The generation of multi-laminar reagent streams for rapid, sequential (bio)chemical reactions on magnetic particles in a continuous flow microreactor

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Abstract We demonstrate a versatile microfluidic system for performing rapid, consecutive (bio)chemical reactions in continuous flow. Surface-functionalised magnetic microparticles are introduced into a chamber and pulled, via a magnet, across a series of laminar flow streams containing different reagents, thus performing multiple sequential reactions on the particles' surface. Such a continuous flow method eliminates many of the inefficiencies associated with batch techniques, such as the time-consuming, laborious sequential reaction and washing steps, to yield a system that can perform analyses far more rapidly and with less reagent volume than conventional methods. This innovative device has been applied to a two-reaction step mouse IgG sandwich immunoassay and one- and two-reaction step DNA hybridisation assays, all of which were completed within one minute. These results pave the way for a multi-purpose microreactor that can perform a variety of analytical and synthetic processes.

Keywords: Multi-laminar Flow, Magnetic Particles, Continuous Flow, Immunoassays, DNA Hybridisation

1. Introduction

Microfluidics, which involves the manipulation of micro- to attolitres volumes of fluids in micron-sized channels, is an exciting and increasingly popular field of research (Whitesides, 2006). One of the inherent properties of microfluidics is the laminar flow regime that is a prominent feature due to the low Reynolds numbers of such systems. This offers fine control over fluid flow, and due to the miniaturised nature of microfluidic devices reactions can be performed faster, there is a significant reduction in reagent consumption and waste production, whilst also demonstrating potential for point-of-care analyses.

Microparticles have been incorporated into microfluidic systems for performing a number of different surface-based procedures (Verpoorte, 2003). Their high surface-to-volume ratio and variety of surface functionalities makes them ideal as solid supports for a range of (bio)chemical reactions. Microfluidic applications utilising microparticles have included immunoassays, DNA hybridisation, affinity assays, and a number of protein, DNA and enzymological studies (Bangs, 1996).

In microfluidics, particles are often trapped in a channel and reagents are flushed over the surface. This has often been achieved using physical barriers such as dams, weirs (Oleschuk et al., 2000; Sato et al., 2000) and pillars (Andersson et al., 2000) to hold the particles in place. However, a more elegant method has been to use magnetic particles, which can be easily manipulated inside channels by an externally applied magnetic field (Gijs, 2004; Pamme, 2006). Magnetic particles contain a core of iron oxide nanoparticles that cause the particle to become magnetised in the presence of a magnetic field, and to behave like a non-magnetic particle when the field is removed. Like conventional microparticles, magnetic particles also feature a variety of surface chemistries, and reactions have been performed where the particles have been trapped in a magnetic field (Fig. 1a), including mRNA isolation (Jiang and Harrison, 2000), DNA hybridisation (Fan et al., 1999) and several bioassays (Bronzeau and

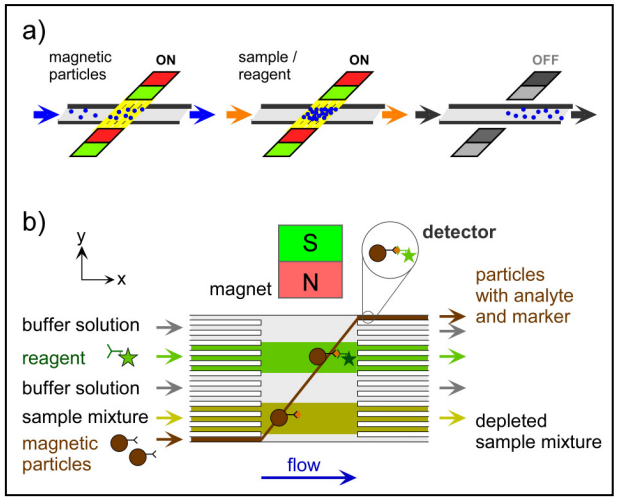

Fig. 1. (a) Conventional trap and release method for magnetic particle handling on-chip. (b) The principle of the continuous flow microfluidic reactor. Magnetic particles are introduced into the reaction chamber and deflected through consecutive reagent and washing streams. The example above demonstrates a sandwich immunoassay, but the technique can be applied to a number of (bio)chemical reactions.

