A Micro-Device for the Mixing of a Highly Viscous Bio-Sample with Water/ Membrane Protein Solution Using Micro-Channel and Centrifugation

Liang Yuan and Yuan F. Zheng
Department of Electrical and Computer Engineering
The Ohio State University
Columbus, OH 43210
{yuanl, zheng}@ece.osu.edu

Abstract

A mechanism for controlling the mixing of highly viscous bio-samples at the micro-liter scale is presented. Existing methods for mixing bio-samples using micro-stirrers or shaking micro-wells are only effective for non-highly-viscous materials. The proposed mechanism mixes monoolein, a highly viscous bio-sample, with water/ membrane protein solution in a micro-device called micro-capsule using a micro-channel and centrifugation. To achieve effective mixing, the design of the micro-capsule along with the micro-channel is presented and so is the hydrodynamic model describing the flow of viscous materials in the micro-channel. The mixing process is analyzed according to the Reynolds number of the bio-samples using computer simulation, which is observed during the experiment using digital images for further analysis. Finally, the new approach is verified by X-ray diffraction experiments with water and the Rh membrane protein solution, which are used to evaluate the effectiveness of mixing. Experimental results not only validate the proposed method but also determine the flow oscillation time in the micro-channel to achieve effective and efficient mixing.

Keywords

Highly-viscous bio-sample; Mixing; Micro-capsule; Micro-channel; Centrifugation; Cubic Phase.

I. INTRODUCTION

In recent years, the technology of micro-mixing has attracted a great deal of attention due to the applications in life sciences, analytical chemistry, and food engineering. Here micro-mixing means that the volumes of the samples to be mixed are at the micro-liter (1 μL =10^{-6} liter) scale. For example, in the field of life sciences, micro-mixing is involved in the process of *membrane protein* crystallization, which is a necessary step for determining the structure of the protein using X-ray crystallography. There are a number of methods which can generate high quality crystals of proteins for the purpose of X-ray crystallography, and in general these methods fall into two categories [1]. The first category is the *in surfo* method that was introduced twenty years ago. The method has been proved difficult to crystallize membrane proteins because it is attempted as for soluble proteins and thus detergents are needed to solubilize membrane proteins, which cause low success rate due to the conformational inhomogeneity of the membrane proteins [2][3]. The second category is those that use a lipidic cubic phase called the *in meso* method as an alternative. In the *in meso* method, an extended bilayer composed of lipid, detergent, and target protein is presumed to form. It constitutes the following two steps for growing crystals [4, 5]: (1) mix two parts protein solution/dispersion with three parts lipid (usually monoolein), and the cubic phase forms spontaneously, (2) add precipitant/salt and incubate, and crystals form in hours to weeks. All operations take place at 20 ºC [5].

The *in meso* method was proved effective for the purpose of crystal growth [1]. The method however requires a highly viscous lipid (monoolein) to be mixed with the protein solution (such as bacteriorhodopsin). Mixing in this manner produces a bio-sample having the *cubic phase* structure which is the medium required for crystal growth. According to the water-composition graph of the monoolein-water system shown in [4], the relevant phases such as the Pn3m cubic phase could form at 20 ºC when the weight of the water is between 39 and 41% of the total (Fig. 1). The cubic phase is then dispensed into an array of wells on a plate as part of the crystallization setup [1, 5-6]. The crystal formed in the wells can be harvested and used to determine the 3D structure of the protein using X-ray crystallography [7-11].

A robotic system for automating the delivery of viscous bio-samples into arrays of wells has been built in our laboratory [10-14] which has significantly increased the efficiency and quality of dispensing. Unfortunately, the mixing of the lipid and protein was still done by hands using two micro-syringes [1]. Even worse is that this manual process must be done one sample at a time which is tedious and time-consuming.

In reviewing the literature, no work was found for mixing highly viscous materials at the micro-liter scale. Conventionally shaking has been a way for mixing at a small volume scale since 1940s [15]. The approach is still widely used in the laboratories today for various purposes. A shaking system includes either shake flasks or micro-well plates. Shake flasks may have a volume ranging from 10 mL to 500 mL [16], and can be made out of glass or plastic. Some have baffles to aid mixing. Liquids in shake flasks can be agitated using either orbital or linear shaking. The factors that affect flask mixing include vessel size, fill volume, shaking frequency, and liquid viscosity. Micro-well plates on the other hand have been widely used in life sciences for more than 50 years. They can handle mixing in parallel and at a scale ranging from 0.1 mL to 5 mL in each well [17-18]. In general, orbital shaking of the entire plate is the most common method even though mixing can be achieved using pipette aspiration or magnetically agitated stirrer bars [19]. The number of wells contained in a micro-well plate is typically 6, 12, 24, 96, 384, 1536, or

3456. Unfortunately plate shaking is still primarily used to handle low-viscous solutions such as bacteria, fungi, and mammalian cells. For highly-viscous bio-samples, shaking is not effective.

Stirring is a common technique for mixing which consists of a motorized agitator to cause the liquid to move or shift with a turbulent motion. The technique has been used in chemistry, food, biology, and pharmaceutical industries. In recent years, efforts have been made to design miniature stirrers and drivers such that the volume of the solutions could be reduced. A number of methods using micro-stirrers for mixing at the milli-liter level were found. Lamping et al. designed a miniature bioreactor for automated bioprocessing, which has three mechanical micro-stirrers (impellers) to mix the samples [20], and the working volume of the bioreactor is 6 mL. Commercially available bioreactors on the other hand can be as small as 10 mL [21-22], which are also equipped with micro-stirrers. Furthermore, the stirrers could be driven by either electric motor or magnetic field. The latter is more attractive for miniature bioreactors since no physical coupling is needed between the micro-stirrers and the drivers [21]. Unfortunately all the reactors as reported in [20-22] are designed for non-viscous solutions (viscosity is significantly less than 3000cP). The major challenge in using the micro-stirrer is that the viscous sample could stick to it when the stirrer is moved.

