Doctoral Dissertation

Development of a Novel In-Vitro Compartmentalization Method for Bias-Free Directed Evolution using Electrostatic Inkjet

Bineet

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Thesis Title	DEVELOPMENT OF A NOVEL IN-VITRO COMPARTMENTALIZATION METHOD FOR BIAS-FREE DIRECTED EVOLUTION USING ELECTROSTATIC INKJET
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patience. It was their love that raised me up again when I got enervated. And finally I would thank all my friends in JAIST who helped me get through this agonizing period in the most positive way, and has been a family away from home.

Bineet

CONTENTS

CHAPTER 3: DEVELOPMENT OF 'SELECTION-IN-A-FL DROPLET' SYSTEM FOR A RAPID AND BIAS-FREE DIRECTED MOLECULAR EVOLUTION

GUIDE TO THE THESIS

Laboratory evolution mimics the natural evolution in the test tube and focused at molecular level for a particular property is termed as directed evolution. Basically the directed evolution experiment comprises of, gene encoding molecule *viz.* DNA/RNA aptamer which is randomized and expressed and linked as genotype-phenotype. A suitable screening or selection methods are used to distinguish the best variant amongst the millions of molecules for a desired trait. Selection method is the imitation of Darwin's theory of survival of the fittest. Over the period of time, many researchers have demonstrated the significance of directed evolution *via* number of biomolecular display technologies like phage display, ribosomal display, mRNA display and so on. But all these technologies lacks the proper handling of millions of molecules, which give rise to the adulterated outcomes (biased). Still today, the current system lacks the detailed knowledge of selection method for evolution experiments because of the possibilities of mismatching genotype to phenotype due to linker molecules (ribosomal display). Since there are millions of molecules present with their expressed proteins that led to existence of the crowding effect and effect of target concentration on the screening steps. Moreover these display technologies does not provide high efficient genotype to phenotype linkage (g-p linkage) due to the batch mode treatment of the molecules. Griffiths et al have shown in vitro compartmentalization (IVC) by encapsulating the biomolecules using water-in-oil emulsion. This technology provided efficient g-p linkage inside water droplets but selection of molecules still have problems discussed above.

A high throughput selection system is need of the hour with bias free screening steps of encapsulated variants. IVC by water-in-oil emulsions have limitations of polydispersity that creates the improper monitoring over evolution. Later droplet based microfluidics has overcome

this problem by generating monodisperse droplets for precise volume, but this technique has limitation of small size droplets production with high throughput droplet generation speed. Furthermore microarray technology provided a platform of small reaction chamber but fabrication of these chambers are limited to kilo-to-mega scale only. So considering the above challenges of directed evolution especially selection method, I planned to develop the bias-free high throughput selection system by IVC using immersed electrosprays.

Chapter first described about the high throughput generation of water-in-oil droplets using immersed electrosprays. The water droplets were generated by dipping the nozzle head of electrostatic inkjet while exploiting the principles of electrosprays. This water-in-oil emulsion system can generate ~1.3 µm size droplets with 10^5 droplets per second generation speed. Further parameters like nozzle size, oil viscosity and bias voltage affecting the droplets size were optimized. Nozzle size and viscosity of the oil directly proportional to the average droplet size while frequency of the inkjet machine inversely proportional to the droplet size.

Chapter second has shown the validity of high throughput water droplet systems for biochemical reactions. Immersed electrosprayed water-in-oil droplets demonstrated successful expression of GFP followed by the time course study in different size droplets. The small sized droplets showed the early saturation GFP expression in 15 minutes of incubation, this can be understood by the fact of miniaturization of compartments enhance the reaction kinetics of protein expression. Furthermore I performed the co-expression of extreme diluted sample of GFP and mCherry and no droplets showed the single fluorescence. This may be explained on the concept of "super concentration effect" in which all the components concentrated in sub-femtoliter droplets and there exists rapid consumption of amino acids.

After demonstration and validation immersed electrosprayed based water-in-oil droplets in previous chapters, I focused on development of "selection-in-a-fL droplet" system in chapter three which includes the generation of agarose-in-oil gel beads, followed by washing steps of unbounded DNA molecules. First of all, target immobilized beads were co-compartmentalized in ultralow agarose gel beads using high throughput immersed electrospray technology. Later, I demonstrated the bias-free washing system comprised of removal of encapsulated Cy-ssDNA (20mer) from the agarose gel beads by acetone and isopropanol wash. Since washing of agarose gel beads involve the centrifugation that can easily break/damage the gel beads, so I also fabricated the PDMS microchip of three inlets which serves the micro-mixer based on vortex technology for better solvent exchange during washing steps.

LIST OF PUBLICATIONS

Patent

- **Japan Patent Application number: 2015-115336**
  インクジェットを用いたＩＶＣ用液滴生成装置及び液滴生成方法
  Bineet Sharma, Manish Biyani, Yuzuru Takamura, Tatsuya Shimoda, (Droplet generation instrument and droplet generation method for IVC by inkjet) – *Chapter 1*.

Scientific Journal

- **Bineet Sharma**, Yuzuru Takamura, Tatsuya Shimoda, Manish Biyani, A bulk sub-femtoliter in vitro compartmentalization system using super-fine electrosprays. (*Sci. Rep.* **6**, 26257; doi: 10.1038/srep26257 (2016))

- **Bineet Sharma**, Yuzuru Takamura, Tatsuya Shimoda, Manish Biyani, Realization of "super-concentration" effect using cell-free system in sub-femtoliter droplets generated by immersed electrosprays (*manuscript in preparation*).

Conference proceeding

- **Bineet Sharma**, Manish Biyani, Yoshiaki Ukita, Yuzuru Takamura, Study on centrifugal force based particle trapping in micro chamber at lower Reynolds number, ***19th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS)***, Texas, USA, October/November 2014 (*conference proceeding*).

Award

- **Best Student Poster Award** in International Joint Symposium on Single Cell Analysis, Kyoto, Japan – *Chapter 3 (micro-mixer device)*.

Presentations

- **Bineet Sharma**, Yuzuru Takamura, Tatsuya Shimoda, Manish Biyani, Cell-free protein synthesis in sub-micrometer electrospray based water-in-oil droplets for *in vitro* compartmentalization, ***8th International Symposium on Microchemistry and Microsystems***, Hong Kong, May/June 2016. (*oral*)

- **Bineet Sharma**, Manish Biyani, Yoshiaki Ukita, Yuzuru Takamura**,** Effect of centrifugal force on vortex-based particle separation in micro chamber at lower Reynolds number, ***8th International Symposium on Microchemistry and Microsystems***, Hong Kong, May/June 2016. (*poster*)

- **Bineet Sharma**, Yuzuru Takamura, Tatsuya Shimoda, Manish Biyani, Super-fine electrostatic inkjetting of monodisperse sub-femtoliter droplets for ultra-rapid single-molecule in vitro compartmentalization, ***The International Chemical Congress of Pacific Basin Societies***, Hawaii, USA, December 2015. (*poster*)

- **Bineet Sharma**, Yuzuru Takamura, Tatsuya Shimoda, Manish Biyani, High throughput monodisperse water-in-oil droplets formation for i*n vitro* compartmentalization using electrostatic inkjet technology, ***28th International Microprocesses and Nanotechnology Conference (MNC)***, Toyama, Japan, November 2015. (*poster*)

- **Bineet Sharma**, Yuzuru Takamura, Tatsuya Shimoda, Manish Biyani, High-speed generation of femtoliter water-in-oil droplets using electrostatic inkjet for in vitro compartmentalization, ***7th International Symposium on Microchemsitry and Microsystems (ISMM)***, Kyoto, Japan, June 2015. (*poster*)

- **Bineet Sharma**, Manish Biyani, Yoshiaki Ukita, Yuzuru Takamura, Study on centrifugal force based particle trapping in micro chamber at lower Reynolds number, ***19th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS)***, Texas, USA, October/November 2014. (*poster*)

- **Bineet Sharma**, Kiyotaka Sugiyama, Yoshiaki Ukita, Yuzuru Takamura, Study on particle trapping by microvortex chamber for single cell washing, ***74th Japan Society of Applied Physics Autumn Meeting (JSAP)***, Kyoto, Japan, September 2013. (*oral*)

- **Bineet Sharma**, Yoshiaki Ukita, Yuzuru Takamura**,** Study on particle trapping by micro vortex chamber for single cell washing, ***International Joint Symposium on Single Cell Analysis***, Kyoto, Japan, November 2012. (*poster*)

- **Bineet Sharma**, Yoshiaki Ukita, Yuzuru Takamura, Study on the vortex behaviour in micro scale chamber for the trapping of particles, ***25th International Microprocesses and Nanotechnology Conference (MNC)***, Kobe, Japan, October/November 2012. (*oral*)

LIST OF FIGURES AND TABLES

Figures

Tables

Abstract

Directed evolution is laboratory evolution by imitating the natural evolution in the test tube and focused at molecular level for a particular property. It comprises of, a library of millions-of-millions molecules and selection method for desired function. Selection of variants has been performed by linking gene encoding molecule *viz.* DNA/RNA and its expressed phenotype as genotype-phenotype (g-p) linkage. Over the period of time, many biomolecular display technologies like phage display, ribosomal display, mRNA display and so on has been discovered for directed evolution, but all these technologies lacks the proper handling of millions of molecules, which give rise to the adulterated outcomes (biased). The current scenario lacks the detailed knowledge of selection method for evolution experiments. Griffiths et al have shown in vitro compartmentalization (IVC) by encapsulating the biomolecules using water-in-oil emulsion. This technology provided efficient g-p linkage inside water droplets but selection of molecules still have problems discussed above. IVC by water-in-oil emulsions have limitations of polydispersity that creates the improper monitoring over evolution. Later droplet based microfluidics has overcome this problem by generating monodisperse droplets, but this technique has limitation of small size droplets production with high throughput droplet generation speed. So I planned to develop new method for monodisperse high throughput IVC droplets using electrostatic inkjet for the bias-free selection system in directed evolution.

The nozzle head of electrostatic inkjet was dipped in oil phase and on voltage application water-in-oil droplets generated termed as immersed electrosprays. A nozzle size of 4 µm was filled with aqueous phase and dipped in oil phase (mixture of ABIL EM 90 (50%), Tegosoft DEC (36%) and mineral oil (14%)) for generation of water-in-oil droplets at 50 V and 100 Hz. When voltage applied to the nozzle water surface started deformation and at threshold voltage it forms a Taylor cone followed by water jet stream which leads to generation of water droplets. The average size of water droplets was found to be ~1.3 µm (CV = 12%) with generation speed of 10^5 droplets per second. By using high speed camera and it is found that ~108 droplets were generated in single pulse. Further parameters affecting the droplets size like nozzle size, oil viscosity and bias voltage were optimized. Increase in nozzle size, increases the droplet size with high degree of polydispersity due to different jet mode of droplet formation. Increase in viscosity also increases the average droplets size due to increase in hydrophobicity of oil phase. High voltage is analogous to the flow rate, so increase in voltage large droplets size were generated while higher frequency reinforced the smaller size. Green fluorescent protein (GFP) – cDNA along with PURE system was taken in inkjet nozzle and biomolecules were encapsulated in water droplets using immersed electrosprays. Then all droplets were incubated for protein synthesis at 37 °C for 2 h. The successful expression of GFP in immersed electro sprayed water droplets was observed. Time course study of GFP expression revealed early saturation of GFP expression within 9 to 15 min for small droplet volume (1.8 fL). This can be understood by the fact of miniaturization of compartments enhance the reaction kinetics of protein expression with rapid consumption of key raw materials like amino acids. Conventional method of selection involves the bulk treatment of variants to the target molecule, this may leads to intermolecular and bias the outcome of selection. Compartmentalized selection by femtoliter droplets provides platform for better understanding of selection system. With the help of immersed electrosprays ultralow agarose droplets (melt over 60 °C and gelled below 10 °C) was generated in oil with average size of ~1.7 µm using 15 µm nozzle size at 500 V and 100 Hz. The successful encapsulation of target beads inside agarose gel-in-oil beads. Similarly encapsulation and washing of Cy5-ssDNA in agarose gel-in-oil beads and performed washing steps using acetone and isopropanol.

In conclusion, sub-femtoliter water-in-oil droplets were generated using immersed electrospray with the generation speed of 10^5 droplets per second. The parameters like nozzle size, oil viscosity and applied voltage were investigated for the droplets size manipulation. Successful GFP expression in water-in-oil droplets with the early saturation between 9 to 15 min. For the minimal volume selection-in-a-fL droplets, ultralow agarose-in-oil gel beads of ~1.7 µm size were generated using immersed electrospray. The washing of Cy5-ssDNA inside agarose gel beads were demonstrated by using acetone and isopropanol.

Keywords: In vitro compartmentalization (IVC), water-in-oil droplets, immersed electrosprays, cell-free protein expression, selection-in-a-fL droplet

Immersed electrosprays for bulk generation of monodisperse sub-femtoliter water-in-oil compartments

CHAPTER 1

~ Highlights ~

- High throughput water-in-oil droplets generation using immersed electrosprays.
- Average size of w/o droplets is found to be 1.3 µm with generation speed of 10^5 droplets per second.
- Increase in nozzle size and viscosity (oil) increase the average size of the droplets.

1.1 Introduction

1.1.1 *In vitro* compartmentalization

The encapsulation of biochemical reactions in pico- to femtoliter-sized microreactors using water-in-oil emulsion technology which offers a means to parallelize biological and chemical assays, termed as *in vitro* compartmentalization (IVC)[1-4]. Initially, IVC (Griffiths et al.) was introduced to generate man-made cell-like compartments for the study of directed evolution. These compartments were used for linkage of genotype (its encoding gene) to phenotype (protein) that is a principle requirement of conventional molecular evolution albeit often a limiting step for directed evolution[5-7]. Directed evolution is evolution at small and fast scale in the laboratory which focused at the molecular level for specific properties from the pool of millions-trillions molecule[8].

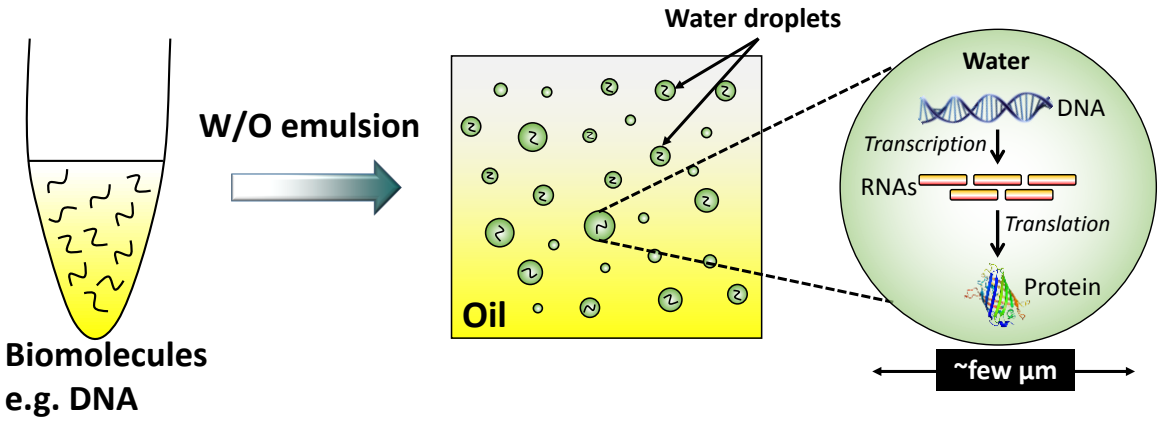


Figure 1.1*: Schematic illustration of in vitro compartmentalization using water-in-oil emulsion.*

1.1.1.1 *Methods of IVC generated system*

Water-in-oil (W/O) emulsion have been extensively exploited as micro-sized reactors which enhanced the reactivity and yield of the reactions. There are many different techniques have been used for generation of w/o droplets with different droplet size manipulation like

homogenizers, extruder, and droplet based microfluidics by which small and stable aqueous compartments are generated in oil phase.

Homogenizer used to generate small size w/o droplets from large droplets by applying shear stress in narrow or physical barrier[9]. This leads to the production of polydisperse droplets with large size distributions and can also result in a loss of biological sample because of the high homogenization speed/pressure. Batch mode emulsion is generated by simple vortexing the water in oil phase which generates 1-100 µm droplets size, resulting in fL-to-nL differences in reaction volume[10,11]. Like homogenizer, vortex method of droplet generation produce polydisperse droplets which has also risk of damaging the sample. The above mentioned emulsion formation technology, w/o droplet shows wide or narrow size distribution. This leads to uneven distribution of reagents into droplets; sequentially causing loss in activity of translated proteins (Miller *et al.*) as a result not all the droplets become active for *in vitro* expression[11]. Although water-in-oil emulsion has been best suited for quantitative screening of molecules using FACS (fluorescence activated cell sorter). IVC using emulsion technology has been successful strategy for directed molecular evolution of enzymes but it also suffers some limitations. After in vitro gene-expression, for the purpose of screening molecules emulsion droplets are re-emulsified in aqueous phase. Due to technical problem, there are two kinds of micro-compartments exists as double emulsion droplets: compartments having gene-of-interest and unrelated gene. Furthermore if two different mutant genes are co-compartmentalized during re-emulsification their genotype-phenotype linkage would be affected during sorting process[12]. Also water-in-oil emulsion cannot applied for in vitro studies of various kind of proteins like membrane proteins.

