# Fabrication of a multiplexed microfluidic system for scaled up production of cross-linked biocatalytic microspheres

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## ABSTRACT

Multiplexed or parallelised droplet microfluidic systems allow for increased throughput in the production of emulsions and microparticles, while maintaining a small footprint and utilising minimal ancillary equipment. The current paper demonstrates the design and fabrication of a multiplexed microfluidic system for producing biocatalytic microspheres. The microfluidic system consists of an array of 10 parallel microfluidic circuits, for simultaneous operation to demonstrate increased production throughput. The flow distribution was achieved using a principle of reservoirs supplying individual microfluidic circuits. The microfluidic devices were fabricated in poly (dimethylsiloxane) (PDMS) using soft lithography techniques. The consistency of the flow distribution was determined by measuring the size variations of the microspheres produced. The coefficient of variation of the particles was determined to be 9%, an indication of consistent particle formation and good flow distribution between the 10 microfluidic circuits.

**Keywords:** biocatalyst, droplet, emulsion, enzymes, monodisperse, microfluidics, microparticles, parallelisation, scale-up.

## 1. INTRODUCTION

Enzymes are biological catalysts that promote 'green manufacturing' in various industrial applications including food and beverage processing[1], manufacturing of pharmaceutical intermediates[2], production of biofuels[3] and textiles processing[4]. Enzymes can be stabilised by rendering them into insoluble form using a process referred to as immobilisation. Enzyme immobilisation helps to improve their functionality and stability in harsh reaction conditions, such as acidic or alkaline pH levels, high temperatures and organic solvents. Enzyme immobilisation also improves the shelf life of enzymes. Various immobilisation techniques that exist include attachment of enzymes onto support beads of various materials such as glass, ceramics, and polymers, and can also be achieved using methods such as entrapment and self-immobilisation[5].

We developed a microfluidic-based method for immobilisation of enzymes on protein microspheres[6]. Microfluidics has, over the past two decades, emerged as a useful tool for preparation of novel biological and chemical products. In particular, droplet-based microfluidics has been demonstrated to be very effective in the production of emulsions and emulsion-based particles with very narrow size distributions. Emulsions are, fundamentally, based on the interaction of two immiscible fluids (often in the presence of a surfactant) where one phase is broken down into discreet elements (droplets) and the other phase is the continuous (carrier) phase. Droplet formation in microfluidics is commonly achieved in flow geometries such as the T-junction[7] and flow focussing or cross junction[8], where the two immiscible fluid phases interact and formation of droplets of one of the phases occurs. During the manufacturing of our biocatalytic microspheres, droplets of an aqueous solution blended with protein and enzyme are utilised as templates for the formation of microspheres as shown in Figure 1. A chemical cross-linking process is utilised to covert the droplets formed in an oil phase into solid particles through a precipitation reaction.

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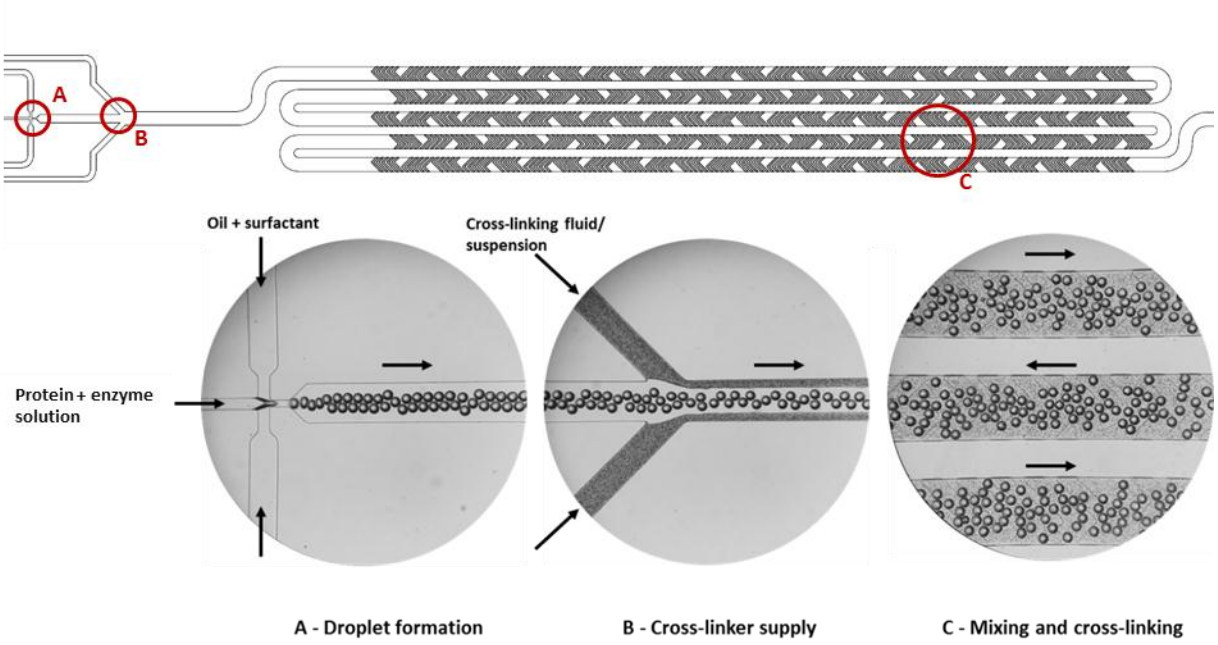