Another method for liquid mixing is by mechanical-channels which employ T-shape [23], Y-shape [24] and slit-type inter-digital homogenization, respectively [25]. Mechanical-channel mixing is designed for mixing low viscosity and high volume liquids which are pumped through the channel continuously. It is not suitable for mixing highly viscous bio-samples at the micro-liter scale. The literature suggests that mixing high viscosity (>3000 cP) and lower volume (<10 μL) bio-samples, such as lipid dispersions for crystallization of membrane proteins in a high-throughput manner, represents a great challenge and has not been seriously addressed by either the life science or control community.

In this paper, we present an approach for mixing a highly viscous bio-sample (monoolein) with water/Rh membrane protein solution at the micro-liter scale. The method makes use of a micro-device which is structured as a two-compartment micro-capsule built in with a micro-channel, through which the water can travel back and forth under centrifugation. The compartments serve as a container for promoting and mixing bio-samples. Due to the centrifugal force, one sample (the water) flows through the micro-channel to mix with the other without any mechanical parts to stir the samples. The combination of these innovative ideas enables the effective mixing of highly viscous bio-samples.

This work is based on our previous effort which has been published as a conference paper [26]. In the previous work, the hydrodynamic model of the flow of viscous materials in the micro-channel was developed and the flow of viscous bio-samples in the micro-channel using centrifugation was described [26]. In this paper, we expand the results in [26] to address the issues related to the control of centrifugation and the mixing process. We will present the physics of the flow of viscous materials in the micro-channel, the control of the centrifugation, the analysis of the mixing process through computer simulation, and the evaluation of the proposed mechanism through experiments including the use of X-ray diffraction.

The paper is organized as follow. The design of the micro-capsule is introduced in Section II. The hydrodynamic model of the flow of viscous materials in the micro-channel and the control of the flow of viscous bio-samples in the micro-channel are described in Section III. The mixing of monoolein and water system in the micro-channel is

analyzed in Section III with the aid of computer simulations. In Section IV, experimental processes and results using X-ray diffraction are presented. Conclusions are presented in Section V.

II. Design of the Micro-Capsule for the High-throughput Mixing

A. Design of the single micro-capsule for mixing

The micro-capsule has two functions. First, it houses the bio-samples in a compartment where the crystal can grow. Secondly, it provides means whereby the contents of the capsule can be mixed in a simple and programmable way. To simplify the fabrication, the micro-capsule is designed to have a cylindrical shape. To accommodate the above two functions, the basic structure of the micro-capsule is shown in Fig. 2. It has two compartments and a micro-channel connecting them. The compartments are for holding the bio-samples while the micro-channel is for the samples to flow back and forth between the two compartments. Mixing is achieved when the materials flow through the micro-channel together as shown in Fig. 2(d), or the turbulence flow of one sample (the water) diffuses itself into the other sample effectively. Each compartment has an access port for loading and unloading samples.

Two considerations determine the dimensions of the micro-capsule. First the size of the compartment should be compatible with the volume of the bio-samples to be handled. Then the length and diameter of the micro-channel should enable adequate flow of the samples for effective mixing. In general there are two types of flow in the micro-channel, laminar or turbulent, which are differentiated by a measure called Reynolds number (R_e). The Reynolds number is the ratio between inertial ($\bar{v}\rho$) and viscous (μ/d) forces:

$$R_e = \frac{\bar{v}\rho}{\mu/d} = \frac{\bar{v}\rho d}{\mu}. \tag{1}$$

In equation (1), ρ is the fluid density, d is the channel diameter, $\bar{v}$ is the mean fluid velocity, and μ is the dynamic fluid viscosity [27]. The transition of the flow between laminar and turbulent is indicated by a critical parameter called Reynolds number, which depends on the exact flow configuration. Within a certain range around this point there is a region of gradual transition where the flow is neither fully laminar nor fully turbulent. In general, the critical Reynolds number is 2300 for flows in circular pipes. For turbulent flows, the Reynolds number should be much greater than 2300 [28].

Laminar flow, which is characterized by smooth and constant fluid motion, occurs at low Reynolds numbers, where viscous forces are dominant, while turbulence is characterized by random eddies, vortices, and other flow fluctuations which the flow generates. Turbulence flow is more effective for mixing since the random eddies or vortices can increase greatly inter facial area of two samples. Generally, micro-mixers are characterized by geometries with a low Reynolds number. In such micro-scale, turbulence flow is hard to achieve, especially, for highly viscous materials or low velocity, and mixing relies on diffusion. However, if a special design is considered, such as a micro-channel, a driving agitator, or a magnetic bar, diffusion time could be reduced or turbulence may even be generated.

In our designed capsule, the long and narrow channels are used for water to generate the turbulence while the force driving the flow is by centrifugation. The turbulence of the water enables it to jet into the monoolein compartment, and monoolein can be smashed by water for diffusing. The viscosity of the mixture such formed in the monoolein compartment is decreased since a low viscosity sample (the water or the membrane protein solution) mixes with a

high viscosity sample (monoolein) [29-32]. When the flow direction is alternated, the remaining water in the monoolein compartment comes back by centrifugation and is ready for a new jet again. After a few times of alternation, the viscosity of the mixture is decreased to such a low level that it can be shaken in the compartment for continuing mixing under centrifugation until the homogeneous mixture is achieved. Fig. 3 shows a micro-array plate which can achieve parallel operation of multiple capsules installed on the plate.