To overcome these problems droplet based microfluidics was introduced[13], which precisely generates highly monodisperse water-in-oil droplets by either T-junction or flow focusing method. The multiple compartments issue can be solved by high throughput droplet-based

microfluidics screening system embraces droplets for gene amplification, coalescence of droplet pairs[12] (droplet with gene and droplet with PURE system) and finally separating the genes of interest. Droplet based microfluidics renovated the whole study of directed evolution by provided a platform to evaluate all micro-compartments for a specific study. But for the library size of million-quadrillion molecules and encapsulating the genes precisely in droplets can be challenging for droplet based microfluidic because of its limitations of droplet production[14-17] (few thousands per minute). Pros and cons of the above discussed droplet generation method can be summarized in table 1.1, which depicts that microfluidics have an upper hand over mini extruder and homogenizer techniques for monodisperse droplets with reasonable high throughput generation speed.

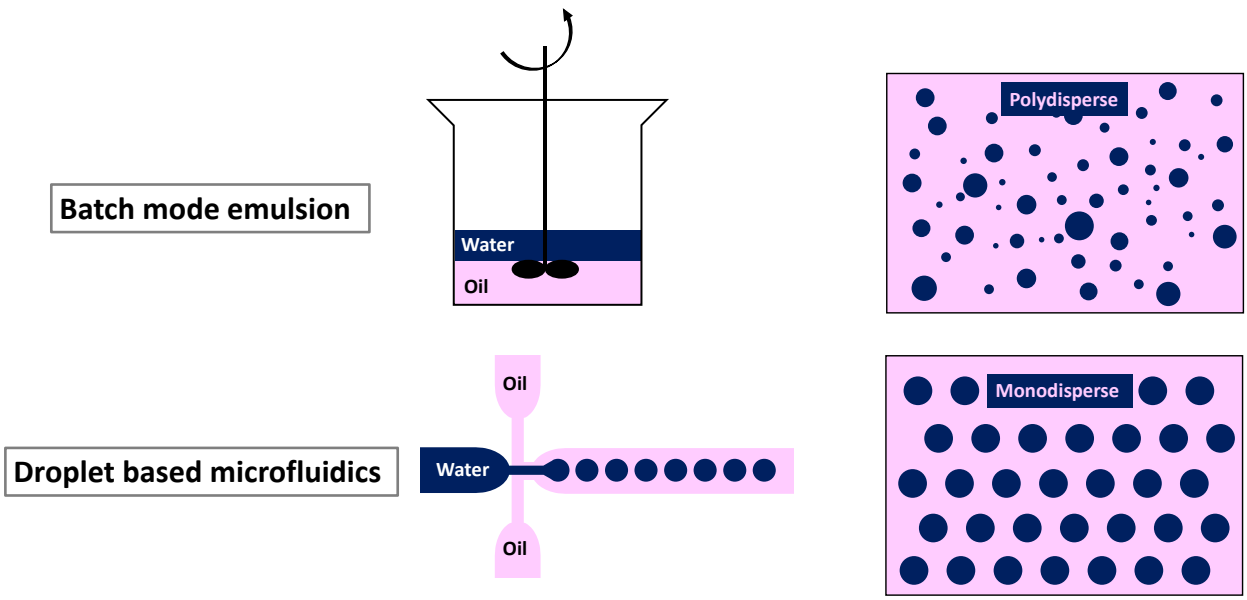


***Figure 1.2**: Methods of water-in-oil emulsion generation system.*

To minimize the volume of the reaction, micro array technology have been used at femtoliter scale and proved to be efficient method for quantitative analysis (Yomo *et al* 2012) but it failed on the ground of IVC because of the non-uniform distribution of sample mixture, difficult to handle the $>10^{15}$ compartments for directed evolution and well defined solid array cannot mimic the cellular compartments[18-21].

Table 1.1: **Comparison of three different droplets generation methods.**

Droplet generation method	Droplet diameter	Generation speed	Uniformity
Homogenizer	~10 µm to mm	~10^9 droplets/min	Poor
Droplet based microfluidic	>50 µm	<10^5 droplets/min	High
Mini extruder	~10 µm	~10^9 droplets/min	Medium

Inkjet printing technology has also used for manipulating the droplet size with precise volume control which proved to be upper hand over microfluidics technology. Recently Yu *et al* (Lab chip 2015) reported droplet in oil for picoliter scale analysis but piezo electric inkjet used till now has limitation of low throughput generation of droplets[22]. Here I present a simple and new platform for ultra-rapid generation of water-in-oil droplets using electrostatic super fine inkjet technology (SIJ Technology, Inc.). The electrostatic based inkjet nozzle is submerged in oil phase and on the applied voltage, this system is capable of continuous droplet production with generation speed of ~3 million droplets/minute which is ideal platform to execute femtoliter-IVC.

~ Objective ~

To develop a new platform for ultra-rapid generation of water-in-oil femtodroplets using electrostatic super fine inkjet technology for the in vitro compartmentalization.

1.2 Methods

1.2.1 Electrospray setup and procedure

The water-in-oil droplet generation system was created by using a prototype head of super-fine inkjet technology [SIJ technology, Japan]. A small hole of ~6 mm was punched on commercial available silicone rubber (3 cm x 3 cm x 3 mm) and placed on glass slide (35 x 55 mm) which represents as chamber for droplet collection (Figure 1.3). Laboratory made glass nozzle (~ 65 µm orifice diameter) and commercially available glass nozzle (~4 µm orifice diameter) [SIJ technology, Japan] were used throughout the experiments. Glass capillary of 1x90 mm size was used to make ~65 µm nozzle by Puller machine PC-10 [Narishige, Japan] using two step mode, keeping heater level 60 and 50. Tungsten wire as an electrode was used in case of large nozzle. Oil phase containing 50% ABIL EM 90 [Evonik Industries], 36% tegosoft DEC [Evonik Industries], and 14% mineral oil [Sigma Aldrich] was used. Different viscosity of oil mixtures was prepared by changing tegosoft DEC from 10% to 90% keeping ABIL EM90 and mineral oil as 3:1. Oil viscosities were measured by Viscomate [VM-10A, Sekonic CBC Co. Ltd]. All the oil mixtures used in the experiments were freshly prepared by vortexing at 2,500 rpm for 5 minutes and incubated at 30°C for 30 minutes.

1.2.2 Water-in-oil droplet generation

The glass nozzle of ~4 µm was filled by 7 µl of nuclease free water and fixed to the inkjet machine. The oil phase (100 µl) was poured to the oil chamber and glass nozzle was immersed in oil, on voltage application through submerged glass nozzle containing aqueous solution was jet into cavity having oil phase succeeding large amount of water-in-oil droplets at the generation speed ~10^5 droplets per second. For droplet size calculation and distribution dynamic light scattering (DLS) [Malvern Zetasizer Nano ZS] machine was used. Droplet average size was also measured using laser scanning confocal microscope [Olympus FluoView

1000] with the help of ImageJ software. High-speed camera [Phantom VR 502 or Kato Koken K5] is used to estimate the water-in-oil droplets generation speed.

1.3 Results and discussion

Table 1.2: **Preparation of different oil viscosities* using tegosoft DEC, ABIL EM 90 and mineral oil.**

	Tegosoft DEC (Vol %)	Mineral oil (Vol %)	ABIL EM 90 (Vol %)	Viscosity (mPa.s)
1	90	7.4	2.6	8.06
2	70	22.2	7.8	12.7
3	50	37.1	12.9	23.3
4	30	51.8	18.1	39.3
5	10	66.6	23.4	73.4

1.3.1 Producing super-fine electrospray based sub-femtoliter in vitro compartmentalization

I report a technique that uses electrospray-based super-fine inkjet (SIJ Technology, Inc.) to generate monodispersed droplet jets with diameters in sub-micrometer range. The principle behind SIJ printing system is similar to electrospray techniques where a conducting liquid is slowly injected through an electrified capillary nozzle. The liquid inside the capillary nozzle attains hemispherical meniscus due to its surface tension. On applied pulsed DC voltage, a tangential electric stress is appeared due to accumulation of ions near the meniscus area. On a particular threshold voltage, Rayleigh instability phenomenon occurred which imbalance the

two forces, electrostatic force and surface tension acting on water surface. This results into formation of a conical shape, commonly referred to as the Taylor cone, and a thin microthread of liquid is issued from the tip of the Taylor cone, which eventually fragments to form a spray of monodisperse droplets. Importantly, the size of generated droplets is independent of the diameter of the capillary tube and thus the droplet size can be obtained below the micrometer range. The electrospray of water-in-oil droplets for IVC can be generated by immersing the nozzle in an immiscible phase, i.e., oil. The basic experimental setup for the technique is

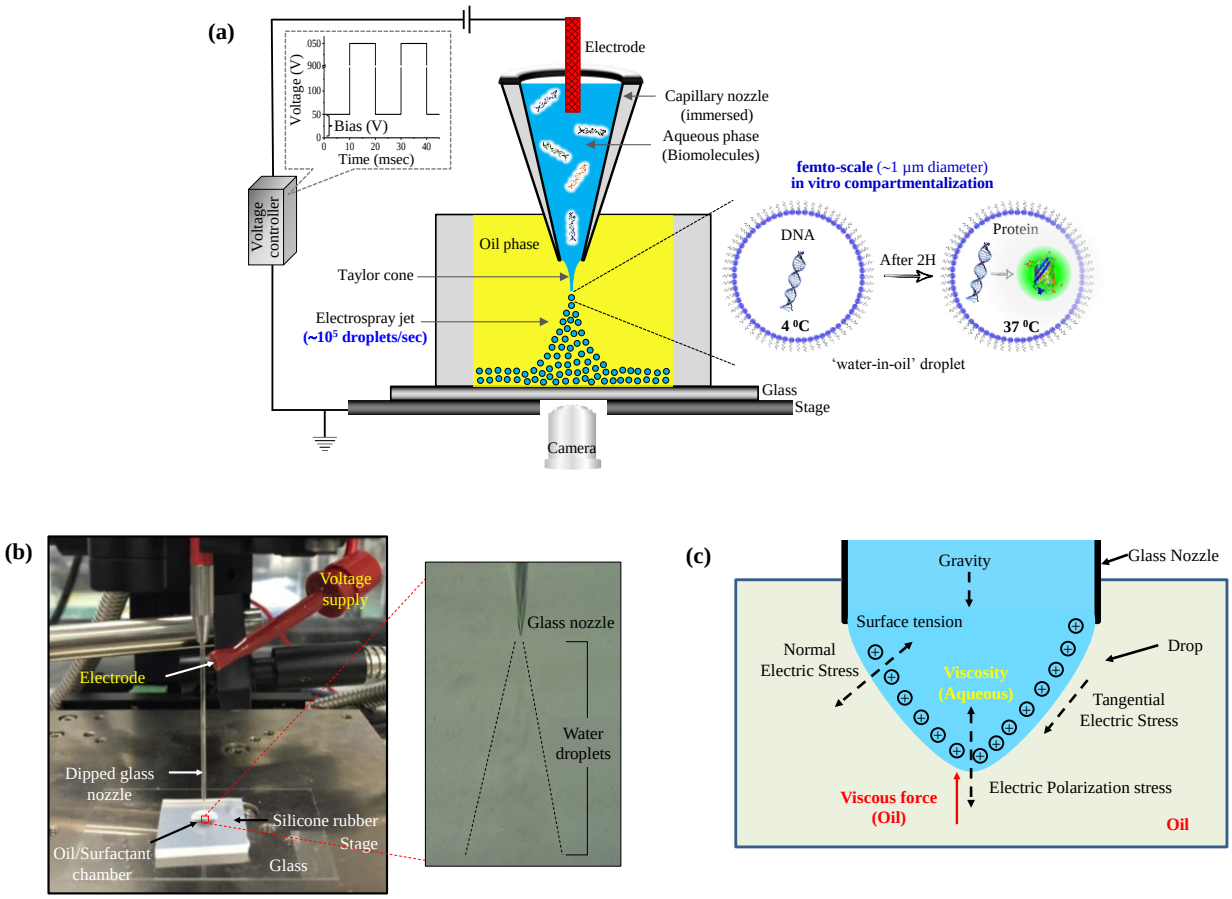


***Figure 1.3**: **Immersed super-fine electrospray based miniaturized in vitro compartmentalization.** (a) Schematic illustration of the experimental setup is shown. A head of inkjet nozzle (filled with nM solution of template DNA and gene expression system) immersed in an immiscible oil-phase can produce ultrarapid monodisperse water-in-oil femtoliter-scale droplets in bulk at mega-scale. (b) Photograph of electrostatic inkjet setup with image of water-in-oil droplet generation. (c) Forces acting on droplet interface under the influence of electric field.*

depicted in figure 1.3a. A capillary nozzle which is connected to a high voltage supply controller unit through a fine electrode wire is immersed in a reservoir filled with a mixture of

oil/surfactant with a clear bottom for visualization. The electrical field is generated by applying a high voltage drop from the capillary nozzle and a liquid consisting of biomolecules was extruded and sprayed through a nozzle. The forces acting on droplets are schematically illustrated in figure 1.3c, at the threshold voltage these forces get unbalanced at the minimum surface area and results in Taylor's cone which further burst as jetstream. The water-in-oil femto-scale compartments were formed by the generation of monodisperse aqueous droplets followed by their mutual diffusion into a mixture of oil/surfactant.

A real time and high-speed camera was used to capture the release of a jet breaking into discrete

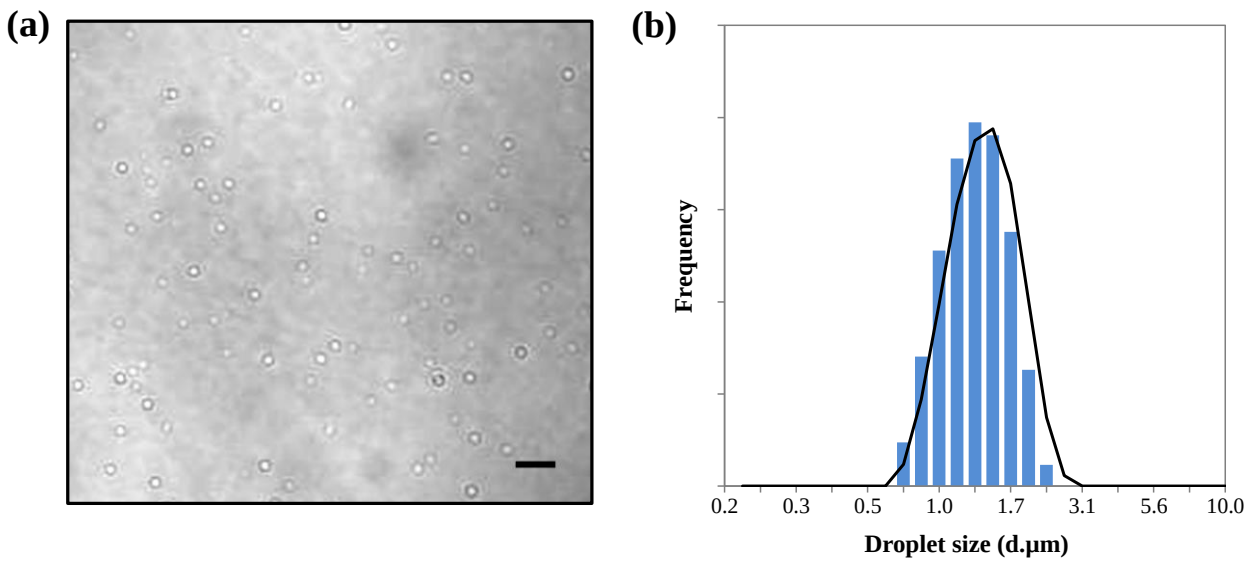


Figure 1.4: Size measurement of femto-scale in vitro compartmentalization. *(a) Confocal microscopic image of water-in-oil droplets. Droplet size distribution measured by dynamic light scattering. (b) Histogram of the droplet size distribution obtained using 4 µm nozzle orifice diameter. The mean average shown is 1.3 µm with a volume of 1.2 femtoliter. Scale bar: 5 µm.*

droplets. Laser scanning confocal microscopy was used to capture the water-in-oil droplet compartments. As shown in figure 1.4a, average size of the droplets was calculated by dynamic light scattering and found to be ~1.3 µm in size using a nozzle with 4 µm orifice diameter. For the calculation of number of droplets generated by electrospray system two types of high-speed

video cameras were used: at 10,000 fps speed (Phantom VR 502, Vision Research) captured

