MICROFLUIDIC PREPARATION OF BIOCATALYTIC PROTEIN MICROSPHERES UTILISING ON-CHIP CROSS-LINKING METHOD
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ABSTRACT
We present an on-chip method for preparing cross-linked protein microspheres. When prepared from a solution of bovine serum albumin (BSA) consisting of lipase, the microspheres showed varying degrees of hydrolytic activity on two p-nitrophenyl ester substrates.

KEYWORDS: Biocatalysis, Bovine serum albumin, Cross-linking, Droplet microfluidics, Enzyme immobilisation, Glutaraldehyde, Lipase

INTRODUCTION
Immobilisation of enzyme provides advantages such as reduced sensitivity and improved stability against environmental parameters such as temperature, pH, shear, oxidation and solvents, thereby ensuring an improved shelf life and handling [1]. Furthermore, enzyme immobilised in particulate form can be recovered easier than solubilised enzymes for reuse. This manuscript reports on preparation of lipase-laden protein microspheres in a microfluidic channel using an on-chip cross-linking method [2]. The capability and suitability of droplet-based microfluidics in producing a wide range of monodisperse microparticles with controllable sizes has, in recent years, been thoroughly demonstrated [3]. In published literature, however, little attention has been given to immobilised enzyme microparticles using droplet microfluidics. Jeong et al., [4] used an ‘on the fly’ microfluidic method to immobilise horseradish peroxidase and glucose oxidase in the matrix of photopolymerised hydrogel microspheres. He et al., [5] produced enzyme functionalised silica nanoparticles using polyethyleneimine (PEI) and a peptide. Xu et al., [6] used a combination of on-chip solvent evaporation and off-chip GLA cross-linking to produce enzyme-loaded chitosan microspheres.

In the current work, we demonstrate the production of bovine serum albumin (BSA) microspheres using on-chip cross-linking method. The monodispersed droplets, which form the templates for microspheres are produced in a flow-focusing junction and cross-linked with an emulsified mixture of glutaraldehyde (GLA) and ethylene diamine (EDA).

EXPERIMENTAL
The microfluidic channels were fabricated from polydimethylsiloxane PDMS (Sylgard 184, Dow Corning) using soft-lithography method and the microchannels were closed by sealing the PDMS onto a glass slide using oxygen plasma bonding technique. Figure 1A shows the planar microfluidic circuit, with a uniform depth of 75 μm, consisting of flow-focusing junction for droplet formation and a second junction for introduction of cross-linking emulsion. The serpentine section consisting of herringbone microstructures allows for mixing and cross-linking of the microparticles.

Dispersed phase was prepared by dissolving 200 mg/ml of lyophilised BSA and 30 mg/ml partially purified lipase (Lipase AK “Amano”) preparation in 250 mM tris-HCl buffer solution (pH 7.2) . The lipase from Pseudomonas fluorescens was purchased in crude form and partially purified before use. Briefly, a suspension of crude Amano lipase (500% m/v) in deionised water was centrifuged at 10 000 rpm. 60% m/v polyethylene glycol 6000 (PEG 6000) was added and dissolved in the supernatant at 4 °C. The mixture was centrifuged in conditions to similar to previously stated and diluted by 1000% volume of deionised water and was washed in ultrafiltration (UF) unit fitted with a 10 kDa molecular weight cut off (MWCO) membrane. The retentate was frozen at ultra-low temperature (-80 °C), lyophilised and kept refrigerated at 4 °C until use. The continuous phase was prepared by dissolving 3% (m/m) Span 80 in mineral oil. The cross-linker reagent was prepared by reacting 100 μl of GLA (25% v/v) solution with 120 μl of EDA (0.33 M, pH 6) solution containing Triton X-100 (9% m/v) surfactant for 45-minutes. A further 100 μl GLA solution was added into the mixture. 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.

