic advection effect is higher, are shown by the darker blue regions in Fig. 3b. The darkest spots (highest Pe) in Fig. 3b are in the centre of the U-bend shapes, indicating a constriction in the flow due to the resistance created by the microchannel walls because of the serpentine geometry. The value of Pe also peaks near to the outlet, because of the sudden release of the flow due to lack of the microchannel wall resistance near to the outlet. As shown in Fig. 3c, the simulated concentration profile is in reasonable agreement with the experimental optical data (an example of which is shown in Fig. 3d) providing confidence in the accuracy and interpretation of our FEM model.

Example concentration profiles for six probes along the serpentine micromixer at a flow rate of 80 µL/min are plotted in Fig. 4. The solutions being mixed can be distinguished by the sharp concentration gradient between them near to the Y-junction. At probe point #3, the effect of the chaotic flow in the microchannel results in an asymmetrical concentration distribution across the y-axis. As the solution progresses towards the outlet, the concentration profiles become more even and flat as the gradient across the y-axis
decreases, indicating homogeneous mixing. At the outlet (probe point #12), the concentration gradient is close to zero, indicating that complete mixing has been achieved.

![Concentration profiles](image)

**Fig. 4.** Concentration profiles at six positions (probe points #1, 3, 5, 7, 9, 12) across the width of the serpentine micromixer. The concentration curve at the inlet (#1, black) shows two distinct solutions with a sharp concentration gradient at the interface. The concentration curves flatten approaching the outlet and are almost completely flat near to the outlet point of the liquid jet (#12, red) indicating enhanced mixing induced by the chaotic advection effect of the serpentine geometry.

### 3. Results

#### 3.1. Mixing Analysis

The degree of homogeneity of the concentration profile in any given cross-section of the mixer microchannel is a measure of the full mixing of the two solutions. The normalised concentration, $c^*$, is defined as:

$$c^* = \frac{c - c_{\text{min}}}{c_{\text{max}} - c_{\text{min}}}$$  \hspace{1cm} (4)

where $c$ is the concentration of the species in solution, and the subscripts indicate the minimum (min) and maximum (max) concentration values. Homogeneity in terms of mixing is characterized by the standard deviation ($\sigma_{st}$) of the sampling points along the concentration profile for a particular probe point\cite{43}, i.e.:

$$\sigma_{st} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (c^*_i - c^*_m)^2}$$  \hspace{1cm} (5)

where $N$ is the number of sampling points, $c^*_i$ is the normalised concentration at point $i$, and $c^*_m$ is the mean normalised concentration. The mixing efficiency ($\eta_{\text{mixing}}$) is thus defined as:

$$\eta_{\text{mixing}} = 1 - \sqrt{\frac{1}{\sum_{i=1}^{N} (c^*_i - c^*_m)^2}}$$  \hspace{1cm} (6)
The mixing efficiency was measured at each of the 12 probe points (shown in Fig. 2) for the serpentine micro-mixer. The experimental mixing efficiencies were calculated using normalised fluorescent light intensities, which directly correlate to the normalised concentration values [43]. Fig. 5 compares some examples of experimental and simulation results for two different flow rates. The overall trend of the experimental mixing efficiency as a function of distance along the serpentine mixer is reproduced in the FEM simulations.

![Fig. 5. Comparison of simulated and experimental results of the mixing efficiency versus the mixing length along the serpentine mixing channel for two different flow rates: 100 and 200 µL/min.]

At a flow rate of 100 µL/min, both the simulated and experimental results reach a peak mixing efficiency of around 95% at the outlet (mixer length = 1200 µm). For the higher flow rate of 200 µL/min, 95% mixing efficiency is already achieved at around 800 µm, indicating that at higher flow rates, a shorter serpentine length is required to achieve uniform mixing. Homogeneous mixing over shorter length scales is critical for solution scattering experiments where it is important that any changes in molecular conformation are triggered at the same time, such that the majority of target molecules are in approximately the same conformational state. The similarity of the simulated and experimental results provides us with confidence that the simulations can be used to predict the geometries and mixing times required to achieve homogeneous mixing of the reactants for a range of input parameters.