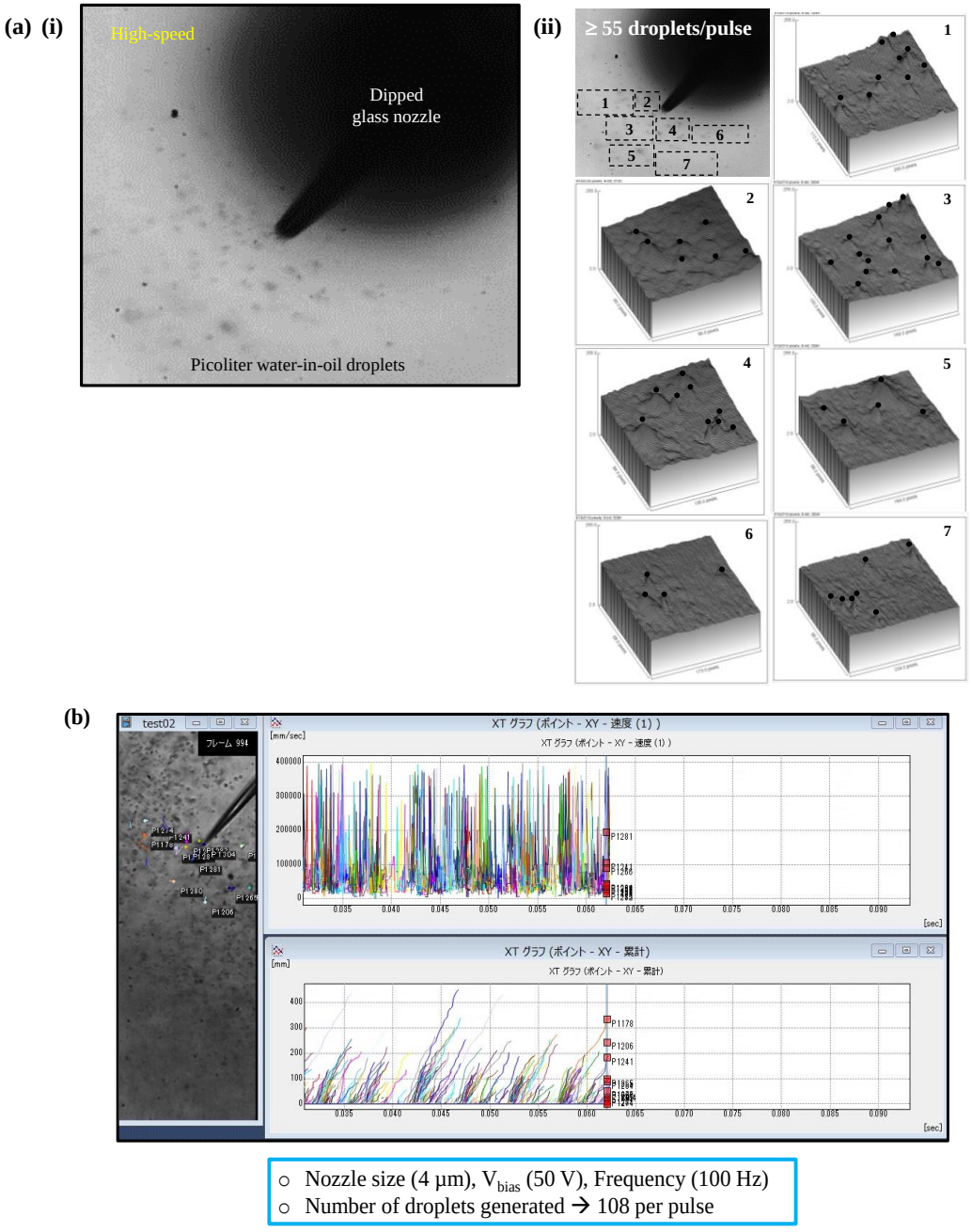


Figure 1.5: Number of droplet generation by high speed camera*. (a-i) High-speed camera movie for water-in-oil droplet produced in very first pulse. (a-ii) Calculation of number of droplets from image (i) using ImageJ software. From 1-7 Surface plot of the different regions showing number of droplets generated by one pulse using high speed camera (Phantom VR 502). More than 55 droplets counted in 1 pulse at 50 V. Nozzle ~65 µm. (b) Another high speed camera at 16,000 fps speed (K5, Kato Koken) with fully automatic 2D motion analysis software (MotionV). Using the experimental setup (Figure 1) ~108 droplets were produced in one pulse. At the maximum frequency in of 1 kHz, this system can produce aqueous droplets at the rate of 10^5 droplets per second. Nozzle ~4 µm.*

the droplets generated in one pulse followed by calculating the number of generated droplets

using ImageJ software (see figure 1.5a) and at 16,000 fps speed (K5, Kato Koken) with fully

automatic two dimensional motion analysis software, MotionV (see figure 1.5b). I calculated 55 to 108 droplets produced in one pulse using the experimental setup (as shown in figure 1.3a) and taking the maximum frequency of 1 kHz, in current setup lead to generate monodisperse aqueous droplets at rates near 10^5 droplets per second which can be further extendable to about 2 orders of magnitude faster using higher-frequency electrostatic waves.

1.3.2 Optimizing parameters for monodisperse femtodroplet generation

In a typical inkjet electric parameters like viscosity and surface tension are key factors which affects droplets size. Since we have used electrostatic inkjet with dipped nozzle which works on electrospray method so the disintegration of jet into different droplets size is predominantly affected by the size of the nozzle, surface tension of aqueous-phase, viscosity of the oil-phase, and various instrumental parameters, including the voltage supply, bias and frequency. Therefore, the optimal conditions for these parameters to generate monodisperse and stable droplets were determined. Firstly, the size of nozzle determines the geometry of Taylor's cone and different types of modes (e.g., cone-jet or oscillating-jet) can be generated in jet spray depending on the orifice diameter of nozzle. Therefore, the size of nozzle plays an important role in the jet spray-mode while optimizing the positioning and precisely controlling the volume of the droplets. Figure 1.6a shows the droplets size distribution of two different size nozzle, 4 µm and 65 µm, keeping other parameters constant. Large size nozzle provides large interface area, so under influence of external electric field liquid drop experienced imbalance stresses on interface and ended up with oscillating jet mode which leads to the wider size distribution of droplets. Whereas small size nozzle provides less interface area so it leads to cone jet-mode. Hence polydispersity is observed in case of bigger nozzle as compare to smaller

one (see figure 1.6b). Secondly, the area of plume is determined by the medium in which spray is occurred. Since air provides negligible friction force, so droplets generation in air by inkjet

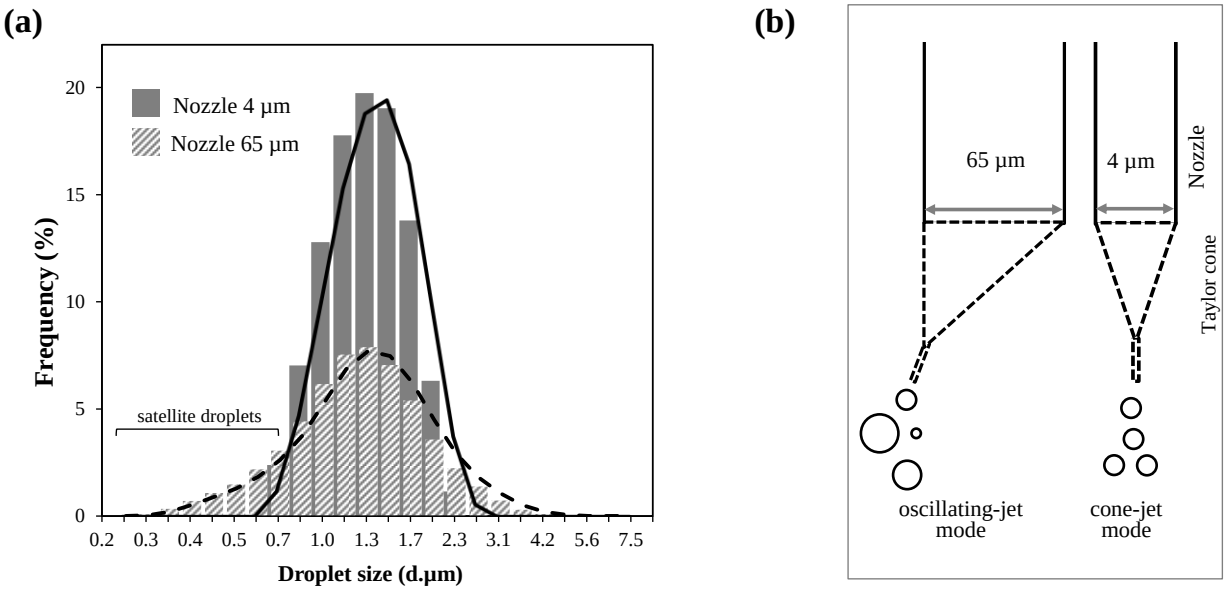


Figure 1.6: Influence of nozzle size on electrospray based water-in-oil droplet size distribution. *(a) Comparison of nozzles with bigger (~65 µm) and smaller (~4 µm) orifice diameter. The bigger results in polydisperse distribution with satellite droplets while small nozzle produces highly monodisperse droplet depending on types of jet mode. (b) Sketch of oscillating-jet mode and cone-jet mode.*

for printing is more convenient than in case of oil where viscous force (or friction force) applies a shear stress at the interface. However, as compare to air, oil medium provides more stable water droplets with less possibilities of generating satellite droplets. Hence, we investigated the effect of viscosity of oil-phase on the average droplets size. The different viscosities of the oil-phase were prepared by mixing Tegosoft DEC, mineral oil and ABIL EM 90 in different ratio (see Table 1.2). I observed that average droplets size increased with increase in the viscosity as shown in figure 1.7.

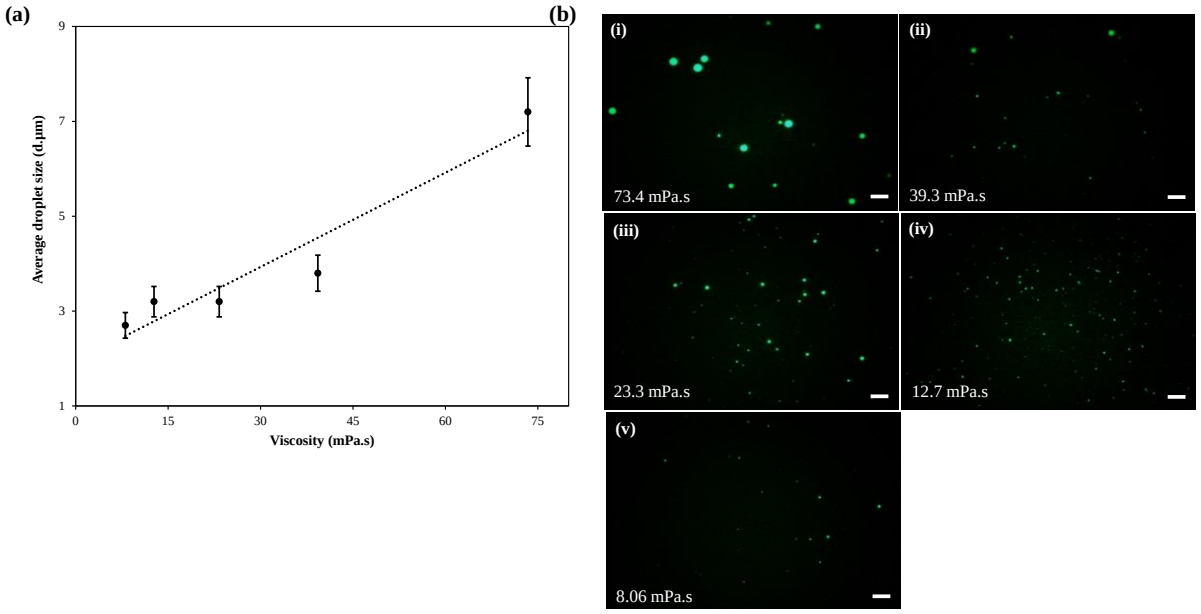


Figure 1.7: Effect of oil viscosity on the water-in-oil droplets size distribution *(a) Comparison of the viscosity of the oil/surfactant mixtures, prepared by changing the tegosoft DEC while keeping ABIL EM 90 and mineral oil in fixed ratio. All observations were carried out at bias voltage 50 V and frequency 100 Hz. (b) Fluorescent images of water-in-oil droplets in different viscosity of the oil-phase. As viscosity decreases from 73.4, 39.3, 23.3, 12.7, 8.06 mPa.s droplets size also decreases as shown in figure (a) – (e), respectively. Scale bar 35 µm.*

Thirdly, it is very obvious to speculate the dependency of voltage and frequency on the droplet size distribution because of the fact that electrospray happens when voltage is applied to the liquid and frequency of the applied voltage can affects the forces acting at interfaces. Similar to flow rate factor in electrohydrodynamic-inkjet[23, 24], voltage also plays the same role in electrostatic inkjet. An increase in applied bias voltage increases the amount of liquid at the interface which results in larger droplets size. Consistent to this, I observed a shift towards larger droplets size while increasing the bias voltage from 100 V to 1000 V at constant frequency of 100 Hz (see figure 1.8a). However, a higher frequency at higher voltage reinforce the smaller droplets size because of the more disturbance at the interface of the aqueous and oil phase which results in small droplets as compare to low frequency (see figure 1.8b). With these observations, the resulting optimal parameters for monodisperse and smaller droplets

were set as follows: nozzle with 4 µm of orifice diameter, a less-viscous (near 8 mPa.s) mixture of oil/surfactant mixture and electrostatic conditions using higher voltage (1000 V_{bias}) and frequency (1000 Hz).

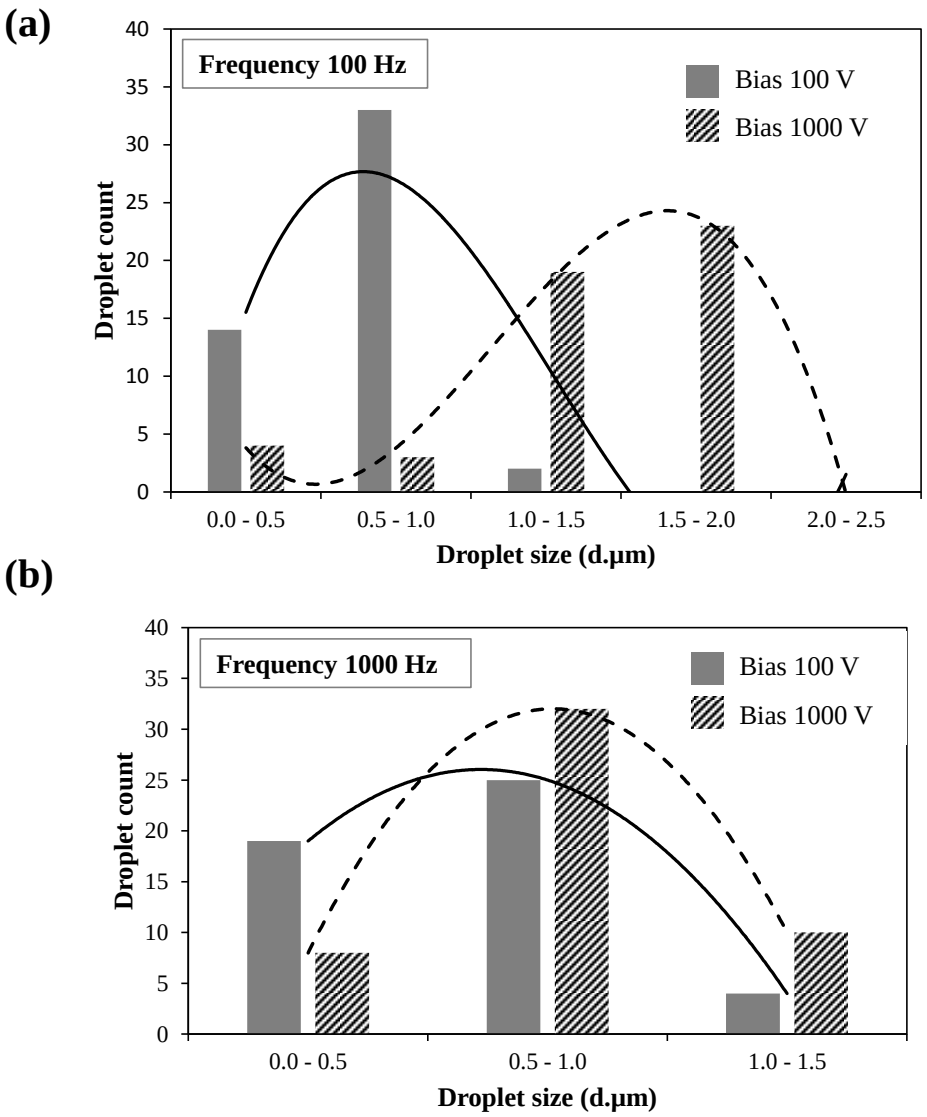


Figure 1.8: Droplet size distribution with change in applied bias voltage and frequency. *High voltage refers to large amount of aqueous phase coming out from nozzle while frequency slightly varies the droplets size. (a) Droplet size increases with increase in voltage from 100 V to 1000 V at 100 Hz frequency. (b) Higher frequency (1000 Hz) reinforce the small size at higher voltage (1000 V).*

1.4 Conclusion and perspectives

I demonstrated the engineered sub-femtoliter-scale aqueous droplet or gel bead compartment with facile approach using electrospray for bulk production of robust artificial cell-like compartments. This electrospray system can generate nearly 10^5 droplets per second and this number can be increased by two order if the frequency of the applied voltage changes. Also the electrospray parameters like nozzle size and continuous phase (oil) viscosity played crucial role for monodisperse aqueous droplets generation.

In perspective, I am considering this high throughput sub-femtoliter droplet generation system for minimal biochemical reaction scale at maximum and accelerated reaction products. Such system are also expected to facilitate not only improvements in the sensitivity-issue in top-down artificial cellular system but also a colossal leap in directed molecular evolution methodologies.

References

1. Kelly, B. T., Baret, J. C., Taly, V. & Griffiths, A. D. Miniaturizing chemistry and biology in microdroplets. *Chem Commun* **18**, 1773-1788 (2007).
2. de Souza, T. P., Stano, P. & Luisi, P. L. The minimal size of liposome-based model cells brings about a remarkably enhanced entrapment and protein synthesis. *Chembiochem* **10**, 1056-1063 (2009).
3. Luisi, P. L. & Stano, P. Synthetic biology minimal cell mimicry. *Nat Chem* **3**, 755-756 (2011).
4. Okano, T., Matsuura, T., Suzuki, H. & Yomo, T. Cell-free protein synthesis in a microchamber revealed the presence of an optimum compartment volume for high-order reactions. *Acs Synth Biol* **3**, 347-352 (2014).
5. Lu, W. C. & Ellington, A. D. In vitro selection of proteins via emulsion compartments. *Methods* **60**, 75-80 (2013).
6. Tawfik, D. S. & Griffiths, A. D. Man-made cell-like compartments for molecular evolution. *Nat Biotechnol* **16**, 652-656 (1998).
7. Griffiths, A. D. & Tawfik, D. S. Miniaturising the laboratory in emulsion droplets. *Trends Biotechnol* **24**, 395-402 (2006).
8. Packer, M. S. & Liu, D. R. Methods for the directed evolution of proteins. *Nat Rev Genet* **16**, 379-394 (2015).
9. Aharoni, A., Amitai, G., Bernath, K., Magdassi, S. & Tawfik, D. S. High- throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. *Chem Biol.* **12**, 1281–1289 (2005).
10. Katepalli, H. & Bose, A. Response of surfactant stabilized oil-in-water emulsions to the addition of particles in an aqueous suspension. *Langmuir* **30**, 12736-12742 (2014).