Figure 1: Illustration of key steps in the formation of microspheres in a single circuit microfluidic device.

For high volume industrial enzyme applications, production rates of kilograms to tonnes per annum of immobilised enzymes are typically required, depending on the enzyme type and its market size. These volumes are much larger than what single circuit microfluidic devices can produce, which is typically a few milligram to gram quantities per day. For the current microfluidic process to be able to produce commercially viable volumes, a scale-up process is necessary. Scale-up of droplet-based microfluidic systems has been demonstrated to work successfully with parallelisation or multiplexing of microfluidic circuits[9-13]. The scalability of the latter systems was demonstrated for single and double emulsions. However, to the best of the authors' knowledge, there have not been similarly demonstrated microfluidic systems for microparticle production. The aim of this work was to demonstrate and evaluate the scalability of our microfluidic process for making the biocatalytic protein particles. The current paper demonstrates a limited scaling up of the process for production of immobilised enzymes using 10 particle formation units or circuits which were operated in parallel as a single unit. The circuits in the system were operated in a parallel using distributed flow approach.

## 2. EXPERIMENTAL

### 2.1 Design and fabrication

The layout of 10 microfluidic circuits is illustrated in Figure 2A, whereby the circuits were laid out in a linear array. In order to achieve equal flow distribution to the microfluidic circuits, micro-wells/reservoirs were utilised[13]. The function of the micro-wells was to provide a central point of fluid distribution from the pump. The microchannels with incoming flow into the reservoirs had double the width of those of the outlet into individual circuits. The fluid distribution reservoir for the cross-linking phase (reservoir 1) was positioned on the same plane as the microfluidic circuits. All the microchannels were fabricated on poly (dimethylsiloxane)(PDMS) using soft lithography[16] and, as such, the microfluidic circuits were moulded in a planar configuration on PDMS layers. Therefore, the fluid distribution reservoirs for the dispersed and the continuous phases could not be placed on the same plane as the 10 microfluidic circuits, as this would have resulted in the intersection of flow channels. This limitation was overcome by positioning of the latter reservoirs on a separate plane (Figure 2B). The microfluidic device was fabricated such that it consisted of two PDMS layers, stacked on top of each other (Figure 3). The PDMS layer with the 10 microfluidic circuits and one reservoir (Figure 2A) was placed at the bottom and the one with two reservoirs (Figure 2B) was stacked on top. The continuous phase and the protein solution were distributed from the respective reservoirs (reservoir 1 and 2) vertically downwards via 0.75 mm

holes through PDMS, created using a biopsy punch. The two-layer approach in the layout of the fluid distribution system resembles that of Romanowksy and co-workers[12].