3.2. Effect of flow rate

Mixing efficiencies were calculated for the 12 probe points along the length of the serpentine micromixer and for the flow rates in the range of 80-200 µL/min (where stable jetting is observed). Fig. 6 shows that there is a steep increase in mixing efficiency for mixer lengths between $x=100$ and $x=600$ µm due to the high concentration gradient in the first half of the serpentine mixer. Within the second half of the serpentine mixer ($x > 600$ µm), the mixing efficiency continues to increase albeit with a gentler slope until uniform mixing is achieved at the outlet.
Fig. 6. Mixing efficiency as a function of the serpentine mixer length for flow rates in the range of 80-200 µL/min. Within this flow rate range, our 1200 µm long serpentine micro-mixers can achieve homogenous (>90 %) mixing whilst producing a stable liquid jet for sample delivery at the outlet.

Even at the lowest flow rate of 80 µL/min, the serpentine micromixer reaches a maximum mixing efficiency of 93 % at the outlet. Whilst for flow rates higher than 100 µL/min, the mixing efficiency at the liquid jet outlet is between 95% and 100%. An increase in the flow rate increases the average velocity magnitude in the mixer microchannel. The increase in the average velocity translates to the dominance of advective mass transport over the diffusive one. For example, for an increase from 100 µL/min to 180 µL/min, the average velocity increases from 2.39 to 4.25 m/s, which leads to an increase in the average value for Pe from 15770 to 29269. Hence, the mixing efficiency is improved at higher flow rates due to an enhanced chaotic advection effect caused by the serpentine geometry. We should note, however, that whilst the higher flow rates enable faster, more homogeneous mixing resulting in a more conformationally uniform mixture, this comes at the cost of higher sample consumption – which can be an important consideration when dealing with expensive or scarce protein solutions.

3.3. Effect of microchannel size

We next used our FEM simulation to explore the effect of the mixer microchannel dimensions on the mixing efficiency. Figure 7 shows the mixing efficiencies as a function of the serpentine mixer length for three different height-to-width ratios \((r = H/W)\): 0.9, 1.8, and 3.6 at a constant flow rate of 100 µL/min. Figure 7a shows the mixing efficiencies for \(H = 45 \mu m\) and \(W = 12.5, 25,\) and \(50 \mu m\), respectively. Figure 7b shows the mixing efficiencies for \(H = 22.5, 45,\) and \(90 \mu m\) for \(W = 25 \mu m\).
Fig. 7. Mixing efficiencies along the length of the serpentine micromixer for a) H=45 µm, W=12.5, 25, and 50 µm, and b) W=25 µm, H= 22.5, 45, and 90 µm at a flow rate of 100 µL/min. The aspect ratios are r = 0.9, 1.8, and 3.6. The simulated results matching the height-to-width ratio used experimentally (represented in a) and b) by a dotted line) has an aspect ratio of r = H/W =1.8 and is included for comparison.

The results in Fig. 7a indicate that for a fixed height of H = 45 µm and a channel width of W=12.5 µm (r= 3.6) the micro-mixer can achieve mixing efficiencies of over 90%, faster than for W = 25 and 50 µm, and that to achieve the same mixing efficiency with a wider channel requires a longer channel length. In addition, wider channels also require more sample to maintain the same flow rate and hence sample consumption can be an issue. However, whilst the narrower microchannels appear to be more efficient at mixing, these also have the disadvantage that when mixing and jetting biological samples, the channels can potentially become blocked.

When examining the effect of height in Fig. 7b for a fixed channel width of W = 25 µm, we find that the most efficient mixing occurs for H = 22.5 µm (r = 0.9). The largest height (H = 90 µm) gives the least efficient mixing, whilst a channel height of (H = 45 µm) provides an intermediate mixing efficiency. The results from both Figs 7a and 7b indicate that a smaller microchannel cross-section results in a uniform mixing faster and with a higher efficiency, due to an increase in the average velocity of the solutions and consequently enhanced chaotic mixing effect.