We consider the length of the micro-channel to be about the same as that of the compartment, and the volume of the compartments around 2-6 times the volume of the bio-samples. These considerations are all ad hoc so long as the compartments can hold the bio-samples, and the micro-channel can produce turbulence flow under the available centrifugation device. In the experimental studies, our prototype micro-capsules have two compartments each with a length of 2.5 mm and a diameter (ϕ2) of 4 mm. That will create a volume of 31.4 μL. The micro-capsule is thus suitable for holding 5-15 μL bio-samples. Subsequently the micro-channel is designed to be 2 mm long, and the diameter is chosen to be 0.4 mm for which the reasons will be discussed in detail in Section IV. The other dimensions of the micro-capsule are shown in Fig. 4.

B. Design for the high-throughput purpose with multiple micro-capsules

Fig. 5 shows the mechanism for high-throughput mixing of a large number of micro-capsules. It consists of a round disc installed with 16 plates along the edge. Each plate has multiple micro-capsules arranged in an array of 4 rows and 12 columns as shown in Fig 3. The question is if each micro-capsule will receive the same driving force when the disc rotates for the centrifugation purpose. To answer the question, the following analysis is in the order.

Consider two capsules on a single row *A* and *B*. *A* is closest to the center of the disc, where the axis of spin is, with a distance R_c, while *B* has a distance R_{c1}. The two distance vectors form an angle θ. The relationship between R_c and R_{c1} can be defined by the following equation:

$$R_{c1} = \frac{R_c}{\cos\theta}. \tag{2}$$

The centrifugal force acting on capsule *A* is:

$$F_c = m\omega^2 R_c, \tag{3}$$

while the centrifugal force acting on capsule *B* is:

$$F_{c1} = m\omega^2 R_{c1}. \tag{4}$$

From F_{c1} one obtains the force F_{c1r} which drives the bio-sample through the micro-channel as:

$$F_{c1r} = F_{c1}\cos\theta = m\omega^2 R_{c1}\cos\theta = m\omega^2 R_c = F_c. \tag{5}$$

Equation (5) indicates that the centrifugal force acting on any micro-capsule of the same row is identical. However the centrifugal forces acting on different rows are different due to the variation of the distance from the center of the disc, which may affect the uniformity of the mixing. To avoid this problem, two approaches can be taken. First, only a few rows are allowed on a plate such that the distance of each row from the center R_c is close to each other. In our design, we use 4 rows in a plate, and the width of the 4 rows is 0.032 m while the centrifugal radius is 0.12 m. The variation of the distance between the rows in such a way is acceptable for mixing. Secondly, in the mixing process

all the plates need to be rotated by 180 degree, after a period of spin, for a few times to repeat the mixing. After each rotation, the row with the shortest distance to the center becomes the longest, i.e., the distance of each row to the center changes alternately. Consequently, the centrifugal force on every micro-capsule is uniform on average in the entire mixing process.

III. HYDRODYNAMIC MODEL OF THE FLOW IN THE MICRO-CHANNEL

The approach we propose is to let the bio-samples travel though the micro-channel such that mixing can be achieved. To understand how the bio-samples flow through the micro-channel under the effect of the centrifugal force, we need to develop a mathematical model to describe the hydrodynamic behavior. Once the model becomes available, the type of flow, laminar or turbulence, can be recognized.

To develop the model, a brief description of viscosity is appropriate. From [26], viscosity can be understood from a viscous material sandwiched between two flat plates separated by a distance y as shown in Fig. 6. When the upper plate moves horizontally with a velocity v and the lower plate remains stationary, the velocity of the material decreases linearly from v at the upper plate to 0 at the lower. To create such a velocity gradient, a shear force F must be applied to the upper plate, which is proportional to the coefficient of viscosity, the velocity gradient (or shear rate) of the material dv/dy, and the area of the plate A:

$$F = \mu A \frac{dv}{dy}. \tag{6}$$

The model of the viscous material flowing through the micro-channel can be built based on the viscosity model of equation (6). Assume a micro-cylinder of length l that is as long as the micro-channel and radius δ within the micro-channel (see Fig. 7). The axis of the micro-cylinder is coincident with the axis of the channel. The centrifugal force acting on the bio-samples in the micro-cylinder can be described as follows:

$$F_c = m\omega^2 R_c, \tag{7}$$

where

m: mass of the bio-sample in the micro-cylinder;

ω: angular velocity of the centrifuge;

R_c: the distance between the center of rotation and the micro-cylinder.

According to equation (6), the sheer force F_u, due to viscosity, is:

$$F_u = \mu A \frac{dv}{dy}. \tag{8}$$

There also exists frictional force between the surface of the channel and the viscous material due to the roughness of the surface. The frictional force F_f based on Bernoulli's principle is [33]:

$$F_f = \pi \rho f l \bar{v}^2 r / 4, \tag{9}$$

where the undefined parameters are:

$\bar{v}$: average velocity of the material;

f: frictional factor which is equal to $f = \frac{112\,\mu}{\bar{v} r \rho}$ [33].