Pamme, 2008; Choi et al., 2002).

Although magnetic particles have been advantageous in performing the above reactions, the methods are still sequential, batch techniques that involve many time consuming and laborious reaction and washing steps. Previously, we presented a microfluidic platform for performing sequential reactions on the surfaces of magnetic particles in continuous flow (Peyman et al., 2008). Such continuous flow methods eliminate some of the inefficiencies of batch techniques by combining multiple reaction and washing steps into a single process. Particles are introduced into a reaction chamber in the x-direction, and deflected in the y-direction via a magnetic field (Fig. 1b) (Pamme et al., 2006). As the particles deflect through the chamber they cross several laminar flow streams containing alternating reagent and washing buffers, and the functional groups on the particle surface react with each reagent as they cross the streams. The proof-of-principle reaction consisted of a one-reaction-step binding assay between streptavidin on the particle surface and fluorescently labelled biotin in the reagent stream, such that when the binding occurred the particle exhibited greater fluorescence intensity than before it had entered the biotin stream.

Here, we explore the versatility of the system by performing a variety of procedures, including immunoassays, DNA hybridisation and peptide synthesis, thus demonstrating its potential for executing more complex processes in the future.

2. Experimental

2.1 Preparation of particle suspensions

2.8 µm diameter superparamagnetic particles featuring either streptavidin or carboxylic acid surface groups (Dynabeads M-270 Streptavidin and Dynabeads M-270 Carboxylic Acid, respectively) were purchased from Invitrogen (Paisley, UK). The particles were prepared by diluting 10 µL of the stock solution in 990 µL of the appropriate buffer solution: PBS buffer (phosphate buffered saline, pH 7.45, Invitrogen) for the two-step IgG sandwich immunoassay, 10x SSC buffer (saline sodium citrate, Sigma-Aldrich, Dorset, UK) for the DNA hybridisation procedures, and 25 mM MES buffer (2-(N-morpholino)ethanesulfonic acid, pH 5, Sigma-Aldrich) for the peptide synthesis. This gave particle concentrations of 6×10^8 particles mL^{-1} for the streptavidin-functionalised particles and 2×10^8 particles mL^{-1} for the carboxylic acid-functionalised particles. Suspensions were diluted prior to use in the multi-laminar flow experiments; the streptavidin particles were diluted to 3×10^8 and 6×10^5 particles mL^{-1} for the immunoassay and DNA hybridisation experiments, respectively, and the carboxylic acid particles to 2×10^6 particles mL^{-1}. BSA (0.01% w/v) was added to the streptavidin particle suspensions, and 0.01 % w/v Tween 20 to the carboxylic acid particle suspensions to prevent particle sticking.

2.2 Fabrication and setup of the microfluidic devices

The microfluidic chip design (Fig. 2) featured an 8 mm long by 3 mm wide reaction chamber, supported by ten posts, and fed by five branched inlet channels. Magnetic particle suspensions were introduced into the chamber

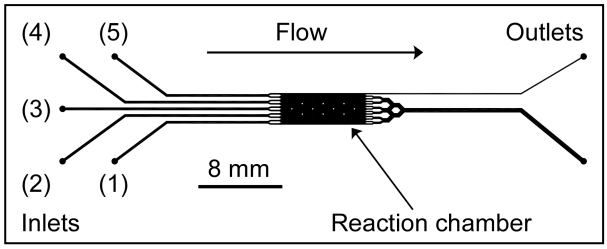

Fig. 2. Schematic of the continuous flow reactor design, featuring a reaction chamber, five inlets and two outlets.

via inlet 1, reagent solutions by inlets 2 and 4, and washing buffer through inlets 3 and 5. The outlet system consisted of a single narrow channel for particle collection, with buffer and reagent solutions exiting via a branched outlet system.