The outlets of all the circuits were combined in a cascade manner following a reverse principle of the biologically-inspired bifurcation rule known as Murray's Law [14,15]. This integration of the outlets allowed for a uniform back pressure in each microfluidic circuit[12,13]. Another consideration in layout of the 10 microfluidic circuits was that planar footprint of the system would would fit on a 5 cm x 7.5 cm microscope glass slide. The glass slide was used to seal the microchannels in the bottom PDMS layer using oxygen plasma bonding (Figure 3B). The glass slides were available commercially, and negated the need to cut up and clean glass for the sealing of the microfluidic channels, which would add extra work in the fabrication process. A distance of 4 mm from the four edges of the glass slide was provided to allow for the surface area for sealing between the glass and the PDMS.

The design drawings were completed using DesignCAD 3D Max (version 21) software package. The drawings were converted into black and white images for printing of transparency masks. The masks were printed using high definition printing (40 000 dpi) on film transparencies. The printed masks were then used in the production of the moulds. The moulds were made on a silicon wafer using the SU-8 development and curing process in a Class 1 000 clean room. The UV exposure of the SU-8 photoresist was achieved using a UV mask aligner (Züss, Type 12100013). The moulds were then used in the casting of the microchannels.

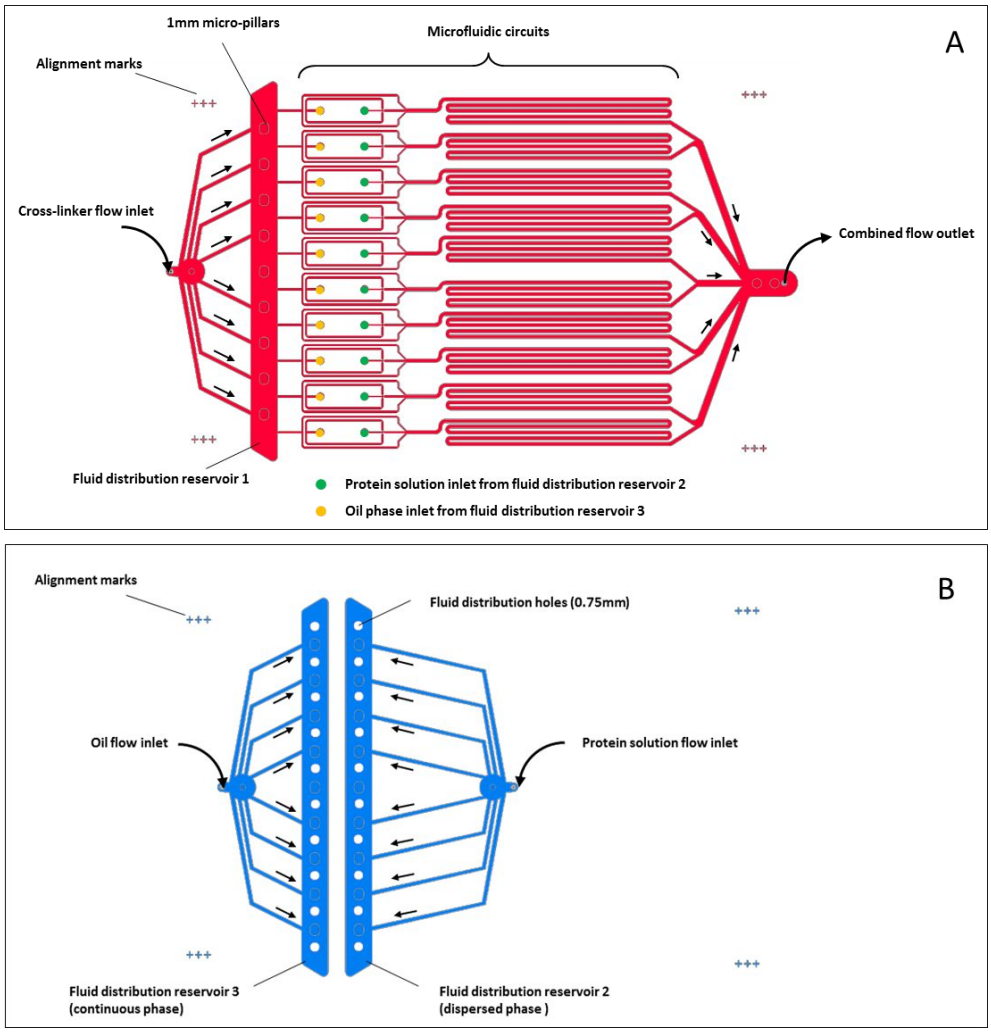


Figure 2: Schematic drawings of the bottom and the top layers of the microfluidic system