Thus, when the material flows, the summation of the frictional force F_f and the shear force F_u must be equal to the centrifugation force F_c to guarantee a constant velocity:

$$F_c - F_f = -\mu A \frac{dv}{d\delta}. \tag{10}$$

From equations (8) and (10), we can obtain the velocity along the radius:

$$dv = -\frac{F_c - F_f}{2\pi l\mu} \cdot \frac{1}{\delta} \cdot d\delta. \tag{11}$$

Based on the boundary condition that $v=0$ when $\delta=r$, we can integrate equation (11) to obtain:

$$v = \frac{F_c - F_f}{2\pi l\mu} \cdot (\ln r - \ln \delta) \cdot \tag{12}$$

Equation (12) shows that the velocity of the flow in the micro-channel has a logarithmic distribution along the micro-channel radius r. The maximum velocity is on the axis and the minimal occurs on the wall of the micro-channel where $\delta=r$.

In Fig. 6, we can take a small circular area of $d\delta$ at a radial position δ to obtain $dB=2\pi\delta d\delta$. The flux through the circular area is:

$$dq = vdB = v \cdot 2\pi\delta d\delta. \tag{13}$$

Substituting v in equation (13) by equation (12), one obtains:

$$dq = \frac{F_c - F_f}{2\pi l\mu}(\ln r - \ln \delta) \cdot 2\pi\delta d\delta \cdot \tag{14}$$

Integrating equation (14), one obtains:

$$q = \frac{F_c - F_f}{4l\mu} r^2. \tag{15}$$

Thus, the average velocity in the micro-channel can be expressed as:

$$\overline{v} = \frac{q}{\pi r^2} = \frac{F_c - F_f}{4\pi l\mu}. \tag{16}$$

Making use of equation (9), equation (16) becomes:

$$\overline{v} = \frac{F_c}{32\pi l\mu}. \tag{17}$$

Equation (17) indicates that the average velocity of the material is directly proportional to the centrifugal force and inversely proportional to the channel length and the liquid viscosity.

IV. Analysis of Mixing Process of Monoolein and Water

As mentioned earlier, one important application of the current method is for crystallizing membrane proteins. It has been shown that the so-called *in meso* method is effective for crystallizing membrane proteins and has been used in the field of bio-chemistry for a long time [4, 5]. The key of the *in meso* method is the homogenous mixing of protein with lipid and the formation of the cubic phase. There are several different types of the cubic phase, for example, Lc, Lα, cubic-Ia3d, and cubic-Pn3m as shown in Fig. 1. Each has its own characteristic low-angle

diffraction pattern under the X-ray [2] which is used to index the corresponding space group (Pn3m, Ia3d, etc). It is not clear which of the cubic phases is required for crystal growth, but the cubic-Pn3m phase predominates which crystals grow and has proved to be a useful medium for the growth of well-structured crystals of membrane proteins [34-36].

In our work, we use pure water as well as membrane protein solution to mix with monoolein since the viscosity of the water is very similar to that of the membrane protein solution. Monoolein on the other hand is a mixture of glycerides of eleic and other fatty acids, consisting mainly of monooleate. Its density is similar to honey, but its viscosity is much higher than the latter. Fundamentally, we need to understand how water or monoolein flows through the micro-channel under centrifugation.

The relationship between the average flow velocity ($\bar{v}$) in the micro-channel and the centrifugal force (F_c) is shown in equation (17). From equation (17), the Reynolds number under the centrifugal force can be acquired:

$$R_e = \frac{\bar{v} d \rho}{\mu} = \frac{F_c d \rho}{32 \pi l \mu^2} \ . \tag{18}$$

The centrifugal force is expressed as below:

$$F_c = m\omega^2 R = \rho V \omega^2 R = 0.010955 \rho V R_c n^2 \, . \tag{19}$$

From equations (18) and (19), the Reynolds number can be calculated:

$$R_e = \frac{0.010955 \rho^2 V R_c n^2 d}{32 \pi l \mu^2} \, . \tag{20}$$

In our work, 200 g (n=800 rpm) of the relative centrifugal force (rcf) and 90 seconds of the duration time are used for each mixing. There are two reasons for this use: one is that 200 g rcf is a low laboratory centrifugation and easy to operate, and the other is that the combination of 200 g rcf and 90 seconds is enough for the samples to flow through the same micro-channel [26]. If these parameters can mix monoolein with water well, a larger centrifugation or longer duration is also qualified for the mixing. For the given dimension of the micro-channel and the spin speed n=800 rpm, the 5 μL water flowing through the micro-channel has a Reynolds number of 3200, but the Reynolds number for monoolein is rather small (less than 100) because of its high viscosity. In fact the velocity of monoolein is close to zero in the micro-channel ($<10^{-6}$) according to equation (17). Thus, our mixing strategy is to let water travel through the micro-channel in a high velocity to mix with monoolein by diffusion as discussed in the previous section.

A. *Analysis of the micro-capsule design*

According to the Reynolds number, the length and diameter of the micro-channel should be optimized in order to guarantee the turbulent flow of water. For the turbulent flow, the Reynolds number of water should be greater than 2300. Based on equation (20), the ratio of the channel diameter and length must satisfy the following equation for the turbulent flow:

$$\frac{d}{l} = R_e \frac{32 \pi \mu^2}{0.010955 \rho^2 V R_c n^2} > 2300 \frac{32 \pi \mu^2}{0.010955 \rho^2 V R_c n^2} = 0.144 \tag{21}$$

In the design of the micro-capsule, the length of the micro-channel is selected using the following principle. It is

fundamentally constrained by the dimension of the micro-capsule, which is determined by the space limitation of the plate that holds the micro-capsules and the volume of the bio-samples to be mixed. According to the space limitation, the size of the micro-capsule is determined. Then the volume of each compartment in the capsule is determined according to the volumes of the two samples to be mixed, i.e., the former should be greater than the latter. As a result, the distance between the two compartments can be determined by the two factors, the volumes of the bio-samples and the size of the plate. For our applications, the length of the micro-channel is selected to be 2 mm as cited earlier. Once the length of the micro-channel is selected, only the diameter of the micro-channel can be optimized to reach effective mixing. In the following, we discuss how the diameter of the micro-channel can be optimally selected.

