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Amplification-free detection of Herpes Simplex Virus DNA†

David A. C. Thomson,ab Krassen Dimitrova and Matthew A. Cooperab

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Amplification-free detection of nucleic acids in complex biological samples is an important technology for clinical diagnostics, especially in the case where the detection is quantitative and highly sensitive. Here we present the detection of a synthetic DNA sequence from Herpes Simplex Virus-1 within swine cerebrospinal fluid (CSF), using a sandwich-like, magnetic nanoparticle pull-down assay. Magnetic nanoparticles and fluorescent polystyrene nanoparticles were both modified with DNA probes, able to hybridise either end of the target DNA, forming the sandwich-like complex which can be captured magnetically and detected by fluorescence. The concentration of the target DNA was determined by counting individual and aggregated fluorescent nanoparticles on a planar glass surface within a fluidic chamber. DNA probe coupling for both nanoparticles was optimized. Polystyrene reporter nanoparticles that had been modified with amine terminated DNA probes were also treated with amine terminated polyethylene glycol, in order to reduce non-specific aggregation and target independent adhesion to the magnetic particles. This way, a limit of detection for the target DNA of 0.8 pM and 1 pM could be achieved for hybridisation buffer and CSF respectively, corresponding to 0.072 and 0.090 femtomoles of target DNA, in a volume of 0.090 mL.

Introduction

The Herpesviridae are a large family of DNA viruses, whose members can cause several diseases including oral and genital herpes, chicken pox, shingles, glandular fever and congenital cytomegalovirus disease. The infections are typically characterised by a long-term latency, which on reactivation, are able to cause significant morbidity and mortality, especially in patients with compromised immune systems, such as organ transplant recipients and HIV infected individuals. In addition, infection of the central nervous system and sensory organs by members of these Herpes viruses can cause severe outcomes for patients.1 Encephalitis caused by Herpes Simplex Virus (HSV) is the most common non-seasonal encephalitis and occurs in an estimated 2.2 people per million, per year.2 Patients with suspected Herpes Simplex Encephalitis (HSE), are usually immediately prescribed with antiviral drugs, as the mortality rate in untreated patients can be as high as 70%.3

Detection of Herpes Simplex Virus (HSV) in human cerebrospinal fluid (CSF) was one of the first clinical applications of Polymerase Chain Reaction (PCR).4 This and other nucleic acid amplification methods were shown to be superior to serological or viral cultural analysis techniques, as they offered a more accurate indication of viral infection or reactivation. Serology can be problematic as antibody titre can be diminished in immune-compromised patients5 and may lead to the risk of false negative results. However, target amplification methods also have their limitations, even though they possess excellent sensitivity6 and specificity. Their limitations are: (a) the risk of false positives arising from contamination during sample preparation, (b) requirement for implementation of temperature-controlled instrumentation, and (c) the risk of false negatives due to sample contamination with PCR inhibitors. Furthermore, many of the PCR based tests for HSV are custom protocols which exhibit considerable variability from one laboratory to another, complicating inter-laboratory comparison.7

In contrast to target amplification, signal amplification involves techniques in which a label is bound directly to the target molecule, creating a signal sufficiently strong that it can be resolved against a system background. Many nanoparticle based detection systems have been developed for nucleic acid assays, for instance (a) DNA functionalised gold colloid which exhibits colour changes upon target induced aggregation,8 (b) oligonucleotide functionalised gold as in the DNA-bar-code assay,9 (c) nanoparticles with time-resolvable fluorescence properties10 and (d) electrochemically detected nanoparticle labels.11 These labels are typically analysed by either a digital single particle method12 or by an analogous approach determining the net aggregate signal. The sensitivities for these assays range across many logs of dynamic range with the highest performance effectively approaching single molecule sensitivity (Table 1).
One key differentiator among these techniques is whether target capture and its detection are conducted on a solid support, similar to a microarray format, or in solution phase. Solid support formats enable extensive multiplexing but are limited by long capture incubation times, in order to compensate limited mass-transport by diffusion. These limitations can be overcome by using particles, and especially nanoparticles with their enhanced surface area and faster diffusion rates.

The methodology reported here comprises a monoplex nanoparticle assay in which DNA capture probes immobilised on 500 nm magnetic nanoparticles are hybridised with the target DNA, in this case synthetic target DNA sequences derived from HSV. The hybridisation with the magnetic nanoparticles was followed by a second hybridisation with a reporter DNA probe coupled to fluorescently labelled polystyrene 200 nm particles (Fig. 1). The resulting sandwich structure, consisting of magnetic and fluorescent nanoparticles linked by target DNA, was magnetically extracted and then washed to remove unbound fluorescent particles. A direct counting method was developed, utilising microfluidic chambers and confocal fluorescence microscopy, which enables the quantitative analysis of the target DNA concentration (Fig. 2). Coupling methods and buffer components were first optimised using an aggregate fluorescence intensity method before detection using the microfluidics chambers, first in a buffer and then in the cerebrospinal fluid.