The chip was fabricated in B270 glass (Telic, Valencia, CA, USA) to a depth of 20 μm using conventional photolithography and wet etching methods, then thermally bonded. Fused silica capillaries (100 μm i.d., 360 μm o.d., Polymicro, Composite Metal Services, Shipley, UK) were glued into the inlet holes and interfaced to glass syringes (SGE, Supelco, USA). The syringes were placed in a syringe pump (Harvard PHD2000, Harvard Applications, USA) for infusion of the solutions into the microfluidic chip. Outlets were connected to a waste vial by fused silica capillary.

A rectangular, 4 x 4 x 5 mm^3 neodymium-iron-boron magnet was placed on top of the chip, beside the reaction chamber (Fig. 3a). The magnetic flux density over the chamber was simulated using FEMM 4.0 freeware (http://femm.foster-miller.net), and is shown in Fig. 3b.

2.3 Visualisation, detection and analysis

Visualisation of the particles was achieved by using an inverted fluorescence microscope (TE2000-U Eclipse, Nikon, Japan) with a 20x objective. Videos and stills were recorded using either a conventional CCD camera with low sensitivity (MTV, Mintron, Taiwan), or a more sophisticated, high sensitivity CCD camera (EXL, Qimaging, UK). The auto-adjustment was disabled on both cameras.

Images and videos of particles were

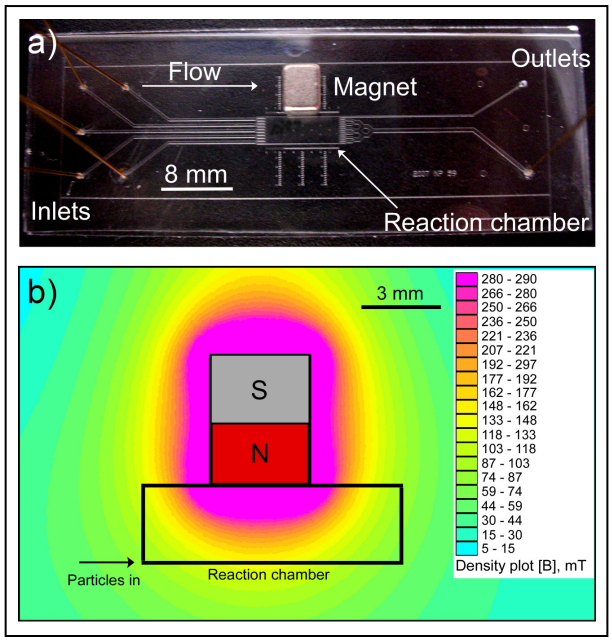

Fig. 3. (a) Photograph of the microfluidic chip and magnet position. (b) Simulation of the magnetic field across the reaction chamber.

analysed using ImageJ software (http://rsbweb.nih.gov/ij/). Fluorescence intensities were determined by measuring the greyscale values of the particles before and after reactions had occurred.

2.4 On-chip experimental procedures

General reaction and assay procedures:

Glass microchips were flushed first with deionised water to remove air from the system, then with potassium hydroxide to render the surfaces negatively charged for the purpose of preventing particles from sticking to the glass. The chip was flushed again with water, before introducing the appropriate reagents and washing buffers via syringes, as described in Section 2.2. Equal pressure was applied to each syringe to generate stable flow streams, and the applied flow rates were 15 μL h^{-1} per syringe, which corresponded to an overall flow velocity of 350 μm s^{-1} in the reaction chamber.