For effective and efficient mixing in the micro-capsule, two factors are critical: turbulence flow and enough smashing time. First the flow of the water in the channel should be turbulence since turbulence flow generates large eddies and vortices which can smash the viscose bio-sample for effective mixing. Secondly, the jet of the water should be as long as possible. The reason is that mixing is more effective when a constant volume of water can smash the viscose material for a longer time. To satisfy the first factor, the diameter of the micro-channel should be *greater* than 0.288mm for turbulence flow according to equation (21). The second factor needs the diameter to be as small as possible since that will prolong the time of flow. Consequently, there is a compromise between the two factors, i.e., the optimal design is to push the diameter to the lower bound as much as possible such that the flow of the water is turbulence while the time of flow is long.

To prove the theory of the optimal diameter of the micro-channel as just described, three different sizes of the diameter, 0.2 mm, 0.4 mm, and 0.8 mm, are used to simulate the flow of the water through the micro-channels by Computer Fluid Dynamics (CFD) simulation package called FLUENT. It is clear that 0.4 mm is closest to the lower bound of the diameter which is 0.288 mm. The results are shown in Fig. 8, Fig. 9, and Fig. 10 respectively. Fig. 8 shows less eddies and vortices because the flow is laminar as the diameter is less than 0.288mm. In comparison, the flows in Fig. 9 and Fig. 10 both are turbulence. Fig. 10 shows a larger flux flowing through the micro-channel than the flow in Fig. 9, which means that the water will be more quickly exhausted in the 0.8 mm diameter. Fig. 9 shows that the 0.4 mm can guarantee eddies and vortices and as the same time the water can jet for a longer time than the 0.8 mm diameter for the purpose of smashing, which means more effective mixing.

In addition, in the multi-micro-capsules plate, the Reynolds number of the shortest centrifugal radius row is 2816 according to equation (20), which still guarantees the turbulence flow of water.

B. *Analysis of the mixing process*

The available literature reveals that the most common and simplest way to determine the quality of mixing is by the flow-visualization technology such as photo or video techniques to monitor the mixing process [37]. To make sure that the mixing progresses according to what has been analyzed, we propose to perform a real mixing experiment in which dyed water is used for clear observation, and observe the process using digital images. The water is initially dispensed into the right compartment and monoolein the left as shown in Fig. 11(a). For convenience each flow motion of the samples under centrifugation is called one time of mixing (TOM), and digital photos are taken at the end of a TOM. Fig. 11(b-j) shows the sequence of the mixing process including 8 TOMs

under centrifugation. The whole mixing process is divided into three stages. In the first stage, the water travels from its compartment through the micro-channel to reach monoolein in the left compartment. Due to the turbulence flow of the water, the water turns itself into eddies and vortices which are jetted into the monoolein compartment and smash monoolein against the wall as shown in Fig. 11(b). Since monoolein has a hydrophilic region in the molecular structure [1], which can easily bond with water, the viscosity of the mixture in the right compartment thus can be reduced.

In the second stage, the mixture of monoolein and water oscillates in the left compartment for mixing as the direction on centrifugation alternates although it cannot flow back to the right compartment which is shown in Fig. 11 (b-f). Meanwhile, some remain water continues to flow back and forth through the micro-channel to mix with the mixture in the left compartment through jetting which is shown in Fig. 11(c) and (e). However the water shown in Fig. 11(c) is less than that in Fig. 11(e), which means that more and more water has mixed with monoolein, and the volume of the remaining water is getting smaller and smaller until all goes into the mixture.

In the final stage, free water is all exhausted after 4 TOMs. The viscosity of the mixture becomes so low that the mixture can be shaken in the left compartment under the alternated centrifugation as shown in Fig. 11(f-i). Even though a little mixture travels to the right compartment under centrifugation as shown in Fig. 11(g), most of them can flow back to the left compartment in the next mixing as shown in Fig. 11(h). In this way, the mixture repeats to shake in the left compartment until the homogeneous mixture is achieved. The question is how many TOMs should be used to achieve the desired mixing, which can be determined by the image analysis of the mixture, while theoretically it is not possible to determine an optimal TOM.

We used image intensity histogram as an effective approach to judge the homogeneity of the mixing [38]. To obtain the intensity histogram, the photos taken at the end of TOM=4, 6, 7, 8, 9, and 10 are used which are converted into an 8-bit grey-scale bitmap (0-255). Fig. 12 shows the intensity histogram of each of the sample images. At TOM=4, 6, and 7 the histograms still have a wide distribution, while at TOM=8, 9, and 10, the distributions become narrower and stable. The corresponding standard deviations of all the TOMs are shown in Fig. 13 which shows that the standard deviation becomes stable after TOM=8, which means 8 is the optimal number of TOM.

V. EXPERIMENTS

In this section, we further test the optimal TOMs by examining the cubic phase of the mixed bio-samples. Fundamentally the mixture has to be homogeneous such that the cubic-Pn3m phase is uniformly formed. The goal is to reach the homogeneity by just enough TOMs. If too few, the desired cubic phase cannot be formed while too many, extra time and energy will be a waste.