Materials and methods

Probe and target sequences

Virus specific DNA sequences for HSV were adapted from the genes quantitatively amplified by Stocher et al.\textsuperscript{13} The probe length was extended by flanking sequences of the virus genome to obtain a 37 basepair target sequence with complementary capture and reporter probes (Table 2). The sequences were analyzed with IDT SciTools Oligoanalyzer\textsuperscript{14} to determine the secondary structure of hairpins and homodimers. All nucleic acids used in the study were purchased from Integrated DNA Technologies (Coralville, USA).

**Fluorescent nanoparticle probe coupling**

Reporter DNA probe coupling studies were conducted to determine optimal concentration of DNA probe in the coupling

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**Table 1** Comparison of sensitivity for various amplification free nucleic acid hybridisation assays

<table>
<thead>
<tr>
<th>Assay format</th>
<th>Detection</th>
<th>Sensitivity</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target dependent aggregation of gold nanoparticles</td>
<td>Evanescent wave induced scattering with colour change indicating target presence</td>
<td>$3.3 \times 10^{-19}$</td>
<td>8</td>
</tr>
<tr>
<td>Target capture on magnetic beads followed by binding dual labelled gold nanoparticles</td>
<td>DNA barcode detection on solid phase using silver precipitation on gold nanoparticles enhanced optical scattering</td>
<td>$5.0 \times 10^{-19}$</td>
<td>9</td>
</tr>
<tr>
<td>Magnetic bead capture of target DNA molecules followed by probe binding</td>
<td>Europium (iii) nanoparticle label with time resolved fluorescence</td>
<td>$1.0 \times 10^{-18}$</td>
<td>10</td>
</tr>
<tr>
<td>Target capture followed by magnetic extraction and labelling with latex particles</td>
<td>Counting of magnetically pulled down 850 nm latex particles</td>
<td>$1.7 \times 10^{-18}$</td>
<td>15</td>
</tr>
<tr>
<td>Target capture and magnetic extraction followed by nanoparticle labelling</td>
<td>Enhanced chemiluminescence with AuNP–luminol–AgNO$_3$</td>
<td>$6.0 \times 10^{-17}$</td>
<td>16</td>
</tr>
<tr>
<td>Magnetic bead capture and extraction (current work)</td>
<td>Fluorescent nanoparticle and aggregate counting</td>
<td>$7.2 \times 10^{-17}$</td>
<td>11</td>
</tr>
<tr>
<td>Solid phase capture with target displacement by oligolabelled nanoparticles</td>
<td>Signal generated by stripping voltammetry of metal sulfide nanoparticle label</td>
<td>$1.0 \times 10^{-18}$</td>
<td>11</td>
</tr>
</tbody>
</table>

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**Fig. 1** Schematic of nanoparticle functionalization with HSV specific capture and reporter probes. (A) Phosphorylated capture probes were first ligated with 166 basepair biotinylated DNA molecules and then subsequently immobilized onto 500 nm streptadivin coated magnetic beads (red). (B) Amine labeled reporter probes were coupled to 200 nm fluorescent polystyrene nanoparticles (green) using EDC as a coupling agent. Subsequently, amine terminated polyethylene glycol molecules were coupled to the bead surface to reduce non-specific particle interactions. (C) Target HSV DNA linked to the magnetic and fluorescent particles resulting in a DNA dependent dose response.
reaction using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Yellow-Green 200 nm, carboxylic acid functionalised polystyrene fluorospheres (Invitrogen, F8811, LOT759339; 4.5 × 10^{12} mL^{-1}) were washed three times into 60 mM 2-(N-morpholino)ethanesulfonic acid (MES Buffer), pH 5.5 with a Quantum Scientific Sigma Centrifuge at 14 000 rpm for 15 min before being resuspended into 100 μL at 1.1 × 10^{12} pr mL⁻¹. Particles were sonicated for 1 min after the final resuspension. The loading of the DNA probe was tested by adding amine terminated HSVp2 reporter probes, at three different concentrations: 6.0 × 10^{12}, 8.0 × 10^{12}, 1.0 × 10^{13} and 1.2 × 10^{13} probes per cm². A second 8.0 × 10^{12} probes per cm² reaction was prepared for the comparison of coupling O-(2-aminoethyl)-O'-methylpolyethylene glycol (Sigma, 07964-250MG, hereafter referred to as amine-PEG) to the particle surface. Freshly prepared 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to the coupling reaction to a final concentration of 5 mM. After 45 mins, another 500 nanomoles of EDC was added with particles incubated on a roller shaker for 105 min in darkness. The amine-PEG was coupled onto the fluorospheres for 30 minutes at 3 × 10^6 molar excess to nanoparticles equivalent to a concentration of 5.7 mM. Finally, any remaining carboxylic acid groups were capped with ethanolamine for 30 min at 100 mM. The particles were then washed four times in 0.4× SSC (60 mM NaCl, 6 mM trisodium citrate), 0.1% Triton X-100 pH 8, with final resuspension to give 7.3 × 10^{10} particles per mL. Batch couplings were conducted with 300 μL of fluorosphere stock using identical incubation times and concentrations with 10^{13} probes per cm² as the coupling density and with amine-PEG treatment.