For the one-step peptide synthesis experiment, the glass surface was treated with a silanising agent to render the surface uncharged and hydrophobic to prevent particles sticking. Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma-Aldrich) was diluted to a concentration of 58 μL mL^{-1} in 2,2,4-trimethylpentane. The chip was first

flushed with acetone and air, followed by 2,2,4-trimethylpentane for 2 min. The silanising solution was pumped through the chip for 10 min, washed out with 2,2,4-trimethylpentane for 2 min, acetone for a further 2 min, and finally water.

Prior to each of the experiments, off-chip tests were performed by mixing the particles with the appropriate reagents and measuring the fluorescence intensity before and after to ensure the reaction proceeded as expected.

Generation of multi-laminar flow streams and diffusion experiments:

To test that laminar flow was achieved in the reaction chamber of the microfluidic device, blue ink was pumped through inlets 1, 3 and 5, and red ink through inlets 2 and 4.

To study the extent of diffusion between streams, iron (III) sulphate (0.5 M, Sigma-Aldrich) and potassium thiocyanate (0.75 M, Sigma-Aldrich) were pumped into the chamber through alternating inlets, and the effect studied by microscopy. Still images of the streams were taken and the greyscale intensities across the chamber were analysed with ImageJ.

The ability to generate multisolvent and multiphase streams was also investigated, with alternating streams of water and organic solvents pumped into the chamber. The organic solvents used, in separate experiments, were: acetone, ethanol, acetonitrile, propan-2-ol and cyclohexane, each purchased from Fisher Scientific (Leicestershire, UK).

Two-step mouse IgG sandwich immunoassay:

Biotinylated mouse anti-human CD4 IgG (AbD-Seratec, UK) and goat anti-mouse IgG labelled with Alexa Fluor 488 fluorescent dye (Invitrogen) were diluted in PBS buffer, with 0.01 % w/v BSA was added to all solutions.

Streptavidin-coated magnetic particles were introduced into inlet 1, biotinylated mouse IgG (10 μg mL^{-1}) in inlet 2, goat anti-mouse IgG with Alexa Fluor 488 fluorescent dye (10 μg mL^{-1}) in inlet 4, and PBS washing buffer in inlets 3 and 5.

One-step DNA hybridisation:

Biotinylated DNA probe primers (oligonucleotide sequence: 5'-CAC TAT TCT TTA CAG AGC CAC GGG-3') and complimentary single-stranded DNA primers labelled with Alexa Fluor 555 fluorophore (5'-CCC TGG GCT CTG TAA AGA ATA GTG-3') were purchased from Eurofins MWG (Germany). All solutions were prepared in 10x SSC buffer, and BSA added to a concentration of 0.01 % w/v.

Streptavidin-coated magnetic particles were reacted with biotinylated ssDNA off-chip, such that the ssDNA was bound to the particle surface. The particles were then introduced into the reaction chamber via inlet 1, complimentary ssDNA labelled with Alexa Fluor 555 fluorescent dye (1 μM) through inlet 2, and 10x SSC buffer through inlets 3, 4 and 5.

Two-step DNA hybridisation:

Complimentary DNA sequence primers (5'-CCC TGG GCT CTG TAA AGA ATA GTG-3') were purchased from Eurofins. PicoGreen© intercalator, a dsDNA (double-stranded DNA) fluorescent stain, was purchased from Invitrogen. Solutions of both were prepared in 10x SSC buffer, and 0.01 % w/v BSA added.

Streptavidin-coated magnetic particles were introduced into inlet 1, biotinylated ssDNA (0.5 μM) into inlet 2, PicoGreen© intercalator (200x dilution of stock solution) in inlet 4, and 10x SSC washing buffer in inlets 3 and 5.

One-step peptide synthesis:

EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Sigma-Aldrich) was dissolved in cold water immediately before use. Fluorescently labelled glycine (fluoresceinyl glycine amide, Invitrogen) was dissolved to a concentration of 20 μg mL^{-1} in 25 mM MES buffer (2-(N-morpholino)ethanesulfonic acid, pH 5, Sigma-Aldrich). Tween 20 was added to each solution to a concentration of 0.1 % w/v.