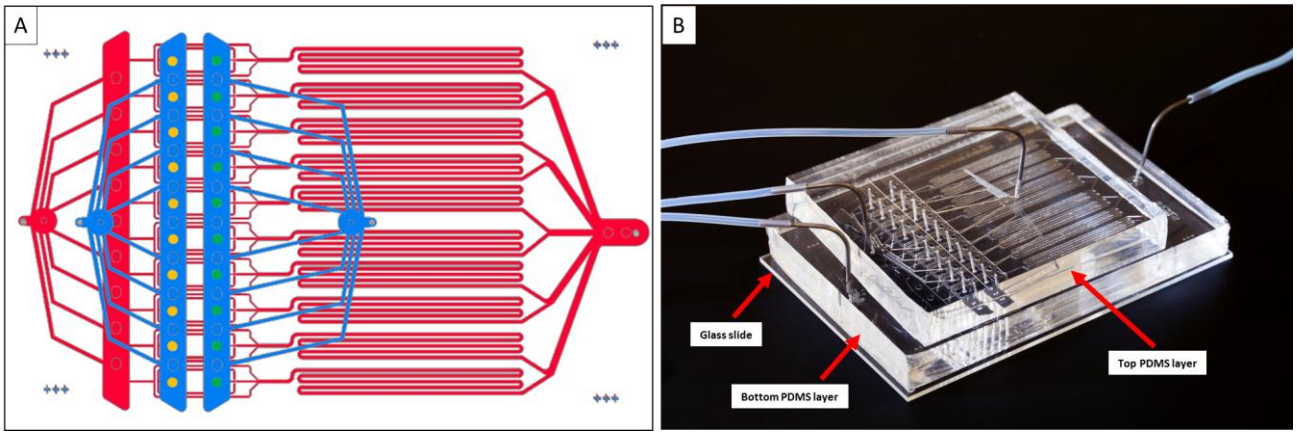


Figure 3: (A)Schematic representation of the alignment between the bottom and the top microfluidic layers; (B) Photograph of a scale-up microfluidic device fabricated in PDMS, with fluid supply connectors and tubing attached. .

The PDMS for casting of the microchannels was mixed in a standard mass ratio of 10:1 of base material to catalyst. The casting for the bottom layer was baked for 60 minutes at 60 $^{o}$C. The bottom layer was then cut in correct dimensions, punched with a 0.75 mm biopsy punch and then sealed onto the glass slide using oxygen plasma bonding. The sealed bottom layer was then baked in the oven at 90 $^{o}$C for 30 minutes. The top layer was baked at 60 $^{o}$C for 30 minutes. This process resulted in an incompletely cured but hardened PDMS. The incomplete curing caused the PDMS to be sticky, an effect which was desired for bonding between PDMS layers. The channel side of the top layer was then sealed, after punching of fluid inlet holes, to the top side of the bottom layer. The sandwich was further baked on a hotplate at 120 $^{o}$C for 12 hours.

### 2.2 Testing

Syringe pumps (Fusion, Chemyx) were used for delivering the fluids to the microfluidic device via Teflon tubing. The flow was actuated using 3 syringe pumps, one for each fluid phase. Mineral oil (Pharma 15, Caltex), containing 5% m/m Span 80 surfactant was used as the continuous phase. Aqueous solution of bovine serum albumin (BSA) was used as the dispersed phase. BSA without an enzyme added was considered sufficient for the proof of concept. The cross-linker fluid was supplied as an emulsion, prepared using a previously described procedure[6]. In brief, 100 µl of glutaraldehyde (GLA) solution (25% m/v) was reacted with 120 µl of ethylene diamine (EDA) solution (0.33 M, pH 6) containing Triton X-100 (9% m/v) surfactant for 45 minutes. A further 100 µl GLA solution was added into the reaction. The reacted mixture, exhibiting a yellowish colour, was then emulsified by magnetic stirring in 1.2 ml of mineral oil containing 5% m/m Span 80 for 15 minutes.