The method we use is to examine multiple samples taken from different parts of the mixture using X-ray diffraction since for a particular cubic phase there is a corresponding pattern of diffraction. If the mixture is homogeneous, every sample from the mixture should produce the same diffraction pattern; otherwise, the diffraction patterns between the samples may be different. According to the equilibrium temperature-composition (T-C) phase diagram [1, 39], Pn3m can form after mixing of 3 parts monoolein with 2 parts water in homogeneity at 20 ºC. For Pn3m the pattern of diffraction is available as reported in [35].

A. *Experimental preparation and method*

A.1 X-ray machine

X-ray diffraction measurements are performed using a rotating anode X-ray generator (Rigaku RU-H3R operating at 40 kV and 90 mA) which produces Cu radiation (wavelength λ=1.5418 Å). Detector is R-AXIS IV++ which combines two large active area (300 mm × 300 mm) imaging plates with fast readout speeds and a wide dynamic range, making it ideal for collecting accurate diffraction data. Sample to detector distance is 450 mm.

A.2 Materials

Nitrosomonas europaea Rh membrane protein was solubilized with crystallization buffer (20 mM TrisCl, 100 mM NaCl, 20 mM Sodium Formate and 30 mM β-octyle glucoside) to the final concentration 6 mg/ml. Monoolein (1-oleoyl-rac-glycerol, lot M-239-M8-R) is purchased from Nu Chek Prep Inc. (Elysian, MN). It has a purity of 99% or greater as determined by thin layer chromatography and is used without further purification. Water is demineralized and distilled.

TABLE I X-RAY DIFFRACTION DATA

Mixing times	d(Å) (º)	Intensity	*hkl*	a_0(Å)	Space group
	76.18 (1.2º)	**6.5**	**110**	**107.7**	
	61.69 (1.4º)	**6.2**	**111**	**106.9**	
TOM=7 (Fig. 16 (d))	**42.93 (2.1º)**	**2.4**	**211**	**105.2**	**Pn3m**
	35.39 (2.8º)	**3.5**	**221/300**	**106.2**	
				106.5 (3%)	
	69.68 (1.3º)	**6.5**	**110**	**98.5**	
	57.12 (1.5º)	**6.1**	**111**	**98.9**	
TOM=8 (Fig. 17 (a))	**40.64 (2.1º)**	**4.2**	**211**	**99.5**	**Pn3m**
	32.96 (2.6º)	**3.8**	**221/300**	**98.9**	
				98.9 (1%)	
	69.92 (1.3º)	**6.5**	**110**	**98.9**	
	57.46 (1.5º)	**6.3**	**111**	**99.5**	
TOM=8 (Fig.17 (b))	**40.16 (2.1º)**	**4.0**	**211**	**98.4**	**Pn3m**
	32.99 (2.6º)	**4.0**	**221/300**	**98.9**	
				98.9 (1%)	

Note: *d* is the spacing of the set of lattice planes, intensity indicates the reflection strength of lattice planes, *hkl* is characterized by the Miller indices, and a_0 is the lattice spacing of the cubic phase.

A.3 Sample preparation for X-ray diffraction

Three parts monoolein is dispensed in one compartment of the capsule and 2 parts water is in another. The capsule is put in the centrifuge (Eppendorf 5702) for mixing which is carried out at room temperature (20 ºC). The homogeneously mixed samples are transferred to 0.3 mm diameter quartz capillaries (Charles Supper, Natick, MA), wax-sealed.

A.4 X-ray diffraction of mixture

The wax-sealed samples are exposed to X-ray for determining the cubic phase of the mixtures. For cubic liquid crystalline phases, one has $d_{hkl} = a_0/(h^2+k^2+l^2)^{1/2}$ in the ratio of $1:\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{5}:\sqrt{6}:\sqrt{8}:\sqrt{9}$ which are indexed as (100), (110), (111), (200), (210), (211), (220), (221)/300 reflections, respectively [40-41]. In these equations, d_{hkl} is the spacing of the set of lattice planes (h, k, l) characterized by the Miller indices h, k, and l and a_0 is the lattice spacing of the cubic phase. For different crystals, the Miller indices are different which denote planes and their corresponding directions in the X-ray crystallography, which is a unique and effective tool to reveal the crystal structures [1]. The reflections of the different planes and their relative positions are displayed in the crystallography. For bicontinuous cubic phases, such as Pn3m and Ia3m etc., their indices have been found. For Pn3m, the sequence of the allowed reflections is *hkl* =110,111, 211, 221/300, etc, and the relative peak positions are in the ratio of $\sqrt{2}:\sqrt{3}:\sqrt{6}:\sqrt{9}$, etc. [42-43] .

B. *Experimental results*

B.1 Mixing with water

TABLE II X-RAY DIFFRACTION DATA WITH MEMBRANE PROTEIN SOLUTION

Samples	d(Å) (°)	Intensity	*hkl*	a_0(Å)	Space group
Fig. 19 (a)	75.78 (1.2°)	7.1	110	107.1	Pn3m
	61.74 (1.5°)	6.1	111	106.9	
	43.72 (2.0°)	1.2	211	107.1	
	35.40 (2.4°)	1.5	221/300	107.4	
				107.1 (1%)	
Fig. 19 (b)	76.51 (1.2°)	7.4	110	108.1	Pn3m
	61.89 (1.4°)	6.8	111	107.0	
	43.96 (2.0°)	2.6	211	107.7	
	35.80 (2.4°)	2.7	221/300	107.4	
				107.6 (1%)	
Fig. 19 (c)	77.85 (1.1°)	6.7	110	110.1	Pn3m
	62.63 (1.4°)	6.3	111	108.5	
	44.28 (2.0°)	2.0	211	108.5	
	36.40 (2.4°)	1.4	221/300	109.2	
				109.1 (2%)	
Fig. 19 (d)	75.91 (1.2°)	6.5	110	107.3	Pn3m
	61.63 (1.4°)	6.3	111	106.7	
	43.86 (2.0°)	3.3	211	107.4	
	36.22 (2.4°)	1.7	221/300	108.6	
				107.5 (2%)	

Note*: d* is the spacing of the set of lattice planes, intensity indicates the reflection strength of lattice planes, *hkl* is characterized by the Miller indices, and a_0 is the lattice spacing of the cubic phase.