Off-chip, 100 μL of 100 mM sodium hydroxide was added to 100 μL of carboxylic acid-functionalised magnetic particles (2 x 10^6

particles mL^{-1}) and incubated with slow rotation for 10 min. The particles were collected via an external magnet, the supernatant removed, the particles washed three times with water and then resuspended in 200 µL of freshly prepared EDC (20 µg mL^{-1}). The suspension was incubated for 30 min, the supernatant was removed and the particles were washed quickly with cold water, 25 mM MES buffer, and finally resuspended in cold MES buffer (25 mM). This particle suspension was introduced into the reaction chamber of a silanised chip via inlet 1. Fluoresceinyl glycine amide was introduced via inlet 3, and 25 mM MES buffer pumped through inlets 2, 4 and 5.

3. Results and discussion

3.1 Generation of multi-laminar flow streams

To demonstrate the stability of the laminar flow regime in the system, alternating streams of aqueous red and blue ink were pumped into the reaction chamber of the microfluidic device (Fig. 4a). The figure shows five well-defined parallel streams as they flow the length of the reaction chamber.

The potential for performing reactions in multisolvent and multiphase systems was also investigated by generating alternating streams of water and organic solvents. Multisolvent systems included the use of water-miscible solvents such as acetone, ethanol, acetonitrile and propan-2-ol, and the multiphase system incorporated the use of water-immiscible cyclohexane (Fig. 4b). In each case, stable laminar flow streams were successfully generated at 350 µm s^{-1}, indicating that many procedures could potentially be undertaken in organic phases in future experiments.

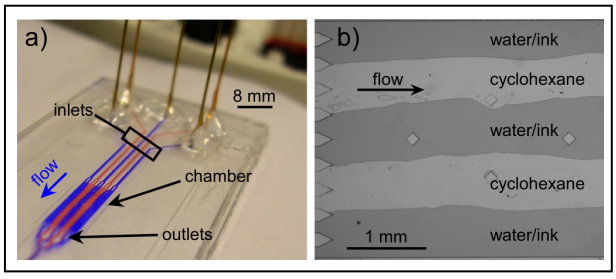

Fig. 4. (a) Generation of aqueous laminar flow streams using red and blue ink. (b) Alternating streams of immiscible fluids; cyclohexane and water.

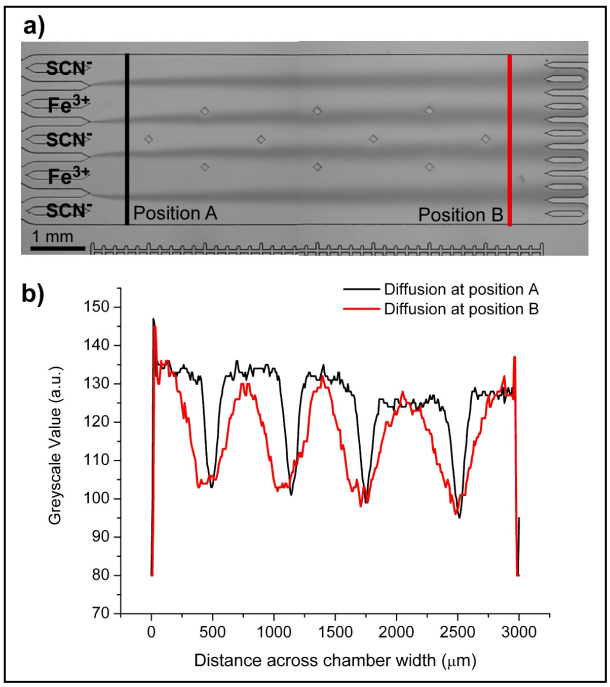

Fig. 5. (a) Diffusion between laminar flow streams. The dark grey regions illustrate the diffusion area. (b) Greyscale intensity plot showing the extent of diffusion at positions A and B. The peaks show that there is no mixing of reagents across the washing streams.

