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