Our first set of experiment is to mix the viscous sample monoolein with water. The results presented below concern the mixing effects at the different times of mixing. Phase characteristics are quantified by low angle X-ray diffraction. Measurements are made at 20 ºC.

When the TOM is 5, the four samples taken from the mixture are different. From the diffraction patterns, one can find four phases including water, Lc, Lα, and Ia3d as shown in Fig. 14. The latter three phases reflect that the water percentage in monoolein is less than 40% which is not surprising since the water has not all been homogeneously

mixed into the monoolein. Fig. 15 shows different phases for another four samples taken from the mixture at TOM=6 including Lα, Lα+Ia3d, and Ia3d which means that water percentage in monoolein is still less than 40%. Fig. 16 shows different phases for four samples taken from the mixture at TOM=7 including water, Ia3d, Ia3d+Pn3m, and Pn3m. One can see that after more TOM, Pn3m starts to form, but the bio-samples are still not evenly mixed. Water still exists which has not been evenly and completely mixed into monoolein such that Ia3d co-exists with Pn3m. Fig. 17 shows the result of the four samples after TOM=8. Again the four samples are randomly taken from the mixture in the micro-capsule, and the X-ran diffraction shows the patterns of Pn3m for all the four samples. This proves that mixing has been evenly achieved. Table I shows the X-ray diffraction data of the three mixing results as shown in Fig. 16(d), Fig. 17(a) and Fig. 17(b) corresponding to a primitive cubic lattice with the unit cell axis. One can see that the spacing of the set of lattice planes (*d*), the reflection strength of lattice planes (intensity), the Miller indices (*hkl*), and the lattice spacing (a_0) are close to each other indicating the formation of the Pn3m cubic phase, while those for Fig. 17(a) and Fig. 17(b) are almost identical. Since Fig. 17(a) and Fig. 17(b) are samples from the same mixing result, the data reveal a very even mixing after TOM=8. Fig. 18 shows the low-angle X-ray diffraction profile at TOM=8.

B.2 Mixing with Rh membrane protein solution

To show that the mixing method can also be used for protein crystallization, the Rh membrane protein solution is used instead of water in our second set of experiments. Fig. 19 shows the X-ray diffraction results of four samples at TOM=8. All the results are Pn3m and the corresponding data are shown in Table II. Meanwhile, Fig. 20 and Table III show the results of four samples from the capsule on the shortest radius row in the multi-micro-capsules plate at TOM=8. In this mixing, the radius of this row becomes longest after a rotation, i.e., the centrifugal radius changes alternately during the entire mixing period. All the results are Pn3m which means that mixing is effective for capsules on the different rows of the plate. One can see that the mixing results for the protein solution are similar to that for the water. The reason is the protein solution has the same viscosity as the water; therefore, the designed mixing method is equally effective.

The experiment results verify that the proposed method of mixing is effective. The optimal TOM was discussed with image histogram analysis and also verified by experiments. Here optimality means just enough TOM to reach an even mixing, nothing less or more. As shown in the image standard deviation and experiments, eight is an optimal number for TOM because it will not get job done if less than eight and waste time and energy if more.

TABLE III X-RAY DIFFRACTION DATA WITH MEMBRANE PROTEIN SOLUTION

Samples	d(Å) (°)	Intensity	*hkl*	a_0(Å)	Space group
Fig. 20 (a)	79.56 (1.1°)	5.2	110	112.5	Pn3m
	65.10 (1.4°)	4.0	111	112.7	
	46.09 (2.0°)	1.0	211	112.9	
	37.16 (2.3°)	1.0	221/300	111.5	
				112.4 (1%)	
Fig. 20 (b)	79.48 (1.1°)	5.2	110	112.4	Pn3m
	64.55 (1.4°)	4.0	111	111.8	
	45.73 (1.9°)	2.0	211	112.0	
	37.49 (2.4°)	2.7	221/300	112.4	
				112.1 (1%)	
Fig. 20 (c)	78.88 (1.1°)	4.4	110	111.5	Pn3m
	64.08 (1.4°)	4.0	111	111.0	
	45.89 (1.9°)	2.6	211	112.4	
	37.11 (2.4°)	1.4	221/300	111.3	
				111.5 (1%)	
Fig. 20 (d)	77.89 (1.2°)	4.8	110	110.1	Pn3m
	62.61 (1.4°)	2.5	111	108.4	
	44.76 (2.0°)	3.2	211	109.6	
	37.33 (2.4°)	0.8	221/300	112.0	
				110.0 (2%)	

Note: *d* is the spacing of the set of lattice planes, intensity indicates the reflection strength of lattice planes, *hkl* is characterized by the Miller indices, and a_0 is the lattice spacing of the cubic phase.