3.2 Diffusion between reagent streams

Cross-contamination of reagents is an important factor that must be avoided in any (bio)chemical procedure. This could be a problem in the continuous flow system if one reagent stream were to diffuse into another. To reduce the possibility of this happening, washing buffer streams were present between each reagent stream to separate them, and also to remove any unreacted reagents from the particle surfaces.

Alternating streams of iron (III) sulphate and potassium thiocyanate were pumped into the reaction chamber at a flow rate of 350 µm s^{-1}. As the two colourless streams diffused into each other, they reacted to form the dark red iron thiocyanate complex (Fig. 5a). Thus, the extent of diffusion between the streams was measured by determining the width of the newly-formed dark red diffusion cone. Still images of the streams were taken near the inlets (position A) and near the outlets (position B), and the greyscale intensity across the width of the chamber was plotted at both positions using ImageJ software (Fig. 5b).

The plot clearly shows that the diffusion

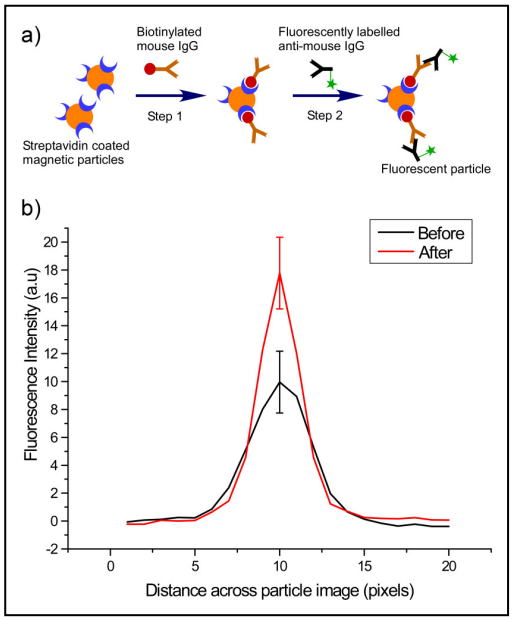

Fig. 6. (a) Reaction scheme for the mouse IgG sandwich immunoassay. (b) Fluorescence intensity of particles before entering the mouse IgG stream, and after exiting the fluorescently labelled anti-mouse IgG stream.

between streams was not great enough to allow cross-contamination between reagent streams in later experiments.

3.3 Two-step mouse IgG sandwich immunoassay

Previously, the on-chip binding of particle-bound streptavidin to fluorescently labelled biotin was demonstrated using our system. Here, we further investigate its applicability to biochemical processes by performing a two reaction step sandwich immunoassay.

Streptavidin-coated magnetic particles were first deflected through a stream of biotinylated mouse IgG (0.1 μg mL^{-1}), before entering a washing stream to remove any unbound reagent (Fig. 6a). The particles were then deflected through a stream of goat anti-mouse IgG that was fluorescently labelled with Alexa Fluor 488, and into a final washing stream. Upon completion of the two reaction steps, the particles showed an increase in fluorescence, as shown in Fig. 6b.

The entire immunoassay procedure, including all reaction and washing steps, was performed within 60 s, a significant decrease from conventional sandwich assays that can take several hours to perform. The reaction was able to occur rapidly as the particle surface was exposed to a constant supply of fresh reagents as it traversed the reagent stream, while any unbound species were washed away. Furthermore, the total reagent consumption during a 10 min assay was only 7.5 μL, a significant reduction in the volumes usually required for such assays.

3.4 One-step DNA hybridisation

The hybridisation of particle-bound ssDNA (single-stranded DNA) to its fluorescently labelled complimentary ssDNA was investigated using the multi-laminar flow system. The particles were deflected through the complimentary ssDNA stream and into an SSC buffer washing stream. Images were taken of the particles before entering and after leaving the labelled ssDNA stream, and the fluorescence intensities of the particles were measured (Fig. 7a).