11. Miller, O. J. *et al.* Directed evolution by in vitro compartmentalization. *Nat Methods* **3**, 561-570 (2006).
12. Fallah, A. A., Baret, J. C., Ryckelynck, M., & Griffiths, A. D. A completely *in vitro* ultrahigh-throughput droplet based microfluidic screening system for protein engineering and directed evolution. *Lab on a Chip* **12**, 882–891 (2012).
13. Abate, A. R. , Thiele, J., Weitz, D. A One-step formation of multiple emulsions in microfluidics. *Lab Chip* **11**, 253–258 (2011).
14. Ma, F., Xie, Y., Huang, C., Feng, Y. & Yang, G. An improved single cell ultrahigh throughput screening method based on in vitro compartmentalization. *PLoS ONE* **9**, 2:e89785 (2014).
15. Guo, M. T., Rotem, A., Heyman, J. A. & Weitz, D. A. Droplet microfluidics for high-throughput biological assays. *Lab Chip* **12**, 2146-2155 (2012).
16. Umbanhowar, P. B., Prasad, V. & Weitz, D. A. Monodisperse emulsion generation via drop break off in a coflowing stream. *Langmuir* **16**, 347-351 (2000).
17. Shim, J. U. *et al.* Ultrarapid generation of femtoliter microfluidic droplets for single-molecule-counting immunoassays. *Acs Nano* **7**, 5955-5964 (2013).
18. Biyani, M., Osawa, T., Nemoto, N. & Ichiki, T. Microintaglio printing of biomolecules and its application to in situ production of messenger ribonucleic acid display microarray. *Appl Phys Express* **4**, 047001-047003 (2011).
19. Rondelez, Y. *et al.* Microfabricated arrays of femtoliter chambers allow single molecule enzymology. *Nat Biotechnol* **23**, 361-365 (2005).
20. Kinpara, T. *et al.* A picoliter chamber array for cell-free protein synthesis. *J Biochem* **136**, 149-154 (2004).
21. Okano, T., Matsuura, T., Kazuta, Y., Suzuki, H. & Yomo, T. Cell-free protein synthesis from a single copy of DNA in a glass microchamber. *Lab Chip* **12**, 2704-2711 (2012).

22. Yingnan, S., Xiaodong, C., Xiaoguang, Z., Jinbiao, Z. & Yude, Y. Droplet-in-oil array for picoliter-scale analysis based on sequential inkjet printing. *Lab Chip* **15**, 2429–2436 (2015).
23. Park, J.-U. et al. Nanoscale, Electrified Liquid Jets for High-Resolution Printing of Charge. *Nano Letters* **10,** 584-591 (2010).
24. Pratikkumar, V. R. & Naresh C. M. A review on electrohydrodynamic-inkjet printing technology. *International Journal of Emerging Technology and Advanced Engineering* **4**, 174-183 (2014).

Study on the use of immersed electrospray for controlled cell-free gene expression in minimal IVC system

CHAPTER 2

~ Highlights ~

- Successful GFP expression in femtoliter water-in-oil droplets using immersed electrosprays.
- Time course study of GFP expression in smaller droplets (1.8 fL) revealed early GFP saturation (15 min).
- Co-expression of GFP and mCherry at extremely diluted concentration explains “super-concentration effect” in minimal IVC.

2.1 Introduction

2.1.1 Cell-free protein synthesis

Cell-free protein synthesis (CFPS) exploits the translational machinery of cellular protein synthesis to produce protein of interest with the help of energy substrates, polymerases, amino acids, nucleoside triphosphate and salts[1]. CFPS drastically improves the development of engineered proteins[2, 3] and has been used as research tool in applied biology as well as fundamental studies[4-7]. Based on CFPS several astonishing methods in directed evolution have been developed such as ribosome display[8, 9], mRNA display[10], and *in vitro* compartmentalization (IVC)[11]. These cell-free display systems have overcome the limitations associated with *in vivo* methods such as genotype-phenotype coupling efficiency, intermolecular interaction (noise) and precise high throughput screening methods having broader library size. Recently Swartz *et al* developed the micro bead display technology using IVC with fluorescence-activated cell sorting (FACS)[12].

The minimal size encapsulation of molecules?

By above understanding it has been clearly depicted that CFPS system is not only for rapid production of desired proteins but also serves as epicenter of many methods in the field of directed evolution. Further advancement in this system was focused on miniaturization of the compartment, encapsulation and distribution of biomolecules/solutes inside cell free system. Miniaturization of the compartment leads to the enhanced interaction between molecules which in turn increases the protein synthesis or enzymatic activity. Standard Poisson distribution method has been used for distribution of molecules inside compartment which is a relevant issue in understanding of the origin of life. First most to realize above query, it is very necessary to build a minimal size compartment to entrap the minimal number of biomolecules (proteins or nucleic acids) which can be helpful for understanding the cellular processes more accurately.

In the league of miniaturization, micro array technology have been used at femtoliter scale and proved to be efficient method for quantitative analysis[13] but it failed on the ground of IVC because of the non-uniform distribution of sample mixture, difficult to handle the $>10^{15}$ compartments for directed evolution and well defined solid array cannot mimic the cellular compartments. Furthermore piezoelectric inkjet printing technology has also used for manipulating the droplet size with precise volume control which proved to be upper hand over microfluidics technology. Recently reported[14] droplet in oil for picoliter scale analysis but piezo electric inkjet used till now has limitation of low throughput generation of droplets.

Many small size compartments have demonstrated the green fluorescent protein (GFP) expression, an easy demonstrable protein using PURE system[15]. Ueda and colleagues has developed the commercially available Protein synthesis Using Recombinant Elements (PURE) system. There are about 80 different macromolecular species along with amino acids, nucleotides, etc. representing the minimal set of the translational machinery derived from E. coli. So to characterize the minimal size of compartment volume Lazzerini-Ospri *et al.* (2011) designed an experimental simulation considering the stochastic factors which affect the reaction progression. The liposomal vesicles of nanometer size was used for investigating the stochastic effect as well as other parameters affecting the protein expression. Since translation process comprises reactions having higher than first order kinetics which likely depends on reaction volume, so it might be possible that encapsulation governed by power-law distribution instead of Poisson statistics. A translation yield was predicted from a pool of smaller liposomes (attoliter range) which states that; protein synthesis yields maximum at 10^{-16} L (840 nm diameter) and 10^{-17} L (275 nm diameter) with Poisson distribution and power-law statistics respectively. So a clear understanding can be acquired that effective protein synthesis cannot be measured at smaller volume than 10^{-17} L. This has been explained on the basis of "super-

concentration" effect[16] which defines the overcrowding of the all protein synthesis components leads to rapid consumption of amino acids and energy substrates.

2.1.2 Droplet based immersed electrospray cell-free expression

Super-fine inkjet (SIJ) technology is based on electrostatic principle which acts on applied pulsed DC voltage to the nozzle having ink which disintegrate into jet stream by well-known Taylor's cone and Rayleigh instability theorem. Inkjet print the solution by desolvation of ink droplets and deposition of solutes on substrate in medium (air) as shown in Figure 2.1a. In the immersed electrospray[17] formation of droplets take place due to presence of oil layer containing

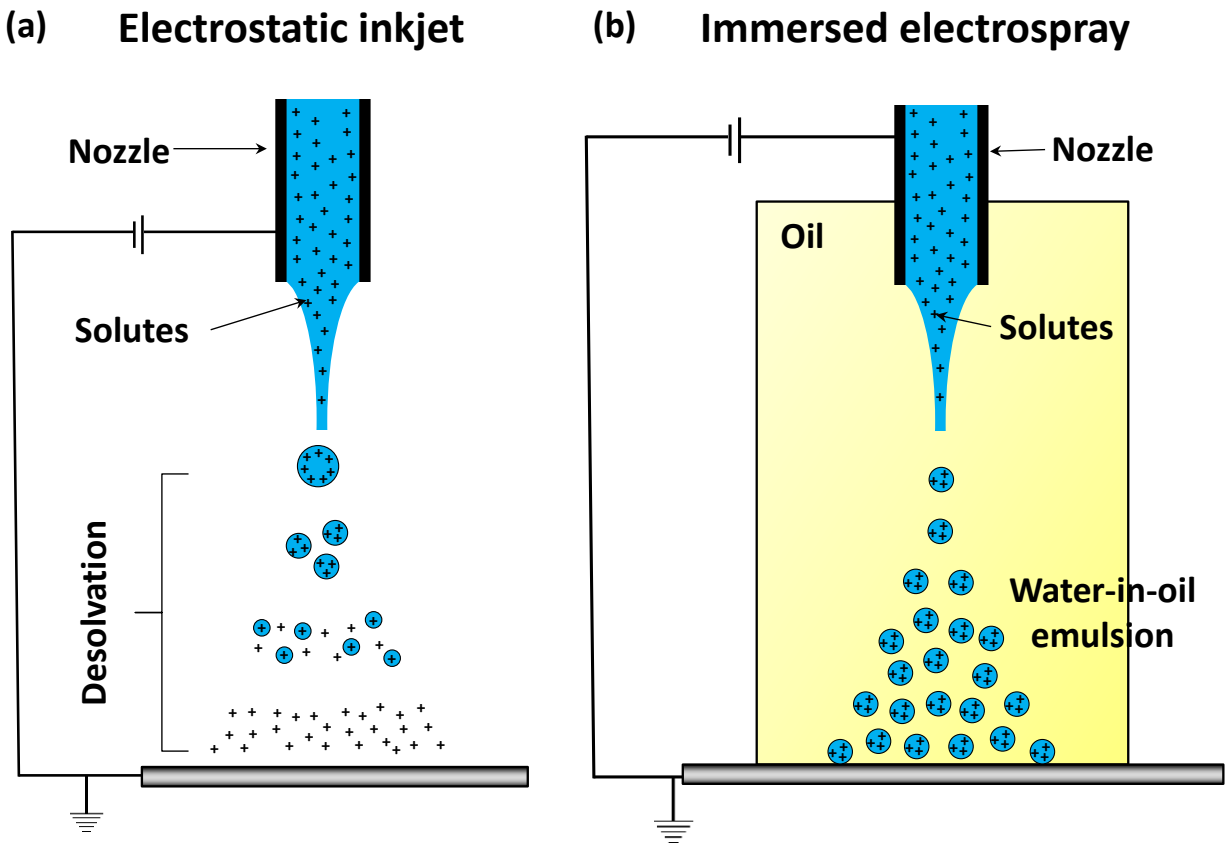


***Figure 2.1: Comparison between electrostatic inkjet and immersed electrospray**. (a) Inkjet printing of solutes on the substrates by the process of desolvation. (b) The encapsulation of molecules by the immersed electrospray based water-in-oil-droplets.*

surfactant which prevents the desolvation phenomenon (see Figure 2.1b). Formation of droplets occurred due to the encapsulation of molecules along with solvents, this encapsulation is stochastic in behavior and totally governed by Rayleigh instability which leads to jet stream of

droplets. Since study of cell-free expression have been most applied for IVC generated system, so it seems to be very interesting to investigate the dynamics of CFPS using immersed electrospray technology based IVC.

~ Objective ~

To carryout protein synthesis (GFP expression) in water-in-oil emulsion system using immersed electrospray technology. Also to precisely observe the time course study of GFP expression in different size droplets. Since inkjet system produce water droplets of nanometer size also so to realize the super-concentration effect, I aimed to carry out co-expression of GFP and mCherry proteins.

2.2 Methods

2.2.1 Electrospray setup for GFP expression

All the set up and procedure are same as described in the previous chapter. Here the water-in-oil droplet generated containing cDNA along with PURE system for study of protein expression.

2.2.2 In vitro protein expression in droplets

The templates used for in vitro expression (coupled transcription/translation) were prepared by PCR amplification using plasmids encoding the GFPuv4 and mCherry [Clontech] followed by purification using a QIAquick PCR purification kit [Qiagen]. The DNA concentrations were determined from the absorbance at 260 nm using NanoDrop 2000 UV-Vis spectrophotometer. A 2.5 µl of purified cDNA template encoding green fluorescent protein [GFPuv4, the brightest variant of GFPuv] or mCherry was gently mixed with a commercialized coupled transcription/translation system [PURExpress, NEB] containing solution-A (5 µl), solution-B (3.75 µl). To avoid the loss of template DNA and other components of cell-free system by possible adsorption onto the inner glass surface of nozzle, the nozzle was pre-treated by rinsing with an aliquot of the reaction mixture followed by pipetting the fresh solution in the glass nozzle for generation of droplets in oil using electrostatic inkjet system. The water-in-oil droplets were generated in 100 µl of oil/surfactant mixture at 50 V and 100 Hz. All the droplets were transferred to an Eppendorf vial and incubated at 37ºC for 2 hours for cell free expression. For microscopic studies, confocal laser scanning microscope (CLSM) [Olympus FluoView 1000 spectral based] was used to capture the bright field and fluorescence images of the droplets. A laser light of Ar 488 nm wavelength was used to excite the GFP and observed through Alexa fluor 488 green dye filter. All the images were captured represents the equatorial section using 60× lens. For time course study above procedure was performed. Different

aliquots were prepared for time intervals. The viols containing droplets with cell free expression were quenched at specific time and observed under CLSM.

2.3 Results and discussion

2.3.1 Quantification of in vitro protein expression in sub-femtoliter droplets

As a first step in the demonstration of utility of super-fine electrosprays for IVC purposes, in which genes are compartmentalized and expressed, GFP synthesis inside femtoliter droplets was carried out by encapsulating GFP encoding gene and PURE system as a cell-free gene expression system in femtoliter droplets. GFP-cDNA of 35 nM concentration along with PURE system was taken in glass nozzle of 4 µm size and water-in-oil droplets were generated using electrosprays and incubated for 2 h at 37°C. As shown in Figure 2.2a, the fluorescence of GFP was successfully monitored using confocal microscopy. Since a negligible amount of fluorescence was detected in the droplets generated in the absence of GFP-cDNA, it seems obvious that the fluorescence were due to the accumulation of synthesized GFP inside the droplets. Fluorescent intensity graph in Figure 2.2b clearly depicts the difference between control (without template) and sample (with template). The smallest droplet size we observed in which GFP was expressed is 0.81 µm in diameter that corresponds to a volume of 0.3 femtoliter. In order to further evaluate whether applied high voltage influence the biological activity or behavior of biomolecules, pre-synthesized GFP were electrosprayed droplets (bulk synthesis; so-called ‘off-droplet’ synthesis) and compared the fluorescence intensity of these droplets with the droplets electrosprayed using GFP encoding gene and in vitro gene expression system (so-called ‘on-droplet’ synthesis).