The microfluidic device was operated on an inverted optical microscope (Olympus) fitted with a digital camera. The flow images recorded were analysed using a commercial software program (Olympus Stream Essentials) and in-house image analysis software.

## 3. RESULTS AND DISCUSSION

Droplet formation in the fabricated multi-circuit device was initially evaluated using different fluid combinations: water - oil-oil, protein-oil-oil and protein-oil-cross-linker. The flow rates for the oil phase and cross-linker phase were kept constant at 40 µl/min and 10 µl/min, respectively. The flow rate for the water or protein solution was varied between 1-40 µl/min (0.1-4 µl/min per circuit), and droplet sizes were measured for each flow rate. Figure 4 shows images recorded

at the droplet generation junction for different water flow rates and droplet sizes generated with protein solution and oil. The concentration of the protein (BSA) solution used for this test was 300 mg/ml.

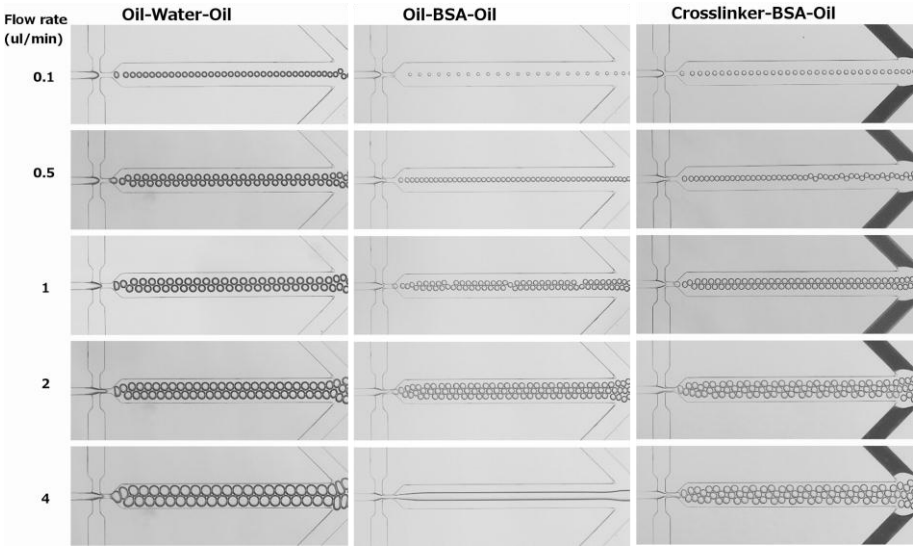


Figure 4: Comparison of droplet formation using various fluids.

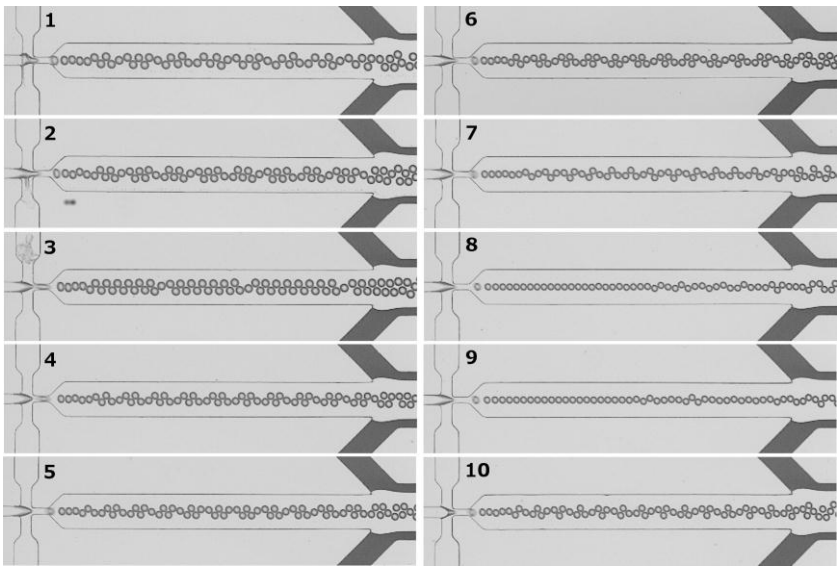


Figure 5: Droplet formation in 10 parallelised microfluidic circuits at a dispersed phase flow rate of 10 µl/min. The concentration of the protein solution (dispersed phase) was 150 mg/ml.