All fluids were freshly prepared before use in microfluidic experiments. Syringe pumps (Fusion, Chemyx) were used for delivering the fluids to the microfluidic device via PTFE tubing. After manufacturing the microparticles were separated from oil by centrifuging at 4000rpm after washing twice with aqueous solution of Triton X100 (1%).

The activity of the resultant immobilised enzyme particles was determined in the hydrolysis of p-nitrophenyl esters, p-nitrophenyl palmitate (PNPP) and p-nitrophenyl butyrate (PNPB) to p-nitrophenol and an aliphatic carboxylic acid. The calorimetric assays were performed using a PowerWave HT Microplate Spectrophotometer (BioTek Instruments) with a temperature-controlled microtitre plate reader. The kinetic measurements were conducted at wavelength of 410 nm and temperature of 35°C. Enzyme activity (U) is the amount of enzyme necessary to produce 1 μmol of p-nitrophenol per minute and the specific activity is activity per unit mass of protein in solution (U/mg). Activity retention was determined as percentage of the specific activity of the immobilised enzyme to the specific activity of the free enzyme in solution, thus:

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\text{Activity retention} \% = \left( \frac{\text{Specific activity of immobilised enzyme}}{\text{Specific activity of free enzyme}} \right) \times 100.
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RESULTS AND DISCUSSION

The droplets of aqueous polymer solution are generated in the continuous stream of mineral oil and the GLA emulsion is introduced in the second junction (Figure 1B). The protein droplets and the emulsion mix in the 120 mm-long serpentine microchannel section where the cross-linker reacts with the proteins via Schiff-base and Michael-type reactions [7]. The primary amines (–NH2) of the protein and the aldehydes (–CHO) of the cross-linker, react in condensation reaction where a water molecule is released and a covalent bond is formed. This reaction leads to denaturing and solidification of the protein. Albumins have an abundance of lysine amino groups their structures which contain two primary amines, making them ideal proteins to cross-link with a di-functional aldehyde molecule. The use of herringbone microstructures has been demonstrated to improve the mixing of protein droplets and the cross-linker emulsion [8]. The flow rate of the dispersed phase could be varied to manipulate the droplet size (Figure 2) while the flow rates of the continuous phase and the cross-linker emulsion were kept constant at 4 μl/min and 1 μl/min, respectively. Control of droplet size by varying dispersed phase was preferable since changing the continuous phase varies the overall residence time of the particles in the microfluidic circuit.

The cross-linker emulsion prepared showed good homogeneity and kinetic stability and could be used in experiment up to 12 hours without showing signs of phase separation or settling.

Figure 1. (A) Photograph of microfluidic circuit (B) Illustration of the microfluidic set-up used for preparation of microspheres using on-chip cross-linking

Figure 2. Manipulation of droplet size by variation of the flowrates of the dispersed phase and the continuous phase.
Figure 3. Micrographs of monodispersed microspheres manufactured at dispersed phase flowrate of 1μl/min: (A) after collection in oil mixed with cross-linker emulsion, (B) suspended in Triton x100 solution with increased average diameter (109 μm) due to swelling and (C) SEM with average diameter of 25μm due to shrinking during drying.

The starting solution had an average specific activity of 577 U/g protein on PNBB and 2507 U/g on PNPP. The immobilized lipase was able to retain on average 50.5% of its initial enzymatic activity on PNPB and only 1% on PNPP. The low activity of the microspheres on hydrolysis of PNPP was not unexpected due to the fact that PNPP has a long acyl (C16) chain, which exhibits diffusional limitations on immobilised enzymes [9]. This is in contrast to the hydrolytic activity of the microspheres on PNPB, which has a shorter acyl (C4) chain has and thus less diffusional limitations.

CONCLUSION
The current paper has demonstrated an on-chip cross-linking method for preparing biocatalytic protein microspheres using emulsified glutaraldehyde-based cross-linker. The lipase in the microspheres could retain at an average 50.5% of biocatalytic activity on PNPB substrate, while effect of diffusional limitation was observed on PNPP substrate.

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REFERENCES

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