The fabrication approach using SU8 developed for our microfluidic devices enables highly flexible geometries to be achieved without requiring any alteration of the photolithography mask design. This means that all of the ratios in terms of height and width simulated here can be readily reproduced experimentally and, according to our previous experimental work, will also result in stable jetting. Ratios are r = 0.9, 1.8, and 3.6. The simulated
Fig. 8. Minimum achievable mixing time (in milliseconds) using a serpentine micromixer for a) $H=45 \, \mu m$, $W=12.5$, 25, and 50 $\mu m$, and b) $W=25 \, \mu m$, $H=22.5$, 45, and 90 $\mu m$ as a function of flow rate. The aspect results matching the height-to-width ratio used experimentally (represented in a) and b) by a dotted line) has an aspect ratio of $r = H/W = 1.8$ and is included for comparison.

The minimum achievable mixing times for the serpentine micromixer as a function of flow rate are shown in Figure 8– the smaller the serpentine, the faster the mixing. The minimum achievable mixing time is defined here as the amount of time required for the reagents to travel in the serpentine micromixer before at least 95% mixing efficiency is achieved. Consistent with the results in Fig. 7, Fig. 8a shows that for a fixed channel height ($H=45 \, \mu m$) as the channel width increases the minimum achievable mixing time also increases (from 0.30 ms for $W=12.5 \, \mu m$ to 1.28 ms for $W=50 \, \mu m$) and that even increasing the flow rate to 300 $\mu L/min$ cannot make up for the difference in mixing times compared to the 12.5 and 25 $\mu m$ width channels. By decreasing the channel width to 12.5 $\mu m$, we can maintain sub-ms mixing (0.85 ms), even with flow rates as low as 120 $\mu L/min$ whilst still maintaining a stable jet. Figure 8b shows that for a fixed channel width ($W = 45 \, \mu m$) as the height of the mixing channel decreases, shorter and shorter mixing times can be achieved for a given flow rate. With the thinnest mixer ($H = 22.5 \, \mu m$) sub-ms mixing times can be achieved with flow rates as low as 80 $\mu L/min$. In fact, by using a micromixer channel of dimensions 22.5 $\mu m \times 25 \, \mu m$ ($H \times W$) it is possible to achieve mixing times in the range of 0.5 to 3.4 ms using flow rates of 40-100 $\mu L/min$. These parameters are readily achievable experimentally and indicate that our mix-and-inject devices could be employed to study a wide range of biomolecular dynamics with minimal sample consumption.
4. Conclusions

Here, we have presented the results of 3D FEM simulations modelling of chaotic advection in passive serpentine-micromixers. We have determined concentration profiles along the length of the serpentine micromixer at 12 different probe points. The results show that our serpentine micromixers can achieve uniform mixing using flow rates as low as 80 µL/min, which is consistent with our experimental data using these devices. The results also show that the chaotic mixing effect significantly increases at higher flow rates, resulting in faster mixing of the reagents. Moreover, we have used simulations to evaluate the impact of the microchannel dimensions on the mixing efficiency of the devices. The results indicate that for a channel width of W = 12.5 µm and height H=45 µm, the serpentine micromixer can achieve uniform mixing within a shorter serpentine length equating to a reduction in the minimum achievable mixing time required to reach > 95% mixing efficiency. Further, we have calculated mixing times for a wide range of different flow rates and different cross-sectional aspect ratios of the serpentine micro-mixer channel. The results show that micromixers with a smaller cross-sectional area can achieve uniform mixing with lower flow rates and shorter mixing times. Both the simulated and experimental results indicate that the serpentine micromixer is an efficient and straightforward method for achieving sub-millisecond and millisecond mix-and-inject devices for studying molecular dynamics. The size of the microchannels can be optimised based on operational factors such as the amount of sample solution available for the experiment, the timescales of the reaction kinetics being probed, the required velocity and diameter of the liquid jet (i.e. to match the size of the X-ray beam), and whether or not the sample is likely to contain particles larger than ~ 0.1 µm. The simulation results reported here will guide future development of rapid mix-and-inject microfluidic devices, providing new insights into biomolecules' structure and dynamics.