The results are shown in Fig. 7b, which clearly shows the increase in fluorescence intensity after the particles have passed through the fluorescent stream, indicating that

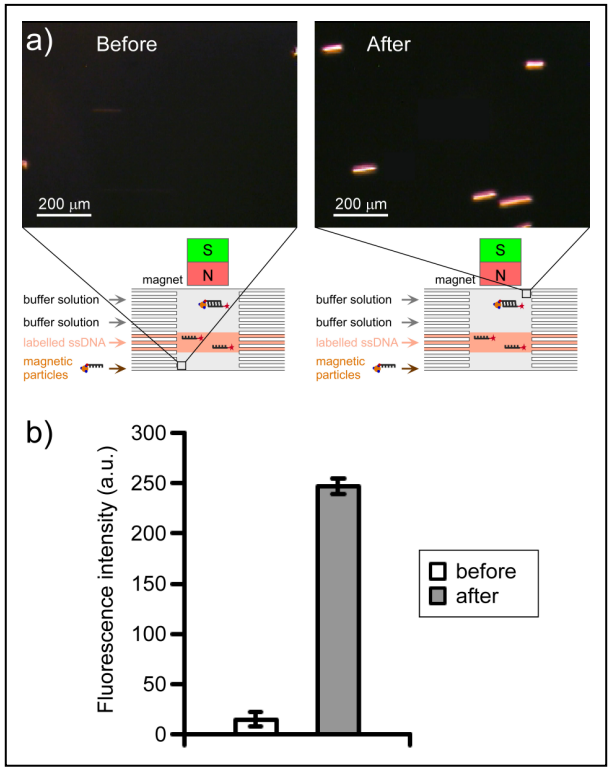

Fig. 7. (a) Photographs of particles before (left) and after (right) DNA hybridisation in continuous flow. (b) Fluorescence intensity of particles after passing through labelled ssDNA stream.

DNA hybridisation was successful. The particles traversed the fluorescent stream in 16 s, which was sufficient time for hybridisation to take place, and the entire process was performed within one minute. This was a significant reduction in procedural time compared to conventional methods that can require at least an hour.

3.5 Two-step DNA hybridisation

The on-chip DNA hybridisation process was further expanded to include an extra reaction step. Here, the complimentary DNA itself was not labelled, as a subsequent reaction step introduced an intercalator, PicoGreen©, that becomes fluorescent upon binding to dsDNA.

The ssDNA-functionalised particles were introduced into the reaction chamber and deflected through the complimentary ssDNA stream to form double-stranded DNA (dsDNA) on the particle surface. The particles entered a washing stream before passing through the PicoGreen©, where the intercalator bound to the dsDNA (Fig. 8a). The particles entered the final washing stream and their fluorescence intensities at this point were determined. These were compared to the fluorescence intensities of ssDNA-functionalised particles that, in a separate experiment, were deflected through a stream of PicoGreen© without having first passed through a complimentary ssDNA stream.

Fig. 8b shows that the particles exhibited an increase in fluorescence after the two reaction steps had occurred, indicating that two-step DNA hybridisation was successful with an intercalator. Again, the entire assay, including both reaction and washing steps, was performed within one minute.

3.6 One-step peptide synthesis

Previous experiments concerned the use of the microfluidic device for bioreactions. Here, we investigated the potential of performing chemical reactions for the synthesis of peptide bonds. Carboxylic acid-functionalised magnetic particles were reacted with EDC off-chip and introduced into inlet 1 in the chip. Fluorescently labelled glycine was introduced into inlet 3, and the particles were drawn through the stream. As the particles traversed the reagent stream, the EDC on the particles was substituted by the glycine molecules, which formed a peptide bond with the carbonyl group on the particle (Fig. 9a). Thus, when the particles left the reagent stream and entered the final washing streams, they showed an increase in fluorescence compared to particles before the reaction (Fig. 9b).