GFP fluorescence increases with the size of droplets and remained near to identical in smaller

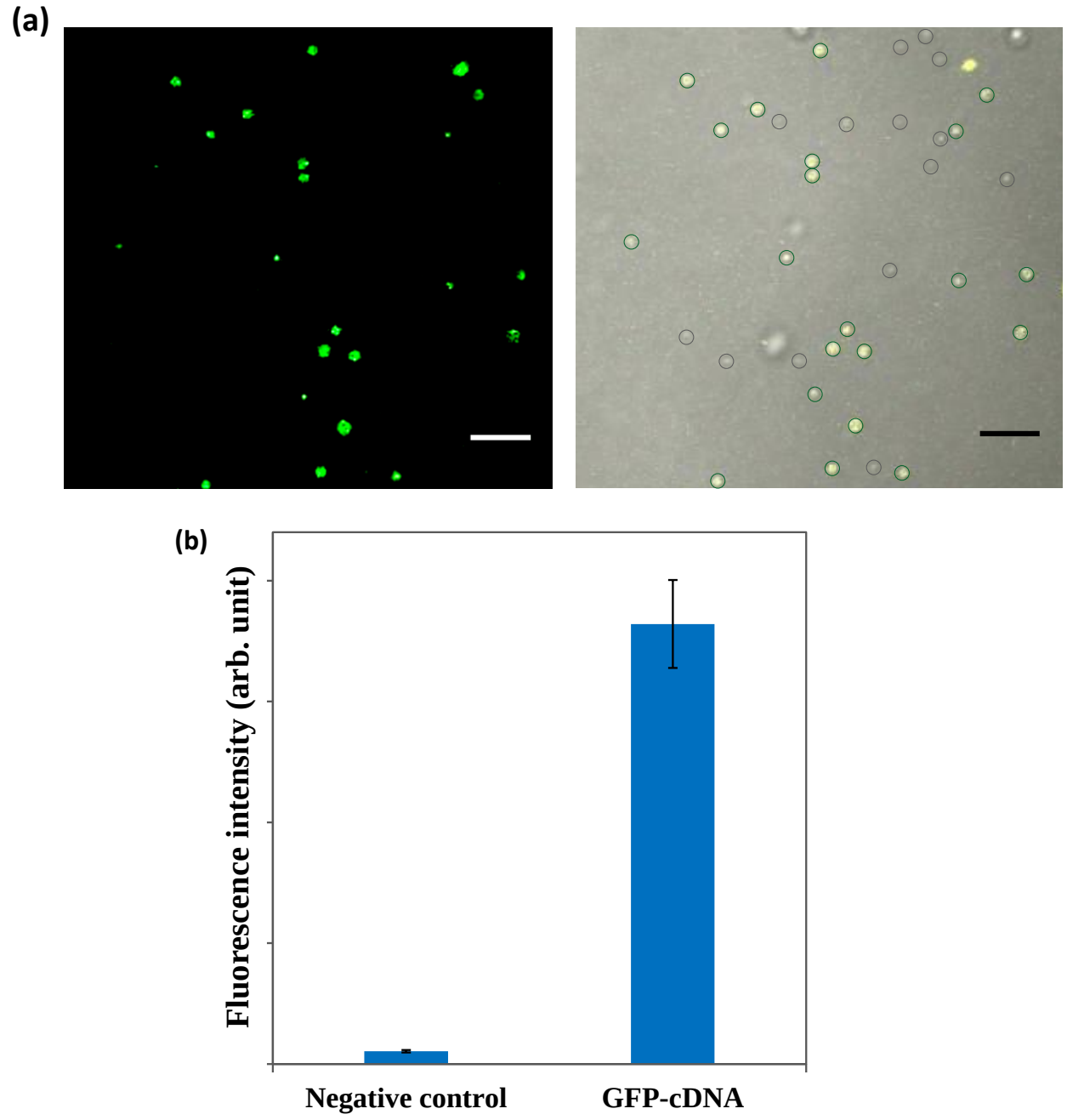


Figure 2.2: In vitro protein expression in sub-femtoliter water-in-oil droplets generated by electrospray*. (a) The figure shows a confocal fluorescence image (left) and the merge image with the corresponding bright field image for fluorescence of Green Fluorescent Protein (GFP) synthesized in femtoliter water-in-oil droplets. Green and grey encircled droplets represent GFP and empty, respectively. (b) Graph showing fluorescent intensity of with and without (negative control) template DNA for GFP synthesis. Scale bar: 15 µm.*

volumes for 'on-droplet' and 'off-droplet' synthesis (see Figure 2.3) which suggest that bio-electrospraying had no effect on protein viability nor the ability of in vitro gene expression

system to synthesize functional protein and thus it is reasonable to neglect any adverse influence of electrosprays onto the efficiency of protein expression for IVC using high voltage in the designed system. Moreover, the production in 'on-droplet' GFP synthesis in larger volumes was comparatively higher than in 'off-droplet' which is similar to the observation reported earlier as confinement of PURE system components in microdroplets can accelerate the turnover reaction of protein synthesis. In our earlier work using microreactor array chip, we observed that the synthesis of GFP inside a microchamber with the diameter and depth of 4 µm that corresponds to a volume of 50 femtoliter reached a plateau after approximately 90 min of synthesis, which is identical with the bulky synthesis at µl-scale volume. Very interestingly, this maturation level was observed to reach a plateau earlier when synthesis occurred inside a smaller fL-scale droplet (<50 fL). The time course study on GFP expression in different size of droplets, from 1.5 µm to 4.4 µm in diameter that corresponds to 1.8 fL to 44.3 fL volume, is presented in Figure 2.4. The increase of fluorescence of GFP stops earlier (within 15 min after the start of protein expression) in the smaller volumes which appears after

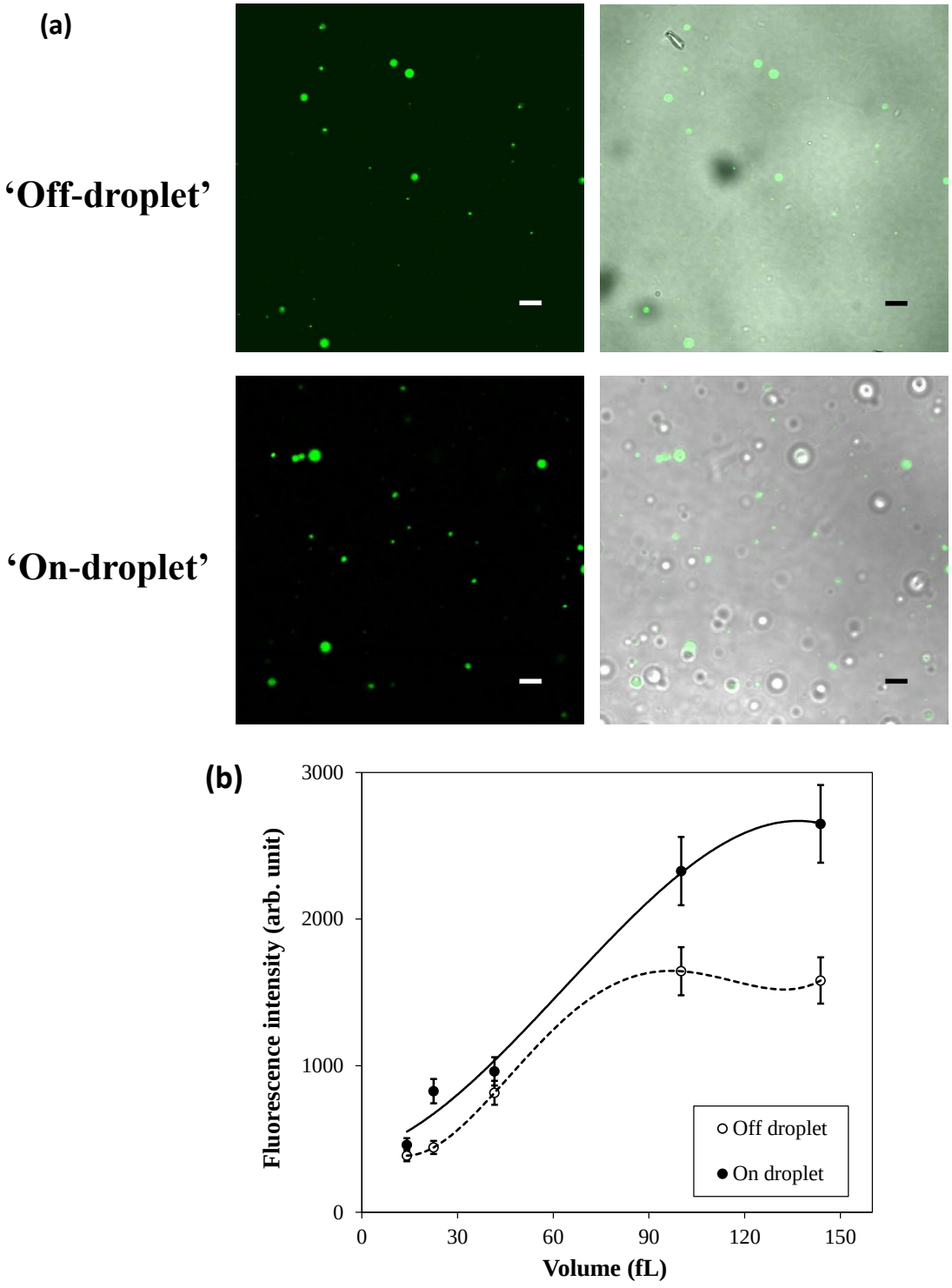
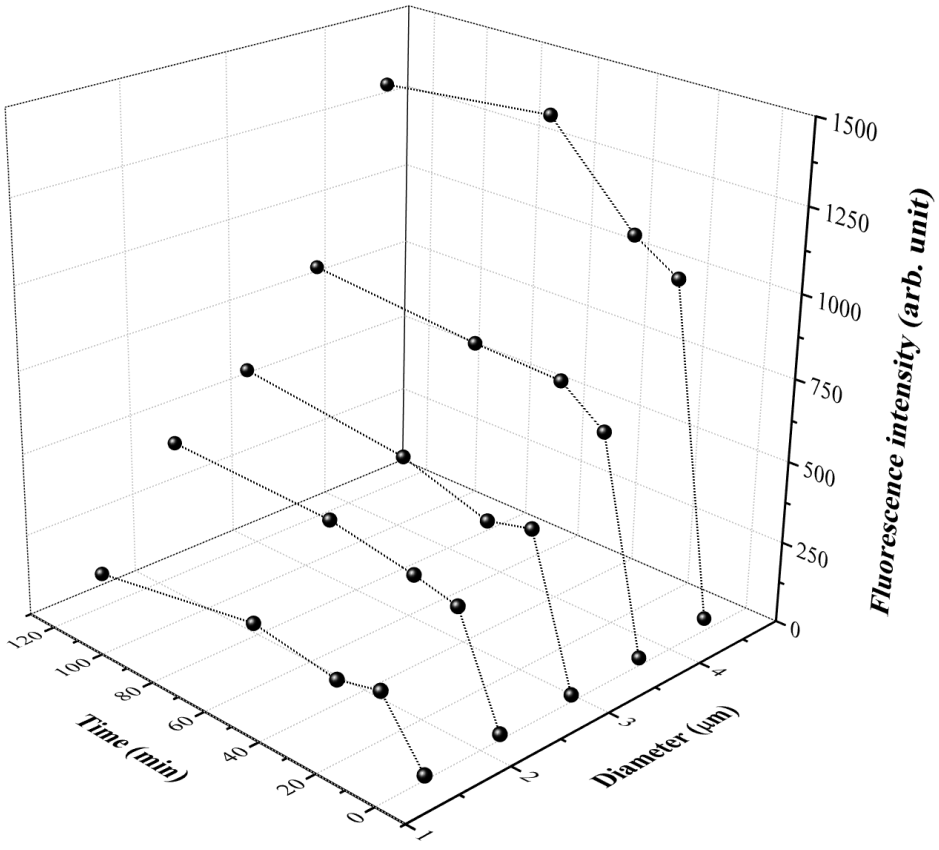


Figure 2.3: *Effect of voltage on DNA and GFP protein. (a) GFP was expressed in bulk using the PURE system before being electrosprayed via a glass nozzle at 50 V and 100 Hz (Off-droplet; top image). Later, GFP-encoding cDNA mixed with the PURE expression system was electrosprayed and incubated for 2 h at 37°C (On-droplet; bottom image). Confocal fluorescence microscopic images alone (left) and merged with the corresponding brightfield images (right) are shown. Scale bar: 15 μm. (b) A comparison of the time courses of GFP expression between off-droplet and on-droplet conditions.*

30 min in the bigger volume. However, the peak of fluorescence intensity was gradually increased with respective to the increase in the volume of droplets. The result of available

PURE system components (e.g., nutrient and energy molecules) for further turnover reactions which is obviously less in smaller droplets because smaller volume have fewer components. As a result, this system can be used to access quantitative information on the rate-determining step of cell-free protein synthesis in femtoliter droplets.

I then attempted to evaluate the expression of GFP in femtoliter gel beads produced by our system. A gel matrix retards diffusional migration of components of cell-free system (about 80 different macromolecular species in the PURE system) and thus can accelerate the reaction because of improved gene stability, higher local concentration and a faster enzyme turnover rate. A hydrogel system has been reported to improve efficiency of protein synthesis about 300



times higher than solution-based system. As expected, we also observed higher fluorescence

Figure 2.4: Time course study of in vitro protein expression in sub-femtoliter water-in-oil droplets generated by electrospray*. (Time courses for the synthesis of GFP in 5 differently sized droplets (1.8 fL, 5.5 fL, 13 fL, 25.5 fL, and 44.3 fL). The fluorescence of GFP reached a plateau at an earlier time (<15 min) in smaller droplets. The results represent the average data from 176 different droplets.*

of GFP in agarose-in-oil gel beads than with the similar size of water-in-oil droplets

(unpublished data). This confirms that the described electrospray system will find many applications based on gel matrix. Very recently, the synthesis of the GFP inside w/o emulsion droplets was reported with the dependence on the droplet interface structure[19-20]. A significant effect of the types of oil/surfactant mixture was observed on the amount of active protein expression inside the droplets and thus such an interfacial phenomena at the membrane surface of droplet can be manifested by minimizing the droplet size using our system since the surface area to volume (S/V) ratio become larger with decreasing the size of droplets. Taken together, cell-free expression in droplets generated by our system can enables not only to control reactions in sub-femtoliter volume but also to study the dynamic effects of volume on protein synthesis.

2.3.2 "*Super-concentration effect"*

Above results show that electrostatic based inkjet system can provides a very facile platform to efficiently produce monodisperse water-in-oil or gel-in-oil droplets in bulk that can be very powerful for applications relying on highly miniaturized and controlled in vitro compartmentalization. After performing the system characterizations, we performed to quantify cell-free protein synthesis dynamics in extreme miniaturized droplets produced by this system at sub-femtoliter volume. A template DNA for GFP expression was extremely diluted, from nM-to-fM, in order to evaluate the effect of template concentration on rate of protein synthesis. Figure 2.5 shows the time course result in droplet size of near 5 µm (~65 fL) for both the concentrations of 35.75 nM and 35.75 fM. It is estimated that near 1300 copies of template DNA molecules can be encapsulated in one droplet at 35.75 nM concentration. Hence a further extreme dilution by order of six would provide encapsulation of near one copy of DNA molecule in one out of 10^3 droplets and thus can leads to a drop of fluorescence about 1000-

fold. Interestingly, it was not the case as both the concentrations showed an identical start of GFP synthesis in initial 15 min of reaction. These results are consistent with our earlier report on the effect of amount of DNA template on the production of GFP inside the microchamber array with diameter range between 20 to 100 μm where no significant difference was observed in the smaller microchambers. In order to further evaluate this phenomena, co-expression of two recombinant genes inside the femtodroplets was performed using two different extremely diluted concentrations (nM-to-fM). For this purpose, we used two fluorescent proteins GFP (green) and mCherry (red) which are commonly used to enable dual protein labelling and co-expressed in the cell-free system. An equal mixture of GFP-cDNA and mCherry-cDNA at 17.87 nM or 17.87 fM concentrations were mixed with PURE system and encapsulated in water-in-oil droplets by electrostray. A concentration of nM would give the encapsulation probability of both cDNAs in each droplet whereas fM concentration will provide either of one

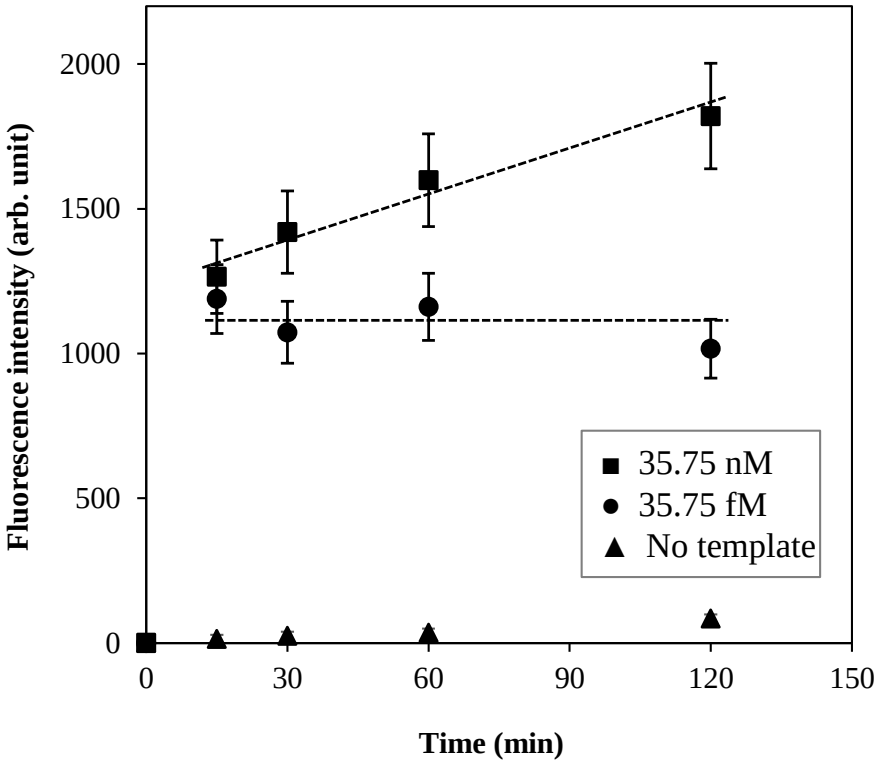


Figure 2.5: 'Super-concentration' effect in sub-femtoliter in vitro compartments. *The extreme template dilution effect, from ~1,300 copies of the GFP encoding gene per droplet (35.75 nM) to 1 copy per 10^3 droplets (35.75 fM). The data were acquired for droplets ~5 μm in size (65 fL).*

copy of cDNA in one out of 10^3 droplets. However, again we could not observe very significant difference in the results for both the two different concentrations except that more empty droplets (no fluorescence) were seen in fM concentration (see Figure 2.6). In comparison to green, a less number of red fluorescent droplets were observed which might be because GFP exhibits a very fast chromophore maturation time (almost 10-fold) than of mCherry. These observations support to this notion that encapsulation of biomolecules in extremely diluted condition, i.e., 17.87 fM cDNA concentration, tends to follow the 'solute entrapment' or 'super-concentration' effect characterized by many empty droplets and a very few (less than 8%) with either green/red or yellow (both) fluorescence of the total number of droplets counted.

