RAPID, MULTISTEP DNA HYBRIDISATION IN CONTINUOUS FLOW.
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ABSTRACT
We present a microfluidic platform for performing fast, continuous flow DNA hybridisation on magnetic particles. Laminar flow streams containing sample oligonucleotides, washing buffer, fluorescently labelled oligonucleotides and washing buffer, were generated in the chamber of a microfluidic chip. Magnetic particles were then introduced into the chamber, and pulled through each of the streams by a permanent magnet placed opposite the particle inlet. DNA hybridisation was successfully performed, as determined by resulting increased fluorescence of the particles. A non-complementary sequence did not yield any signal, showing non-specific binding was minimal. The whole procedure required only several minutes, a significant reduction compared to conventional methods. A detection limit of 20 nM was achieved.

KEYWORDS: DNA hybridisation, continuous flow, magnetic particles

INTRODUCTION
DNA hybridisation detection is an important tool for clinical or forensic diagnostics and is often performed on DNA microarrays, also called gene chips. Such a method requires expensive instrumentation and further amplification for verification of the results. Another approach is to use particles as solid supports for the hybridisation, where the capture probe is immobilised on the surface of the particles. A high density of the probe can be achieved on the particles, resulting in high sensitivity. However, such particle based methods involve many repetitive reaction and washing steps, rendering the whole procedure laborious and time-consuming. Many different microfluidic systems have been developed for DNA hybridisation, either homogeneous (in solution) [1] or heterogeneous (immobilised capture) [2]. The limitation of the heterogeneous systems is that they are all batch procedures, and therefore often have long procedural times. The homogeneous systems found in the literature require careful microfluidic design and fabrication to provide sufficient mixing, and also showed high detection limits.

We have developed a microfluidic platform for continuous flow particle based processing, using magnetic particles as solid supports [3]. Previously, we have demonstrated the performance of the device for one-step DNA hybridisation with a detection limit of 20 nM for fluorescently labelled oligonucleotide [4]. Here, we present a two-step DNA hybridisation using a “sandwich” format, in which the sample DNA is unlabelled. When the particles enter the chip via inlet 1 (see Fig. 1), they are attracted to a magnet placed on the opposite side of the chamber and move through laminar flow stream containing sample oligonucleotides, washing buffer, labelling oligonucleotides (modified with AlexaFluor555) and final washing buffer, respectively. Consecutive hybridisation and washing can thus be performed in continuous flow on particle surface.

THEORY
The principle of the continuous flow system is shown in Fig. 1. The deflection of magnetic particles across the chamber depends on the magnetic field strength and gradient, the magnetic content of the particles and their radius, and on the velocity of the laminar flow streams. Here, monodispersed commercial particles were employed, hence there should be little variation in the magnetic content of the particles. Without the presence of a magnetic field, the particles simply follow the laminar flow streams along the chamber. However, in the presence of a field, they are pulled across

Figure 1: Principle of the continuous flow DNA hybridisation procedure using magnetic particles. Particles with immobilised capture probe are introduced into the chip via inlet 1, reagents and washing buffer via subsequent inlets. Particles were deflected across the chamber, towards a magnet placed on the opposite side of the chamber. Fluorescence measurements were taken in the final stream.
the chamber through the different laminar flow streams. The resulting deflection velocity of a particle at any point is given by Equation (1).

\[ u_{\text{res}} = u_{\text{hyd}} + u_{\text{mag}} \]

Eq. (1)

where \( u_{\text{hyd}} \) is a velocity of the flow and \( u_{\text{mag}} \) is the magnetically induced velocity of a particle, given by Equation (2).

\[ u_{\text{mag}} = \frac{V \Delta \chi (\mathbf{B} \cdot \nabla \mathbf{B})}{6 \pi \eta d \mu_0} \]

Eq. (2)

where \( V \) (m³) is volume of the magnetic material in a particle, \( \Delta \chi \) is the difference of magnetic susceptibility between the magnetic material and the media, \( \mathbf{B} \) (kg m⁻² A⁻¹) is the magnetic flux density, \( \eta \) (kg m⁻¹ s⁻¹) is the dynamic viscosity of the medium, \( d \) (m) is the hydrodynamic radius of the whole particle and \( \mu_0 \) (4\pi \times 10⁻⁷ kg m s⁻² A⁻²) is the permeability of free space. Equation 2 arises as a result of the equilibrium between magnetic and hydrodynamic drag forces acting on a particle.