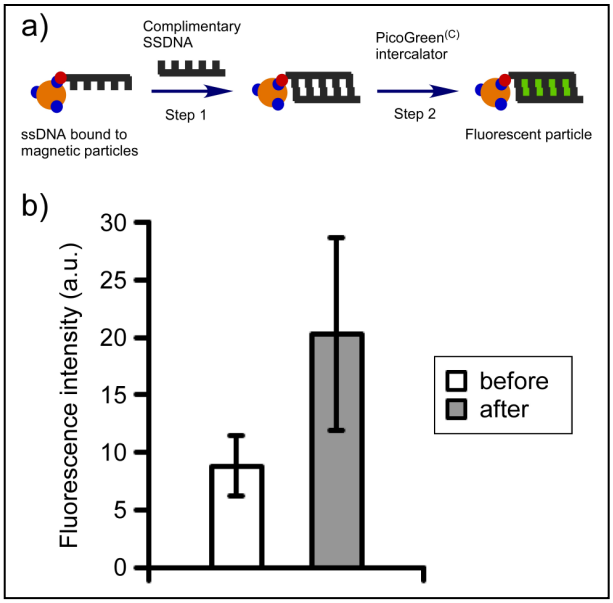

Fig. 8. (a) Reaction scheme for the two-step DNA hybridisation. (b) Fluorescence intensities of particles before entering the complimentary ssDNA stream, and after leaving the intercalator stream.

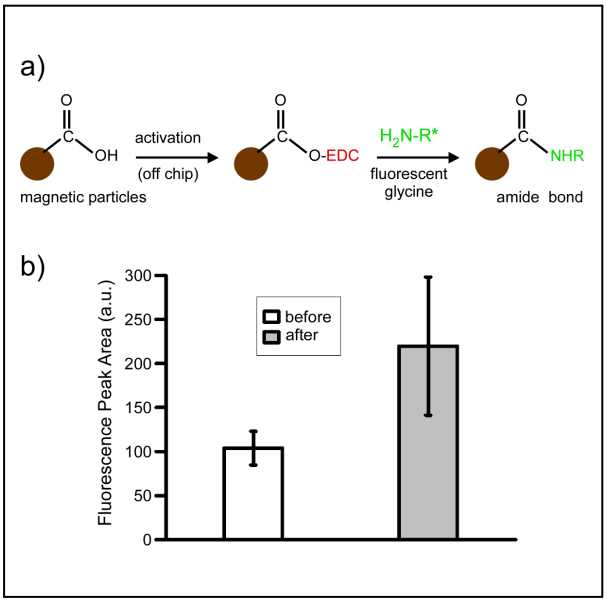

Fig. 9. (a) Reaction scheme of the one-step peptide synthesis procedure. (b) Fluorescence intensity of particles before entering and after leaving the fluorescent glycine stream.

One-step peptide synthesis, involving a chemical reaction rather than a binding step, was performed within one minute using the system, a significant improvement on the 30 min required using conventional methods. The method could be used to create longer peptide sequences by simply increasing the number of amino acid reagent streams. These results also show the potential for performing a range of other organic syntheses using our system.

4. Conclusions

We have demonstrated a microfluidic platform in which fast, sequential (bio)chemical reactions can be performed on the surfaces of magnetic particles in continuous flow. We have so far used the system for mouse IgG sandwich immunoassays, one- and two-step DNA hybridisation. In addition to these biochemical processes, we have also demonstrated the application of the system to the synthesis of peptide bonds. This demonstrates a highly versatile microfluidic platform capable of greatly reducing procedural times compared to conventional batch methods, whilst only consuming nanolitres of potentially expensive reagents.

Acknowledgements

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