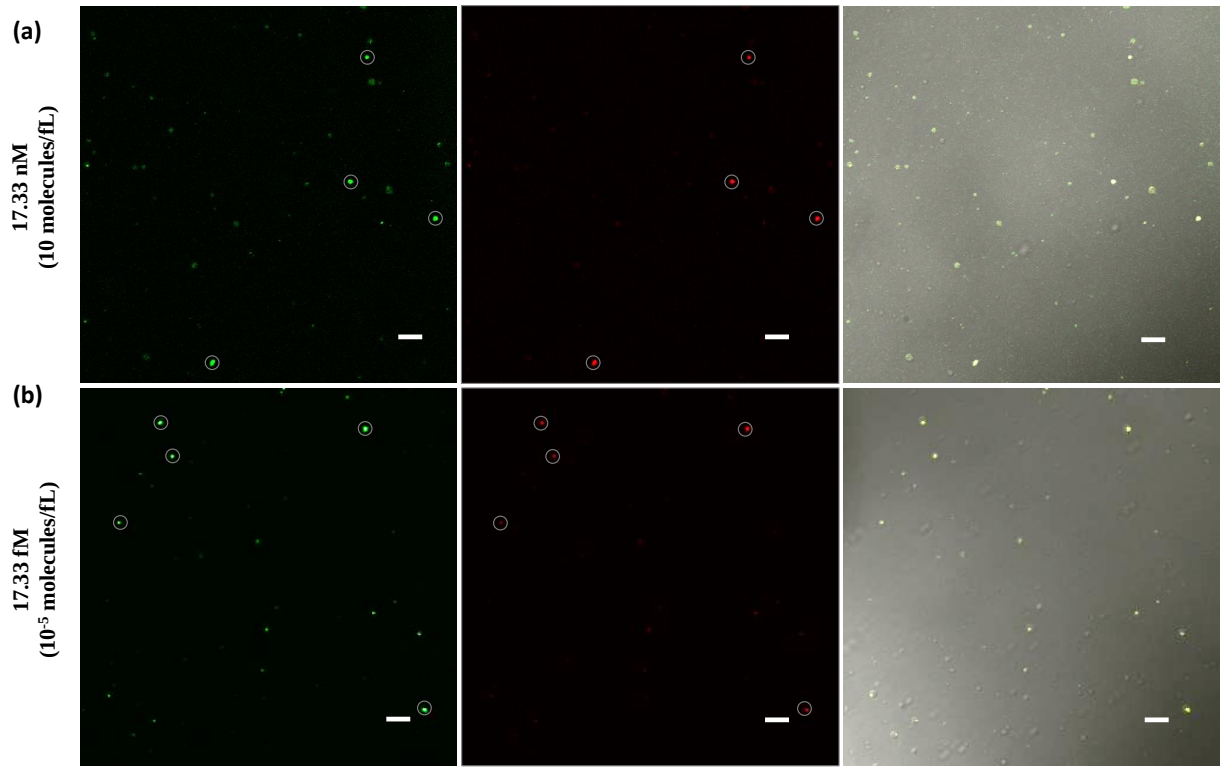


Figure 2.6: Co-expression of GFP and mCherry inside water-in-oil droplets at 2 different DNA template concentrations*. Fluorescence confocal microscopic images were captured using an Alexa Fluor 488 filter for GFP (left) or an Alexa Fluor 594 filter for mCherry (center); the merged images with the corresponding bright field images are shown at the right. Encircled droplets represent the co-expression of both GFP and mCherry, which appeared as a yellow color in the merged image. Scale bar: 15 μm.*

This suggests that extreme miniaturized femtoliter compartments generated by our system can be useful to induce 'super-concentration' effect and thus can lead to produce a remarkable rate acceleration effect in a complex biochemical reaction in sub-femtoliter IVC-based artificial cellular system.

2.4 Conclusion and perspectives

This chapter describes a facile approach using electrospray for bulk production of robust artificial cell-like compartment at minimal biochemical reaction scale for maximum productivity. I showed that engineered sub-femtoliter-scale aqueous droplet successfully expressed the GFP and mCherry even with very small concentration. Furthermore time scale study of GFP expression reveals that smaller volume droplets reached early saturated with respect to large size droplets.

In perspective, this high throughput system of small droplet generation present a platform for understanding the minimal volume for precise investigation of complex reactions other than protein expression.

References

1. Nirenberg, M., W. & Matthaei, J., H. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc Natl Acad Sci USA* **47**, 1588–1602 (1961).
2. Katzen, F., Chang, G. & Kudlicki, W. The past, present and future of cell-free protein synthesis. *Trends Biotechnol* **23**, 150–156 (2005).
3. Swartz J. Developing cell-free biology for industrial applications. *J Ind Microbiol Biotechnol* **33**, 476–485 (2006).
4. Goerke, A., R. & Swartz, J., R. Development of cell-free protein synthesis platforms for disulfide bonded proteins. *Biotechnol Bioeng* **99**, 351–367 (2008).
5. Kanter G, Yang J, Voloshin A, Levy S, Swartz JR, Levy R. Cell-free production of scFv fusion proteins: an efficient approach for personalized lymphoma vaccines. Blood **109**, 3393–3399 (2007).
6. Yang, J. et al. Rapid expression of vaccine proteins for B-cell lymphoma in a cell-free system. *Biotechnol Bioeng* **89**, 503–511 (2005).
7. Zawada, J., F.et al. Microscale to manufacturing scale-up of cell-free cytokine production—a new approach for shortening protein production development timelines. *Biotechnol Bioeng* **108,** 1570–1578 (2011).
8. Mattheakis, L., C., Bhatt, R., R. & Dower, W., J. An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proc Natl Acad Sci USA* **91**, 9022–9026 (1994).
9. Zahnd, C., Amstutz, P. & Pluckthun, A. Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target. *Nat Methods* **4**, 269–279 (2007).
10. Roberts, R., W. & Szostak, J., W. RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci USA* **94**, 12297–12302 (1997).

11. Griffiths, A., D. & Tawfik, D., S. Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. *EMBO J* **22**, 24–35 (2003).
12. Stapleton, J., A. & Swartz, J., R. Development of an in vitro compartmentalization screen for high-throughput directed evolution of [FeFe] hydrogenases. *PLoS One* **5**, e15275 (2010).
13. Okano, T., Matsuura, T., Kazuta, Y., Suzuki, H. & Yomo, T. Cell-free protein synthesis from a single copy of DNA in a glass microchamber. *Lab Chip* **12**, 2704-2711 (2012).
14. Yingnan, S., Xiaodong, C., Xiaoguang, Z., Jinbiao, Z. & Yude, Y. Droplet-in-oil array for picoliter-scale analysis based on sequential inkjet printing. *Lab Chip* **15**, 2429–2436 (2015).
15. Shimizu, Y., Kanamori, T. & Ueda, T. Protein synthesis by pure translation systems. *Methods* **36**, 299-304 (2005).
16. Lorenzo, L., O., Pasquale, S., PierLuigi, L. & Roberto, M. Characterization of the emergent properties of a synthetic quasi-cellular system. *BMC Bioinformatics* **13**, (Suppl 4) S9 (2012).
17. Park, J.-U. et al. Nanoscale, Electrified Liquid Jets for High-Resolution Printing of Charge. *Nano Letters* **10,** 584-591 (2010).

Development of 'Selection-in-a-fL Droplet' system for a rapid and bias-free directed molecular evolution

CHAPTER 3

~ Highlights ~

- Demonstration of high throughput selection system in femtoliter agarose-in oil gel beads of ~1.7 μm size.
- Successful co-compartmentalization of target immobilized beads inside agarose-in-oil gel beads.
- Fabrication of micro-mixing device to apply "selection pressure" for selection-in-a-fL droplet.

3.1 Introduction

3.1.1 Directed evolution

Directed evolution is evolution at small and fast scale in the laboratory which focused at the molecular level for specific properties from the pool of millions-trillions molecule. Directed evolution is composed of generation of diversity and selection of desired phenotypes. Since the driving force for the evolution is the result from the linkage of genotype-to-phenotype, then for precise selection of probe it is very logical to tune between library size and suitable selection procedure.

The different biomolecular display technologies have been introduced that mimic the natural evolution. Basically display technology refers to the "display" of the encoded gene of interest with linker like cell-free type or cell-based type. A typical display technique comprised of encoded gene, linker and displayed article. Over the past two decades many display technologies have been developed and contributed substantially in the field of molecular evolution and combinatorial chemistry for drug discovery. Cell based display technologies engage phage, bacteria and yeast to display[1] while the cell free module exploits the translational machinery as tool for linkage *viz.* ribosome[2], mRNA[3, 4], IVC[5], cDNA[6] and so-on[7, 8]. But the first and most widely adopted technology is phage display George Smith in 1985 for the discovery of drugs[9] by. This technology utilized the bacterial virus related filamentous phage, as cell based physical coupling of gene and its protein. In spite of this, cell-based technologies have some technical limitations like transformation efficiency of the host that limits the library size ~10^8, takes few days to weeks for sample preparation. While cell free display technologies are fast, easy to express the proteins, larger diverse library size, increased affinity between gene and its product. Herein, I am briefly mentioning the limitations of ribosomal display (first cell free display) and mRNA display. But cell-free display methods also have some limitations like

in ribosomal display[10, 11], there is non-covalent ribosomal linkage between mRNA and expressed peptide. Due to large size of ribosome, inter molecular interactions between different peptides is not controlled which could be result in misleading results. Then mRNA display was introduced that has covalent linkage of genotype to phenotype for the first time. It utilizes the puromycin linker (mimic of aminoacyl tRNA) between peptide and mRNA. It also suffers from limitation of pre-mature translation since puromycin can attached to peptidyltrasferase site and stops the expression[12]. Later to overcome these problems an evolutionary Rapid Panning Analysis System (eRAPANSY)[13, 14] has been developed by Nishigaki's group[15, 16], apart from it, many display technologies have been developed, Griffiths et al. in 1998 have introduced the idea of in vitro compartmentalization for the effective binding of genotype to phenotype which has proved as a revolutionary technique not for enzyme evolution but also for other biomedical applications[17-20].

3.1.2 Selection-in-a-tube vs selection-in-a-droplet

The generation of diversity has been developed over the period of time as described above but the method of selection are less developed which is makes it the limiting step in directed evolution. Selection process includes the screening of probe molecule of millions molecules of library which shows high affinity towards a specific target (biomarker). Conventional method of selection includes the bulk treatment of all the variants to the specific target in a test tube followed by several rounds of washing[21]. This type of selection method has drawbacks of probe-probe interactions, molecular crowding due to target concentration and non-molecular interactions as shown in figure 3.1. All these undesired interactions critically affect the selection pressure and could results in mismatching and misleading probe sequence. A suitable selection method for directed evolution should be highly specific to the desired property with

low limit of detection towards any enzymatic activity. The selection technique must be simple, robust and easy to reproduce. High throughput screening system provides a platform to increase the library size to billion/quadrillion for finding the best variant towards target molecule.

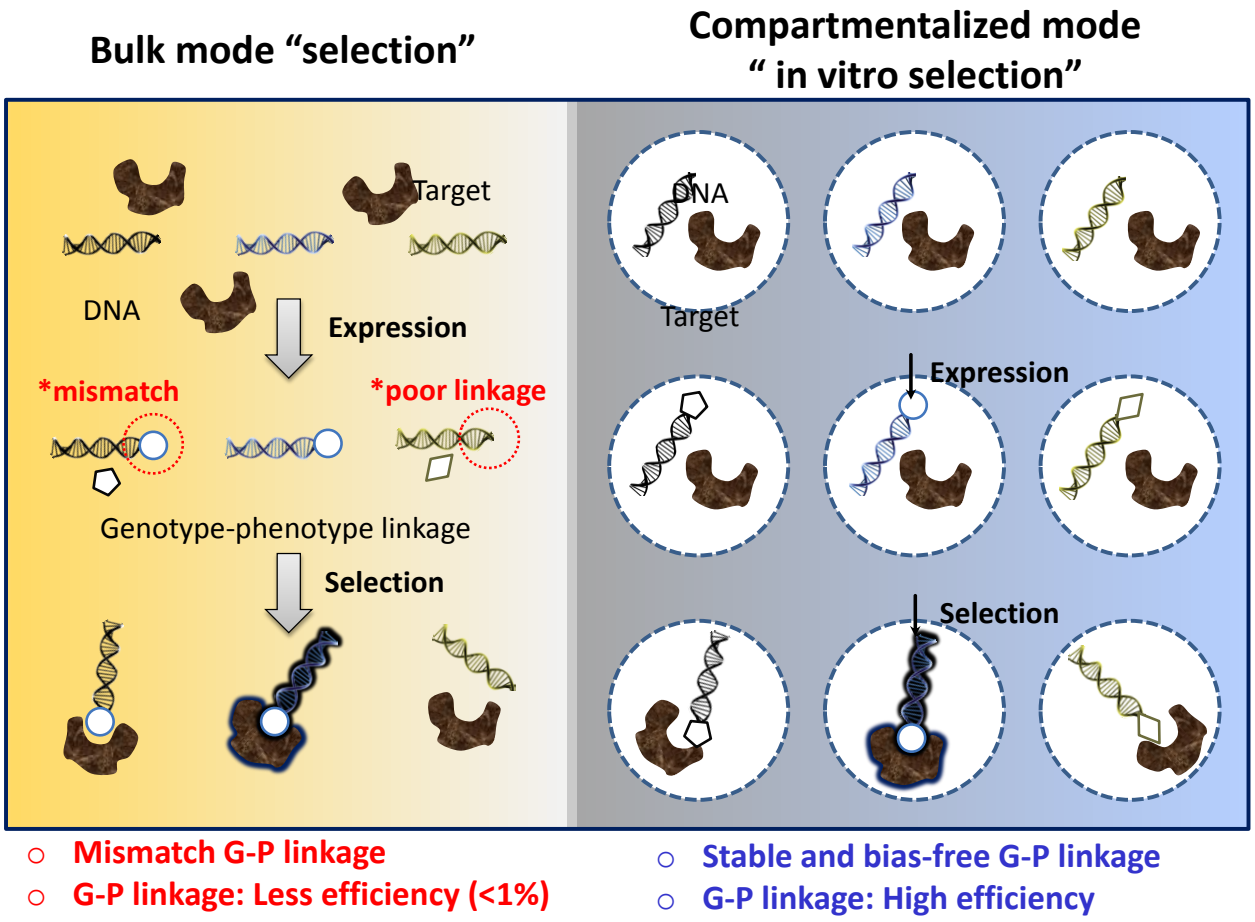
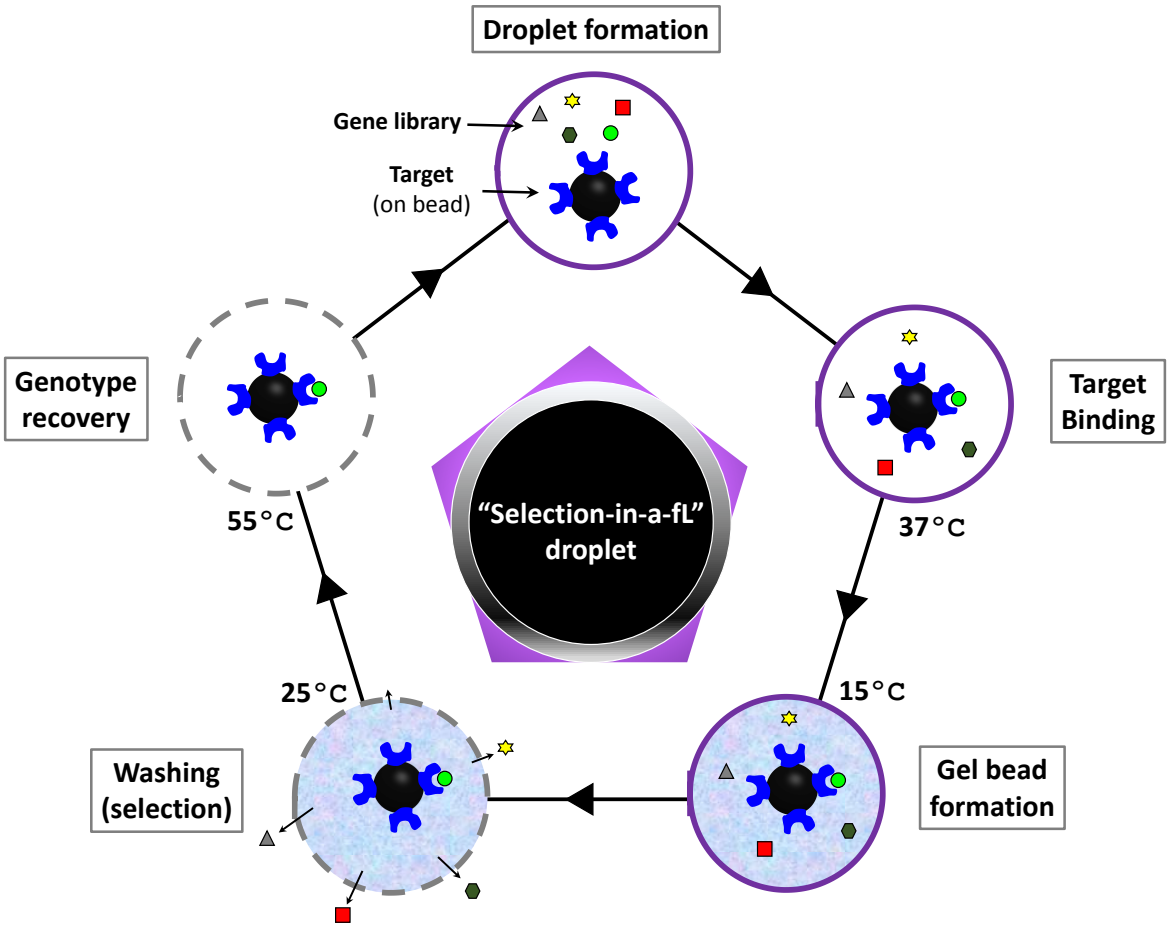


***Figure 3.1**: Comparison between conventional bulk selection method and selection-in-a-fL droplet. Explaining the limitations of conventional method and advantage of selection in droplets.*

~ Objective ~

With all the pros and cons of above discussed selection method and display technology, I aimed to develop an ultrahigh throughput system termed as "selection-in-a-fL droplet" which can overcome the problems associated with conventional selection methods (see Scheme 1).