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Authors contributions: M.H. conceived of the presented idea, developed the theory, performed the simulations and post-processing, developed the structure of the paper, and wrote the manuscript. B.A and E.B supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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References

1. Neutze, R. and K. Moffat, Time-resolved structural studies at synchrotrons and X-ray free electron lasers: opportunities and challenges. Current opinion in structural biology, 2012. 22(5): p. 651-659.

2. Schulz, J., et al., A versatile liquid-jet setup for the European XFEL. Journal of synchrotron radiation, 2019. 26(Pt 2): p. 339-345.

3. Grunbein, M.L. and G. Nass Kovacs, Sample delivery for serial crystallography at free-electron lasers and synchrotrons. Acta Crystallographica Section D, 2019. 75(2): p. 178-191.

4. Martiel, I., H.M. Muller-Werkmeister, and A.E. Cohen, Strategies for sample delivery for femtosecond crystallography. Acta Crystallographica Section D, 2019. 75(2): p. 160-177.

5. Zhao, F.-Z., et al., A guide to sample delivery systems for serial crystallography. 2019. 286(22): p. 4402-4417.

6. Steinke, I., et al., A liquid jet setup for x-ray scattering experiments on complex liquids at free-electron laser sources. Review of Scientific Instruments, 2016. 87(6): p. 063905.

7. Ward, K. and Z.H. Fan, Mixing in microfluidic devices and enhancement methods. Journal of micromechanics and microengineering : structures, devices, and systems, 2015. 25(9): p. 094001.
8. Lee, C.-Y., et al., Microfluidic mixing: a review. International journal of molecular sciences, 2011. 12(5): p. 3263-3287.
9. Lee, C.-Y., et al., Passive mixers in microfluidic systems: A review. Chemical Engineering Journal, 2016. 288: p. 146-160.
10. Chen, X. and J. Shen, Simulation and experimental analysis of a SAR micromixer with F-shape mixing units. Analytical Methods, 2017. 9(12): p. 1885-1890.
11. Zare, P. and S. Talebi, Numerical simulation of geometry effect on mixing performance in L-shaped micromixers. Chemical Engineering Communications, 2020. 207(5): p. 585-597.
12. Chen, X. and J. Shen, Numerical and experimental investigation on splitting-and-recombination micromixer with E-shape mixing units. Microsystem Technologies, 2017. 23(10): p. 4671-4677.
13. Chung, C.K., C.K. Chang, and C.C. Lai, Simulation and fabrication of a branch-channel rhombic micromixer for low pressure drop and short mixing length. Microsystem Technologies, 2014. 20(10): p. 1981-1986.
14. Wu, C., et al., Concentration-dependent viscous mixing in microfluidics: modelings and experiments. Microfluidics and Nanofluidics, 2016. 20(6): p. 90.
15. Razavi Bazaz, S., et al., Obstacle-free planar hybrid micromixer with low pressure drop. Microfluidics and Nanofluidics, 2020. 24(8): p. 61.
16. Tsai, R.-T. and C.-Y. Wu, An efficient micromixer based on multidirectional vortices due to baffles and channel curvature. 2011. 5(1): p. 014103.
17. Melin, J., et al., A fast passive and planar liquid sample micromixer. Lab on a Chip, 2004. 4(3): p. 214-219.
18. Chen, X. and S. Zhang, 3D micromixers based on Koch fractal principle. Microsystem Technologies, 2018. 24(6): p. 2627-2636.
19. Shi, X., et al., Numerical analysis of passive micromixer with novel obstacle design. Journal of Dispersion Science and Technology, 2019: p. 1-17.
20. Wu, S.-J., H.-C. Hsu, and W.-J. Feng, Novel design and fabrication of a geometrical obstacle-embedded micromixer with notched wall. Japanese Journal of Applied Physics, 2014. 53(9): p. 097201.
21. Bernacka-Wojcik, I., et al., Experimental optimization of a passive planar rhombic micromixer with obstacles for effective mixing in a short channel length. RSC Advances, 2014. 4(99): p. 56013-56025.
22. Shi, X., et al., A novel passive micromixer with array of Koch fractal obstacles in microchannel. Journal of Dispersion Science and Technology, 2019: p. 1-12.
23. Chen, X. and Z. Zhao, Numerical investigation on layout optimization of obstacles in a three-dimensional passive micromixer. Analytica Chimica Acta, 2017. 964: p. 142-149.
24. Solehati, N., J. Bae, and A.P. Sasmoto, Numerical investigation of mixing performance in microchannel T-junction with wavy structure. Computers & Fluids, 2014. 96: p. 10-19.
25. Chen, X. and T. Li, A novel passive micromixer designed by applying an optimization algorithm to the zigzag microchannel. Chemical Engineering Journal, 2017. 313: p. 1406-1414.
26. COMSOL Multiphysics® v. 5.6. www.comsol.com. COMSOL AB, Stockholm, Sweden.
27. Liu, C., Y. Li, and B.-F. Liu, Micromixers and their applications in kinetic analysis of biochemical reactions. Talanta, 2019. 205: p. 120136.
28. Jiang, L., et al., Visualizing millisecond chaotic mixing dynamics in microdroplets: A direct comparison of experiment and simulation. 2012. 6(1): p. 012810.
29. Song, H. and R.F. Ismagilov, Millisecond Kinetics on a Microfluidic Chip Using Nanoliters of Reagents. Journal of the American Chemical Society, 2003. 125(47): p. 14613-14619.
30. Chen, C., et al., Improvement of silver azide crystal morphology and detonation behavior by fast mixing using a microreaction system with an integrated static micromixer. Reaction Chemistry & Engineering, 2020. 5(1): p. 154-162.
31. Pal, S., Chapter 7 - Protein folding, in Fundamentals of Molecular Structural Biology, S. Pal, Editor. 2020, Academic Press. p. 149-169.
32. Lindorff-Larsen, K., et al., Picosecond to Millisecond Structural Dynamics in Human Ubiquitin. The Journal of Physical Chemistry B, 2016. 120(33): p. 8313-8320.
33. Sekhar, A., P. Vallurupalli, and L.E. Kay, Defining a length scale for millisecond-timescale protein conformational exchange. 2013. 110(28): p. 11391-11396.
34. Strack, R., *XFELs probe protein dynamics*. Nature Methods, 2015. **12**(2): p. 109-109.