DNA hybridisation relies on Watson-Crick base pairing to recognise specific sequences. The specificity however depends on conditions such as temperature, salt concentration or addition of other compounds e.g. formamide. Generally, temperatures close to the melting point of the sequence are used. However, varying salt concentrations can provide the same effect even at room temperature.

**EXPERIMENTAL**

The chip design (Fig. 2 (A)) featured five inlets, two outlets and a 3 mm x 8 mm reaction chamber, supported by 10 diamond shaped pillars. The microfluidic chip was fabricated in glass by conventional photolithography and wet-etching techniques with an etch depth of 22 μm. The chip was placed in a chip holder and fused silica capillaries were attached into the inlet and outlet holes by NanoPort Ferrules and Super Flangeless TinyTight PEEK nuts (both from Presearch, UK). At the inlets, tubing was dipped into Eppendorf tubes containing particle suspension, oligonucleotide solution or buffer solutions. The remaining outlet was interfaced to a gas-tight syringe (SGE, Supelco, USA) mounted on a syringe pump (PHD 2000, Harvard Apparatus, USA) operating in withdrawal mode at a flow rate of 50 μL h⁻¹, corresponding to a velocity of 210 μm s⁻¹ in the reaction chamber. The magnetic field was generated by a NdFeB magnet (4 mm x 4 mm x 5 mm, grade N48H, Magnet Sales, UK) placed next to the chip, 1 mm from the side of the reaction chamber as shown in Fig. 2 (B). The chip was mounted on the stage of an inverted fluorescence microscope (TE2000-U, Nikon, Japan) equipped with a CCD camera (Retiga-EXL, QImaging, UK) and a G-2A epifluorescent filter (excitation window: 510-560 nm, dichroic mirror: 575 nm, emission filter: 590 nm). For fluorescence intensity measurements the exposure time of the camera was set to 300 ms. Video sequences of single magnetic particles were recorded near the exit of the chamber closest to the magnet using ImagePro 6.2 software, and particle grey scale intensities were measured using ImageJ software (http://rsbweb.nih.gov/ij). Agglomerated particles were excluded from the measurements. All experiments were conducted at ambient temperature (24 °C).

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Saline sodium citrate buffer (Sigma, UK) 20x concentrated stock was diluted once to yield 10x concentrated working stock, and pH was adjusted to 7.4 for typical experiment. Oligonucleotides (Table 1) were purchased from Eurofins MWG Operon, Germany. Capture probe was biotinylated and the labeling oligonucleotide was modified with Alexafluor555. Sample oligonucleotide was unmodified. Superparamagnetic particles (M-270 Streptavidin Dynabeads) were purchased from Invitrogen, and were diluted 100 times in SSC buffer prior the experiments.

RESULTS AND DISCUSSION
As the magnetic particles entered the reaction chamber, they were deflected through each of the reagent and washing streams by the external magnet. As they traversed the second stream, capture probe immobilised on particles surface hybridised to the sample oligonucleotide contained in the stream. After being washed in the following buffer stream, labelling oligonucleotide became hybridised to the sample oligonucleotide that was now bound to the particle. Fluorescence measurements of the particles taken in the final washing stream showed an increase in fluorescence intensity of the particles, indicating that non-specific DNA binding was negligible. Fluorescence signals for fully complementary and 3-base mismatch sequences were not resolved. The fluorescence signal also slightly decreased, when lower buffer concentration was used. Sodium chloride is added to facilitate the hybridisation, as the DNA molecules are negatively charged and the sodium ions help to overcome the electrostatic repulsion between the DNA molecules. A too high concentration of salts can however cause denaturation of DNA, hence optimal conditions have to be determined.

CONCLUSION
We have demonstrated a microfluidic, multilaminar flow procedure for performing two-step DNA hybridisation on magnetic particles in continuous flow. No labelling of the sample oligonucleotide prior to the experiment was required, and no manual intervention was required throughout the hybridisation procedure. The specificity of the hybridisation will be improved as various conditions such as temperature, salt concentration or addition of denaturing agents such as formamide are evaluated.

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REFERENCES

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