***Scheme 1**: Cartoon demonstration of selection-in-pL droplet method by ultralow agarose gel beads-in-oil using immersed electrospray.*

3.2 Methods

3.2.1 Agarose-in-oil droplet using immersed electrospray setup and procedure

The agarose-in-oil droplet generation system was created by using immersed electrospray using super-fine inkjet technology [SIJ technology, Japan] as described in previous chapter. Ultralow agarose solution (1.0%) was used for encapsulation of cDNA and components of the PURE system. Agarose solution was melted over 60ºC and after entrapment by agarose droplets using electrostatic inkjet transferred to 4ºC for 30 min for gelation. As previously described Tungsten wire as an electrode and same oil phase containing 50% ABIL EM 90, 36% tegosoft DEC, and 14% mineral oil were used. Since ultralow agarose has been used so before droplet formation, agarose solution, inkjet nozzle were kept at 60ºC for 30 minute while oil phase and collecting chamber were kept at 4ºC for 30 minute.

3.2.2 Transmission electron microscopy (TEM) observation

Agarose solution (1.0 %) with or without gene expression system was pipetted in glass nozzle of ~15 µm for generation of agarose-in-oil gel beads. On the voltage (500 V) application, agarose droplets in oil were generated by dipped nozzle following by a quick transfer of the oil chamber to 4ºC for 30 min for polymerization and conversion of agarose droplet in gel bead. For TEM observation, sample was prepared by washing the gel beads twice by acetone for removal of oil boundary and centrifuged at 1000 rpm for 2 min. The washed gel beads were then dropped onto a copper grid and dried at room temp in vacuum overnight. TEM images were recorded by H-7100 machine (Hitachi, Ltd.) operating at 100 kV.

3.2.3 In vitro protein expression in agarose-in-oil gel beads

Cell-free protein expression was also performed in agarose gel beads using immersed electrospray. A 5 µl of purified cDNA template encoding green fluorescent protein [GFPuv4, the brightest variant of GFPuv] was gently mixed with a commercialized coupled transcription/translation system [PURExpress, NEB] containing solution-A (10 µl), solution-B (7.5 µl) along with agarose solution (2.5 µl, 1.0%). As previously described droplets were generated using immersed electrospray and transferred to 4ºC for 30 min. After gelation all the droplets were transferred to an Eppendorf vial and incubated at 37 ºC for 2 hours for cell free expression. For microscopic studies, confocal laser scanning microscope (CLSM) was used to capture the bright field and fluorescence images of the droplets.

3.2.4 Design of microchip for washing of agarose gel beads

A PDMS microchip was prepared using photolithography technique. Photoresist SU8 3050 was used to create the mold on Si-substrate using UV rays. SU8 developer was used for removal of non-molded photoresist followed by isopropanol washing. Then PDMS monomer and catalyst were thoroughly mixed in ratio of 10:1 and degassed on vacuum and poured on the mold for casting microchip. Substrate was transferred at 75ºC for 90 min. for PDMS polymerization. Figure 3.2 shows the fabrication steps of microchip.

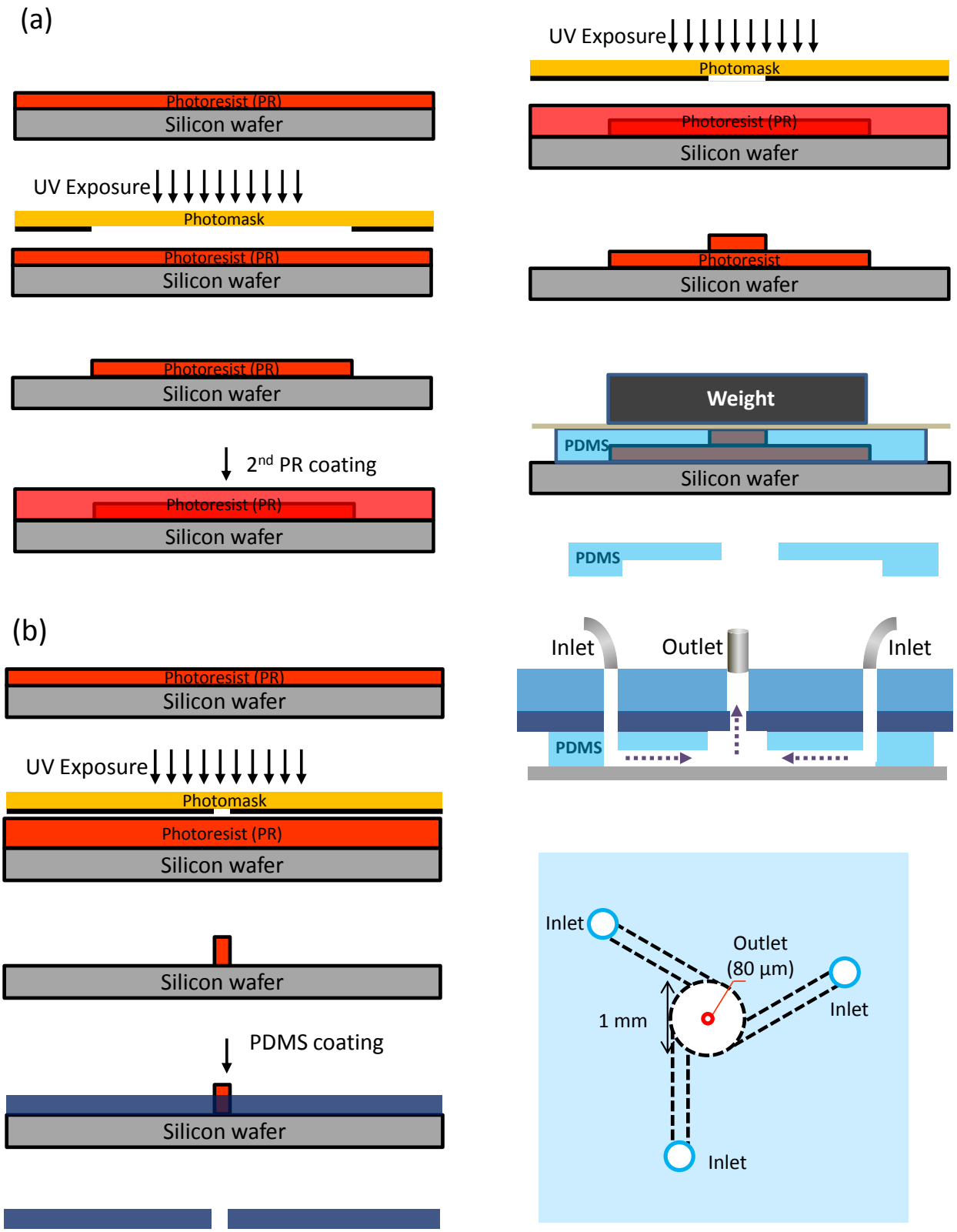


***Figure 3.2**: (a-b) Photolithographic steps of fabrication of PDMS microchip showing the 3D circular chamber for micro mixing using vortex technique.*

3.2.5 Encapsulation of fluorescent beads in agarose gel-in-oil

Fluoresbrite® Yellow Green Microspheres polystyrene beads (1µm) [Polysciences, Inc] was used to encapsulate in agarose droplets. Fluorescent beads solution (0.25 µl) was dissolved in ultralow agarose solution (0.5%) and pipetted in nozzle of ~65 µm. After agarose droplet formation in oil, sample was transferred to 4ºC for 30 minute for gelation and observed under inverted fluorescence microscope.

3.2.6 Washing for bias-free selection in agarose gel-in-oil

Washing steps are usually performed for the removal of unbounded molecules and the reactions inhibitors from the pool of millions of molecules targeted upon desire biomarker. For this purpose I planned to demonstrate the removal of Cy5-ssDNA and Biotin-4-Fluorescein (B-4-F) from the agarose gel beads. First most Cy5-ssDNA (5 µl, 100 pM) was mixed with ultralow agarose solution (5 µl, 1.0%) and agarose droplets were generated transferred at 4ºC for gelation. For washing steps, first acetone wash was performed to remove the oil phase and later isopropyl alcohol (IPA) was added to the agarose gel beads containing ssDNA and both steps were centrifuged at 1000 rpm for 2 minute. Similarly the removal of molecules like phosphates ions etc. which inhibits the reactions inside agarose gel was also demonstrated. Biotin-4-Fluorescein (1 µl) was added to ultralow agarose solution (99 µl, 0.5%) and droplets generation and gel beads formation took place as described previously. Same washing steps were followed for B-4-F molecules.

3.3 Results and discussion

3.3.1 Characterization of agarose-in-oil gel beads

In order to perform directed molecular evolution inside these tiny femtoliter droplets, it is desirable to exchange the solution or add multi-washing steps for removal of undesired molecules. However, this can be problematic with freestanding oil layer in water-in-oil droplets. Recently, the application of hydrogel matrix, such as agarose, inside the droplet is greatly anticipated as a matrix support for multistep processes, in which small molecules can be exchanged as the gel matrix can hold bigger and desired molecules such as proteins. Moreover, the effect of the support (hydrogel) matrix on enzyme activity can be more pronounced than relative to unconfined enzyme in water[26]. In this stream, the use of low-melting-temperature agarose provides a flexible sol-gel switching property. Therefore, in next I evaluated our system to produce monodisperse femtoliter hydrogel-in-oil gel beads that are difficult to obtain by using conventional microfluidic processes. I encapsulated ultra-low gelling agarose, which

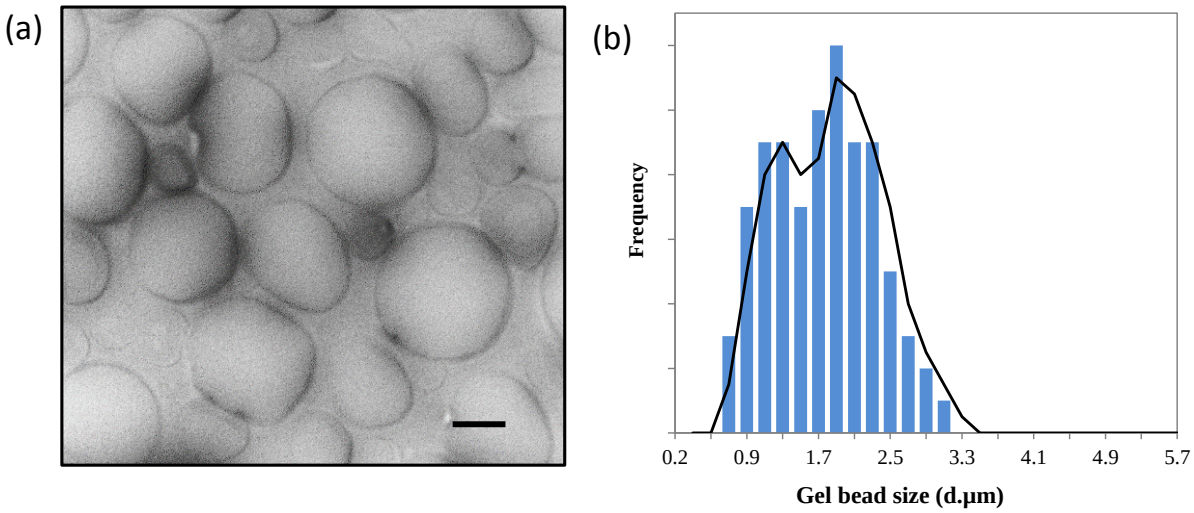


Figure 3.3*: (a) TEM image of agarose gel beads-in-oil. (b) Droplet size distribution obtained using a 15-µm nozzle orifice diameter and calculated by ImageJ. The mean diameter is 1.75 µm, with a volume of 2.8 fL. Scale bar: 1 µm.*

has a melting point of about 55°C and a gelling point below 15°C, with fluorescent dye inside water-in-oil droplets. Following to the electrospray, the generated agarose-in-oil droplets were

converted into agarose-in-oil gel beads upon cooling (from 37°C to 4°C). The oil boundary on agarose hydrogel bead can be removed by breaking the emulsion in the presence of the acetone (twice). Figure 3.3 shows the transmission electron microscopy (TEM) images of the agarose hydrogel beads produced by our system using a nozzle with 15 µm orifice diameter. Average size of the beads after removal of oil boundary was found to be ~1.75 µm in size that corresponds to a volume of ~2.8 fL.

3.3.2 GFP in vitro protein expression in agarose beads-in-oil

GFP encoded genes along with PURE system were encapsulated in agarose droplets-in-oil by nozzle (~65 µm) using immersed electrosprays. Then all the droplets were transferred to 4°C for 30 min for gelation, after that incubated for 2 h at 37°C for expression of GFP. Figure 3.4 demonstrate the GFP expression inside agarose beads-in-oil, different sized agarose beads were shown because of nozzle size and swelling of beads after polymerization. This results is just to validate the utility of immersed electrospray for agarose beads expression in oil since agarose

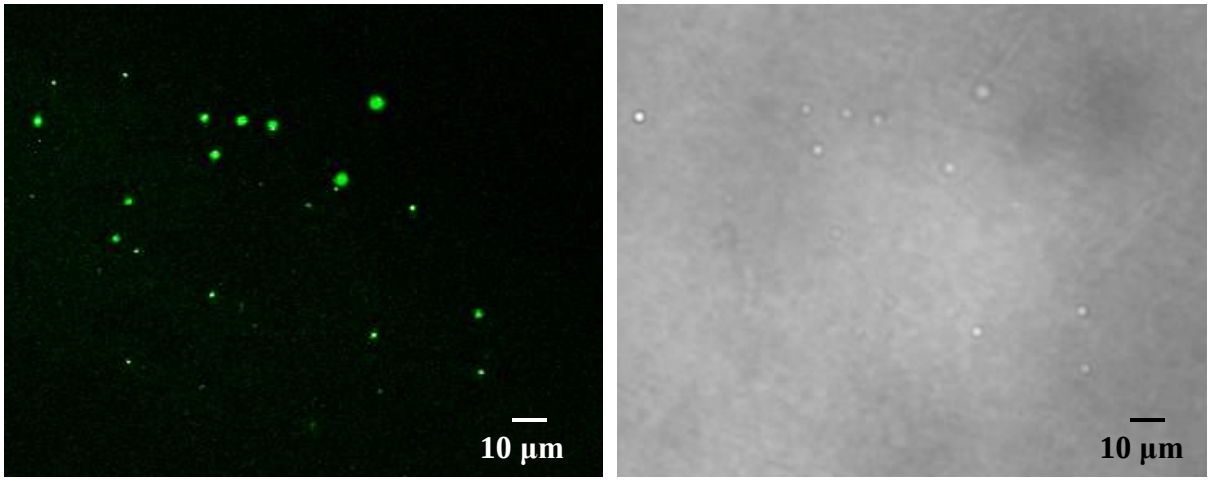


***Figure 3.4**: Successful expression of GFP in agarose-in-oil gel beads using immersed electrosprays. Nozzle size (~65 µm), bias (500 V), frequency (100 Hz).*

has been extensively used for biological samples and reactions like PCR, whole genome amplification.

3.3.3 Development of a microchip for improved bias free washing of agarose gel beads

Agarose gel beads (0.5%) was used for encapsulation of fluorescence beads, Cy5-ssDNA, and biotin-4-fluorescein and all the gel beads were centrifuged at 1000 rpm for 2 min for washing. But this process has limitations of damage of gel beads at high centrifugation speed since 0.5 % agarose gel doesn't have strength to stand with high centrifugal force. For better solute-solvent exchange a PDMS microchip has been fabricated using photolithography technique. As shown in Figure 3.5 circular trajectory of fluorescent particles confirmed the proper circulation of solvents inside trapping chamber which could be applied to demonstrates the mixing of solvents at low Reynolds number. This PDMS microchip can help in improved solute-solvent exchange without damaging the agarose gel beads.

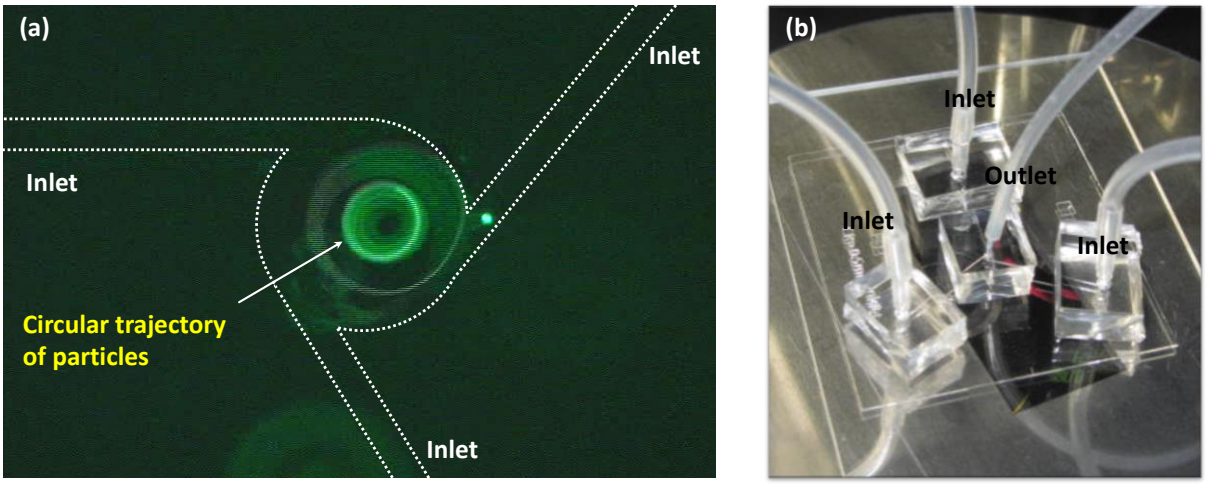


***Figure 3.5**: (a) A PDMS microchip showing the circular trajectory of the fluorescent beads in trapping chamber via vortex method. (b) Photograph of the fabricated microchip, this microchip can help in washing of agarose gel beads at low Reynolds number. Outlet at the center, circular chamber (~1 mm), flow rate (800 µl/min).*

3.3.4 Co-compartmentalization of fluorescent beads in agarose-in-oil gel beads

The immobilized target on the beads used for selection of desired probe molecule from the library of million molecules. So I demonstrated co-compartmentalization of the same size of fluorescence polystyrene beads in agarose gels. Figure 3.6 shows that single bead encapsulation of fluorescence beads in agarose-in-oil gel beads using inverted fluorescence microscope.