The collected microspheres were analysed for size distribution and were found to vary by 9%. This figure varied from the size distribution of particles obtained from a single circuit device, which had a variation of less than 5% [6]. This deviation was caused by the variation in droplet formation between the 10 microfluidic circuits (Figure 5). The droplet size variation was observed at various flow rates of the dispersed phase but no specific trend was observed (Figure 6).

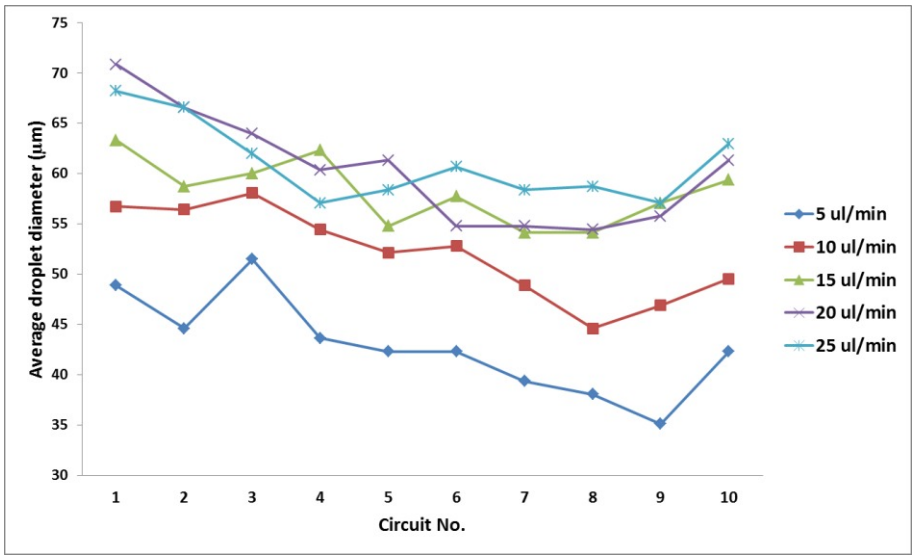


Figure 6: Comparison of average droplet sizes formed at different flow rates.

The advantages of parallelised or multiplexed microfluidic production system are[12]: (1) minimal number of pumps and devices, (2) smaller foot print than multiple systems, (3) ease of handling, and (4) reduced cost of scaling up. However, there are some disadvantages such as possible non-uniformity of the particles formed and complexity in assembly and fabrication that can emanate during microfluidic scale-up. Ultimately, a scaled up microfluidic system that was easy to operate, simple and cost effective to manufacture and was able to produce monodisperse particles would be considered successful. The current microfluidic device was able to produce 10 times the volume of a single microfluidic device without extra ancillary equipment being added. Although the size distribution was not as good as the single-circuit device, 9% is considered acceptable. The two layer device approach proved to function optimally under the flow conditions tested without sealing issues. Previous attempts to bond PDMS layers using oxygen plasma process had been unsuccessful. The seals between the layers had been found to be inconsistent, often causing leakages at high flow rates. The method utilised herein proved to result in consistently bonded seals between two PDMS layers. The disadvantage with the method, however, is that care is required during the removal of the partially cured PDMS from the mould.

Blockages during testing were experienced due to three main causes. The first cause of blockages was when foreign particulate material entered the system and lodged in the microchannels. The second cause was due to cross-linking of the protein by the cross-linker before protein droplets were formed. This problem was eliminate by a start sequence whereby the microfluidic system was first primed with oil and the protein droplets formed before starting the flow of the cross-linker. The last cause was due to congestion in the outlet microchannel by the microparticles formed. This was eliminated by punching a bigger outlet hole (1.2mm instead on 0.75mm) attached to external tubing for particle collection..

## 4. CONCLUSION

We have demonstrated a multiplexed microfluidic system consisting of 10 circuits. The device forms a basis on which further design improvements can be made. Furthermore the system was easy to prototype using PDMS and also easy to test. The coefficient of variation of the particles was less than 10%. For a commercial system, a similar design approach could be adopted.

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