VI. CONCLUSIONS

In this paper, we have presented a mechanism for mixing highly-viscous bio-samples using a micro-device called micro-capsule, which is structured with a micro-channel connecting two compartments, and centrifugation. We first present the design of a micro-capsule for holding and mixing the bio-samples in which a micro-channel is built. The size of the micro-capsule is designed in such a way that it is enough to hold bio-samples as well as suitable for building a micro-array plate for the high-throughput purpose. According to the requirement of turbulence flow and effective mixing, the diameter of the micro-channel is determined once the length of the micro-channel is selected. To further facilitate micro-channel mixing, the flow of viscous materials in the micro-channel is studied. A mathematical model which governs the behavior of the flow is developed. To understand micro-channel mixing with centrifugation, Reynolds number is derived based on the structure of the micro-capsule and the dimension of the micro-channel. Then the mixing process is analyzed according to the Reynolds numbers of water and monoolein, respectively. It is shown that the flow of the water is turbulence. Turbulence flow of the water generates eddies and vortices which smash the monoolein in the other compartment to achieve effective diffusion. As the water continues to diffuse into the monoolein by alternating the direction of centrifugation, the viscosity of the mixture becomes lower and lower. Consequently, shaking of the mixture in one compartment becomes possible which further

improves the effectiveness of the mixing. Experimental results have shown that the invented mixing method is effective for mixing two bio-samples with low and high viscosities, respectively. The paper has also shown that the optimal times of mixing (TOM) can be determined by visual observation method. Finally, this paper has been using the cubic phase required in the *in meso* method as the target of study. The method however can be employed to mix viscous bio-samples of other kinds at the micro-liter scale.

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Figure Captions

Fig. 1 Water-composition phase diagram for the monolein and water system [1].

Fig. 2 Micro-capsule for mixing two bio-samples cell suspension and monoolein: (a), (c) and (d) are the top view, (b) is the side view, and (e) shows the actual micro-capsule. Note that a micro-channel connecting the two chambers and each chamber has a mouth to serve as the access port.

Fig. 3 The plate with an array of micro-capsules spins in a centrifuge.

Fig. 4 The dimension of the micro-capsule: (a) front view, and (b) side view where the units are in mm.

Fig. 5 The centrifugal forces applied to multiple plates for high throughput mixing.

Fig. 6 Two parallel plates move with relative velocity v when the space between is filled with viscous material.

Fig. 7 The hydrodynamic model of the flow of viscous bio-sample in the micro-channel.

Fig. 8 The water flowing through the micro-channel of 0.2mm diameter at: (a) 0s, (b) 0.0001s, (c) 0.0002s, (d) 0.0003s, (e) 0.0004s, and (f) 0.0005s., which show laminar show.

Fig. 9 The water flowing through the micro-channel of 0.4mm diameter at: (a) 0s, (b) 0.0001s, (c) 0.0002s, (d) 0.0003s, (e) 0.0004s, and (f) 0.0005s, which show turbulence flow with large eddies and vortices.

Fig. 10 The water flow process through the micro-channel of 0.8mm diameter at: (a) 0s, (b) 0.0001s, (c) 0.0002s, (d) 0.0003s, (e) 0.0004s, and (f) 0.0005s, which show turbulence flow, but the flux of the water is quicker than the 0.4mm diameter as the water level is lower in (f).

Fig. 11 The mixing process: (a) monoolein and dyed water are dispensed in the left and right compartments respectively, (b) the first mixing (centrifugation: from right to left), (c) the second mixing (centrifugation: from left to right), (d) the third mixing (centrifugation: from right to left), (e) the forth mixing (centrifugation: from left to right), (f) the fifth mixing (centrifugation: from right to left), (g) the sixth mixing (centrifugation: from left to right), (h) the seventh mixing (centrifugation: from right to left), (i) the eighth mixing (centrifugation: from left to right), and the ninth mixing (centrifugation: from right to left).

Fig. 12 The 2D-histograms at different TOMs with water: (a) TOM=4, (b) TOM=6, (c) TOM=7, (d) TOM=8, (e) TOM=9, (g) TOM=10. The unit of the horizontal axis is pixel intensity, and that of the vertical axis is the number of pixels.

Fig. 13 The corresponding 2D-histograms standard deviations at different TOMS.

Fig. 14 Low angle X-ray diffraction patterns of the four samples taken from the mixed cubic phase in the micro-capsule after five TOMs: (a) water, (b) Lc, (c) Lα, and (d) Ia3d.

Fig. 15 Low angle X-ray diffraction patterns of the four samples taken from the mixed cubic phase in the micro-capsule after six TOMs: (a) water, (b) Lα, (c) Ia3d+Lα, and (d) Ia3d.

Fig. 16 Low angle X-ray diffraction patterns of the four samples taken from the mixed cubic phase in the micro-capsule after seven TOMs: (a) water, (b) Ia3d, (c) Ia3d+Pn3m, and (d) Pn3m.

Fig. 17 Low angle X-ray diffraction patterns of the four samples taken from the mixed cubic phase in the micro-capsule after eight TOMs. All shows Pn3m.

Fig. 18 Low-angle X-ray diffraction profile obtained from monoolein and water mixing at TOM=8.

Fig. 19 Low angle X-ray diffraction patterns of the four samples taken from the mixed cubic phase with the Rh membrane protein solution in the micro-capsule after eight TOMs. All shows Pn3m.

Fig. 20 Low angle X-ray diffraction patterns of the four samples taken from the mixed cubic phase with the Rh membrane protein solution in the micro-capsule, which is on the row with alternately changed centrifugal radius after eight TOMs. All shows Pn3m.