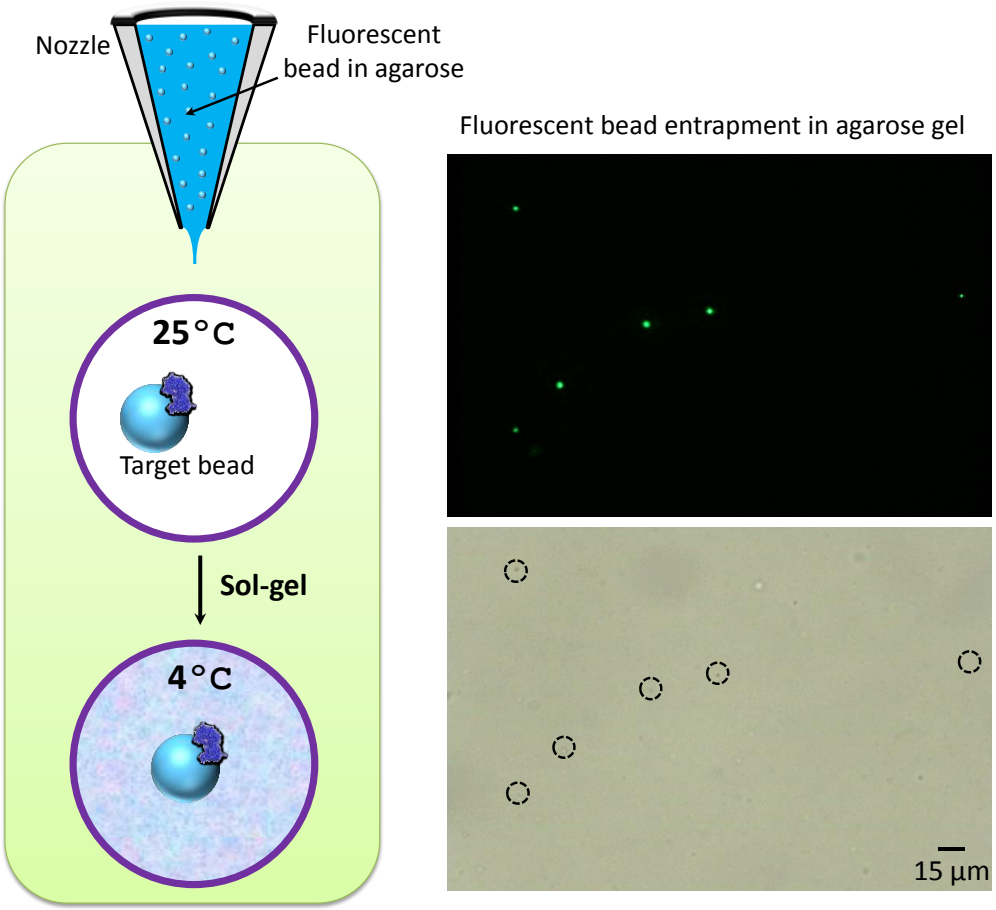


***Figure 3.6**: Co-compartmentalization of target fluorescent beads in agarose gel beads-in-oil using immersed electrosprays at 500 V, 100 Hz using nozzle size of 15 µm. Inverted fluorescence microscope was used to capture the images.*

3.3.5 Demonstration of washing steps for bias-free selection

Washing is the most crucial step of direction evolution since it helps in selection of probe from million molecules. Conventional washing steps face the problems of miss-matching binding, intermolecular interaction which can mislead the selection procedure. So first most I demonstrate the system which can remove the unbounded aptamers from agarose gel beads.

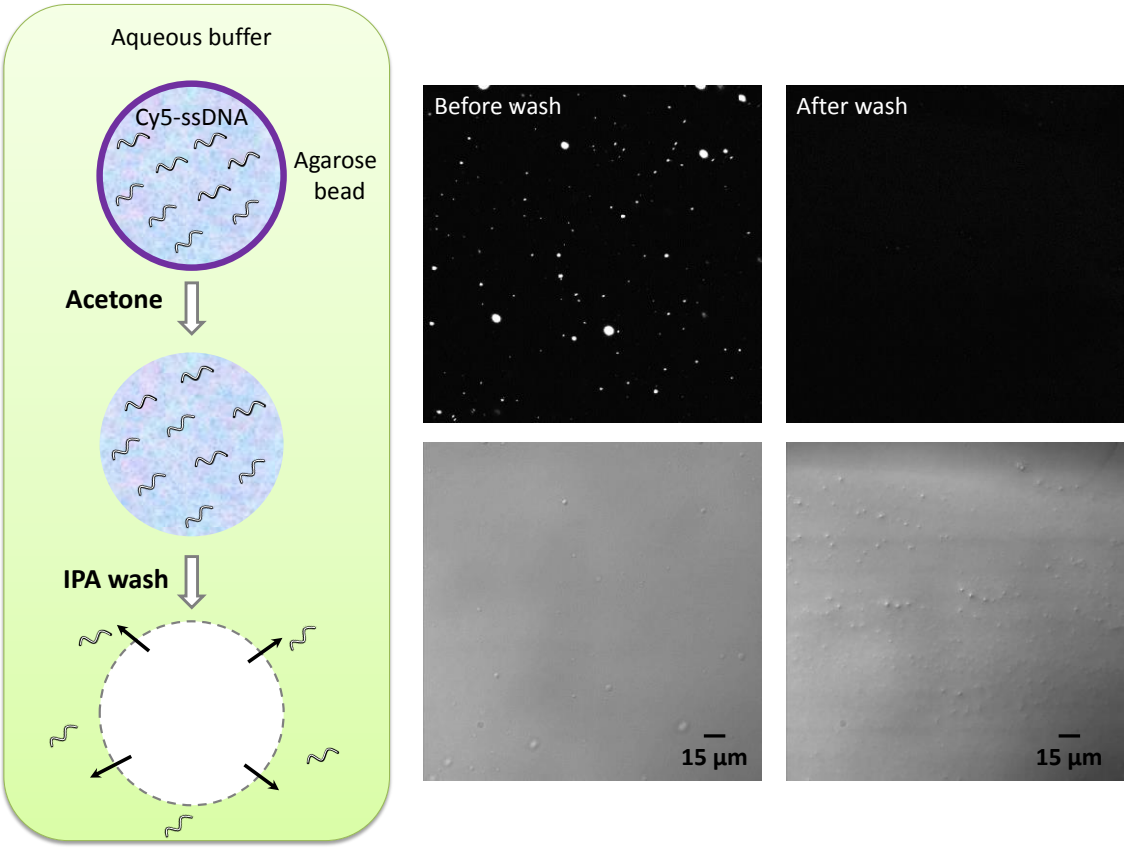


***Figure 3.7**: Washing of encapsulated Cy5-ssDNA in agarose gel beads-in-oil using acetone and isopropanol. This washing step is to demonstrate the removal of unbounded molecules from agarose gel.*

Figure 3.7 shows the centrifuged washing of the encapsulated Cy5-ssDNA (20mer) in agarose gel beads with acetone and isopropanol for 2 min at 1000 rpm, which washed out almost all the ssDNA from agarose gel beads. This demonstrate the significance of the agarose gel washing system which is truly bias free.

Additionally I also considered the elimination of reaction inhibitors of in vitro expression like phosphate ions or so. Figure 3.8 shows the entrapment of biotin-4-florescein (B-4-F) in agarose gel beads of same strength and similar washing steps were followed as described before using acetone and isopropanol wash.

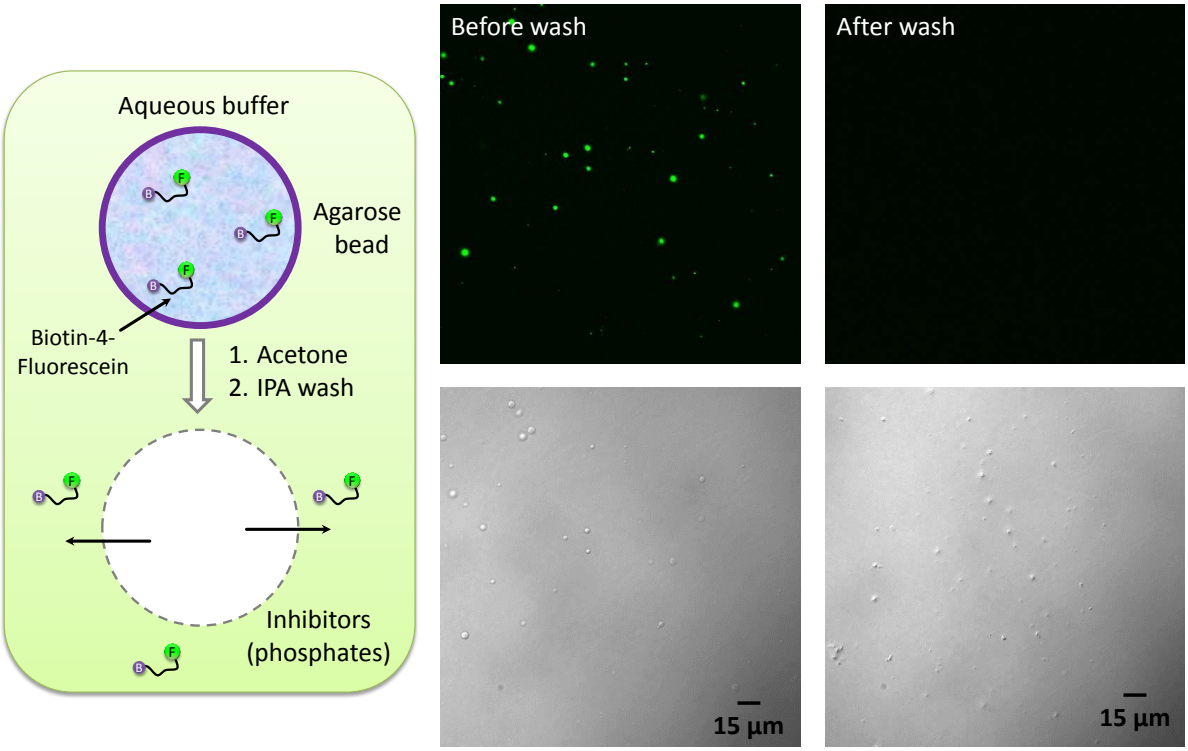


***Figure 3.8**: Removal of inhibitors from agarose gel beads-in-oil. Demonstration of this washing shown by entrapment of biotin-4-fluorescein in agarose gel beads and washed out using acetone and isopropanol using centrifugation.*

3.4 Conclusion and perspectives

This chapter successfully demonstrated the selection-in-a-fL droplet system by demonstrating the encapsulation of target fluorescent beads, removal of unbounded ssDNA from the agarose-in-oil gel beads using acetone and isopropanol washing steps.

In perspective, this high throughput system of small agarose gel beads can further improved the selection quality with large diverse variants library. Microfluidics washing of agarose gel beads can further improve the selection pressure by provided better solute solvent exchange in microchip.

References

1. Smith, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315–1317 (1985).
2. Mattheakis, L. C., Bhatt, R. R. & Dower, W. J. An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proc Natl Acad Sci USA* **91**, 9022–9026 (1994).
3. Nemoto, N., Miyamoto-Sato, E., Husimi, Y., & Yanagawa, H. In vitro virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro. *FEBS Letters* **414**, 405–408 (1997).
4. Roberts, R. W. & Szostak, J. W. RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci USA* **94**, 12297–12302 (1997).
5. Griffiths, A. D. & Tawfik, D. S. Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. *EMBO J* **22**, 24–35 (2003).
6. Yamaguchi, J. et al. cDNA display: a novel screening method for functional disulfide-rich peptides by solidphase synthesis and stabilization of mRNA-protein fusions. *Nucleic Acids Research* **37**, e108 (2009).
7. Odegrip, R., Coomber, D., Eldridge, B., Hederer, R. & Kuhlman, P. A. CIS display: In vitro selection of peptides from libraries of protein-DNA complexes. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2806–2810 (2004).
8. Bertschinger, J., Grabulovski, D., & Neri, D. Selection of single domain binding proteins by covalent DNA display. *Protein Engineering, Design & Selection* **20**, 57–68(2007).
9. Smith, G. P., & Petrenko, V. A. Phage display. *Chemistry Reviews* **97**, 391–410 (1997).
10. Lamla, T., & Erdmann, V. A. Searching sequence space for high-affinity binding peptides using ribosome display. *Journal of Molecular Biology* **329**, 381–388 (2003).

11. Gersuk, G. M. et al. High-affinity peptide ligands to prostate-specific antigen identified by polysome selection. *Biochemical and Biophysical Research Communications*, *232*, 578–582 (1997).
12. Litovchick, A., & Szostak, J. W. Selection of cyclic peptide aptamers to HCV IRES RNA using mRNA display. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15293–15298 (2008).
13. Kitamura, K. et al. Development of systemic in vitro evolution and its application to generation of peptide-aptamer-based inhibitors of cathepsin E. *Journal of Molecular Biology*, **387**, 1186–1198 (2009).
14. Kitamura, K. et al. Proven in vitro evolution of protease cathepsin E inhibitors and activators by a paired peptides method. *Journal of Peptide Science*. http://dx.doi.org/10.1002/ psc.2453 (2012).
15. Kitamura, K. et al. Peptide aptamer-based ELISA-like system for detection of cathepsin E in tissues and plasma. *Journal of Molecular Biomarkers Diagnostic* **2**, 104 (2011).
16. Biyani, M. et al. *In vitro* selection of cathepsin E-activity enhancing peptide aptamers at neutral pH. *International Journal of Peptides*, 834525 (2011).
17. Kelly, B. T., Baret, J. C., Taly, V. & Griffiths, A. D. Miniaturizing chemistry and biology in microdroplets. *Chem Commun* **18**, 1773-1788 (2007).
18. Griffiths, A. D. & Tawfik, D. S. Miniaturising the laboratory in emulsion droplets. *Trends Biotechnol* **24**, 395-402 (2006).
19. Miller, O. J. *et al.* Directed evolution by in vitro compartmentalization. *Nat Methods* **3**, 561-570 (2006).
20. Tawfik, D. S. & Griffiths, A. D. Man-made cell-like compartments for molecular evolution. *Nat Biotechnol* **16**, 652-656 (1998).

21. Takehiro, N., Takeshi, S., Tomoaki, M. & Tetsuya, Y. Directed evolution of proteins through in vitro protein synthesis in liposomes. *Journal of Nucleic Acids* **2012**, 1-11 (2012).

General Conclusion

A high throughput water-in-oil emulsion system has been developed by exploiting the electrosprays technology, in which nozzle head of electrostatic inkjet was dipped inside oil phase. For the best of my knowledge our group has reported the first one to use immersed electrosprays to generate water-in-oil emulsion for in vitro compartmentalization. The average size of water droplets is found to be 1.3 µm with generation speed of 10^5 droplets per second (high speed camera captured). Moreover this high throughput system was optimized on the grounds of nozzle size, oil viscosity and inkjet parameters and it can be concluded that nozzle size drastically affected the droplet size distribution. Larger the nozzle size, high degree of polydispersity. While oil viscosity is directly proportional to average droplets size.

The small sized water-in-oil droplets were successfully performed the cell free protein expression for GFP. Time course study of GFP expression with different droplets size concluded that increase in the droplet size, increases fluorescence intensity but smaller droplets (~1.0 µm) showed the early saturation of GFP expression in 15 min of incubation. Later on, extreme diluted co-expression of GFP and mCherry does not show very clear results of single expression, so I would like conclude this results on the speculation of "super concentration effect" which caused the rapid consumption of important translationary factors like amino acids.

After confirmation of cell free expression in small droplets, this immersed electrosprays system generated agarose gel beads-in-oil of ~1.75 µm size. For the demonstration of selection-in-pL droplet system, first and foremost successful co-compartmentalization of fluorescent beads in agarose gel beads-in-oil was shown. Secondly, successful washing process of the encapsulated Cy5-ssDNA was demonstrated with acetone and isopropanol. This washing step shown the removal of unbound/free molecules from agarose gel beads that inhibits the intermolecular interaction and led to the idea of bias-free selection-in-pL droplets. Lastly, reaction inhibitors like phosphate ions which produced during the in vitro expressions need to remove for the

improved expression. So similar washing steps were followed as described above for the entrapment of biotin-4-flourescein in the agarose gel beads.

Finally I would like to conclude that high throughput water-in-oil droplet generation by using immersed electrosprays provide highly robust and easy to handle platform for the molecular protein/enzyme evolution, IVC based reactions, minimal cell volume study for the origin of life and so on.