35. Liu, H. and W. Lee, *The XFEL Protein Crystallography: Developments and Perspectives*. International journal of molecular sciences, 2019. **20**(14): p. 3421.

36. Kang, Y., et al., *Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser*. Nature, 2015. **523**(7562): p. 561-567.

37. Knoška, J., et al., *Ultracompact 3D microfluidics for time-resolved structural biology*. Nature Communications, 2020. **11**(1): p. 657.

38. Kim, H., et al., *Submillisecond organic synthesis: Outpacing Fries rearrangement through microfluidic rapid mixing*. Science, 2016. **352**(6286): p. 691.

39. Mäeots, M.-E., et al., *Modular microfluidics enables kinetic insight from time-resolved cryo-EM*. Nature Communications, 2020. **11**(1): p. 3465.

40. Hejazian, M., et al., *Mixing and jetting analysis using continuous flow microfluidic sample delivery devices*. RSC Advances, 2020. **10**(27): p. 15694-15701.

41. Rani, S.A., B. Pitts, and P.S. Stewart, *Rapid Diffusion of Fluorescent Tracers into <em>Staphylococcus epidermidis</em> Biofilms Visualized by Time Lapse Microscopy*. 2005. **49**(2): p. 728-732.

42. Simonnet, C. and A. Groisman, *Chaotic Mixing in a Steady Flow in a Microchannel*. Physical Review Letters, 2005. **94**(13): p. 134501.

43. Nguyen, N.T., *Micromixers: Fundamentals, Design and Fabrication*. 2012: Elsevier/William Andrew.