**Magnetic nanoparticle probe coupling**

Streptadivin coated 500 nm diameter Masterbeads from Ademtech (Pessac, France, ~6.4 × 10^{10} particles per mL) were used throughout the study (StreptaDivin is neutralized form of avidin without carbohydrates and tripeptide sequence Arg–Tyr–Asp (RYD), these features provide for very low non-specific binding). The particles were coupled to a 166 basepair (bp) double stranded DNA linker with a single stranded capture probe (HSVp1) ligated to its terminus. Ligation of the capture probe before or after coupling the linker to the particle was compared. This was followed by the determination of optimum double stranded linker to magnetic bead biotin binding capacity. The double stranded linker was first hybridised using equimolar quantities of the single stranded ultramers, Ultra_DT_002 and Ultra_DT_004 (Table 2) in 200 μL of 300 mM NaCl, 30 mM trisodium citrate (pH 8), heating to 95 °C and passively cooling within a heat block over 120 min. Linker loading titrations were conducted at 0.6, 0.8, 1 and 1.2 molar excess of the biotinylated oligonucleotide binding capacity (2.4 × 10^{12} biotins per cm²) of the particles for both on and off bead ligation. The linker coupling was performed in 5× SSC, 0.1% Triton X-100 (750 mM NaCl, 75 mM trisodium citrate, pH 8) for 180 min on a roller. For the on-bead ligation, particles were washed three times into T4 DNA Ligase Buffer (50 mM Tris–HCl, 10 mM MgCl2, 1 mM ATP, 10 mM dithiothreitol, pH 7.5, New England Biosciences). The HSVp1 capture probe was added at 10 molar equivalents to the linker and ligated with 0.5 μL of 400 000 per mL cohesive end units of T4 DNA ligase (NEB) at room temperature, on a roller shaker for 180 min. Off-bead ligation...
was conducted for 120 min with 10× molar excess of HSVp1 to the linker with 0.5 μL of T4 DNA ligase in 50 μL of DNA ligase buffer. The product of the ligation reaction was added directly to the magnetic beads for coupling via the biotin. Following ligation and coupling the beads were washed with 0.4× SSC, 0.1% Triton X-100, pH 8. It was assumed that 20% of the particles were lost in the linking process. Particles were stored ready-for-use at 4°C.

Batch couplings were conducted with 1.2 times molar excess of linker to biotin binding capacity with ligation conducted prior to coupling to the particle surface with 10 molar equivalents of capture probe HSVp1.

Table 2 Target, capture and reporter probe sequences for Herpes Simplex Virus

<table>
<thead>
<tr>
<th>DNA molecule</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSVTar</td>
<td>NC_001865:6505-65569 (UL30 DNA Polymerase Gene) CAG CTG GCT GAA CGT CTT TTC GCA CTC GAG TTT GAT G, Mw 11 377.4, Max Hairpin –0.95 kcal mol⁻¹, Max Self Dimer –12.64 kcal mol⁻¹</td>
</tr>
<tr>
<td>HSVp1</td>
<td>/5Phos/CTG ACC TCC CTC GTT GGC GCC ATC AAA CTC GAG TGG ACA TTA GTG GCG TAT TGG GAC GAG GAC G</td>
</tr>
<tr>
<td>HSVp2</td>
<td>AAG ACG TTC ACC AAG CTG AAA AAA AAA AAA AAA/3AmMO/(54°C), Mw 10 401.9, Max Hairpin –0.2 kcal mol⁻¹, Max Self Dimer –6.34 kcal mol⁻¹</td>
</tr>
<tr>
<td>Ultra_DT_004</td>
<td>/5Bios/CCT GCT TCA AAT ACC CCA CGT ATA GGT CTA TCA CGA TAC TCT CTC AAG TTG CCT AGG ATT GAC AGT CAG GTC CGC GGG AGT TTA CCG TTT TAT ATG TCT ACC GAC GAA GTA TTA GCC ACA CGG CTC CGG TAG AGA CCT GTC TT, Mw 45 257.4</td>
</tr>
<tr>
<td>Ultra_DT_002</td>
<td>GCG CCA ACG AGG GAC ATC AGA AGA CAG GTC TCT ACC GGA GCG GTG TGC GAA ATA CTT CGT CGG TAG ACA TAT AAA AGC GTA AAC TCC CGC GGA CCT GAC TGT CAA TCC TAG GCA ACT TCA GAG AGT ATC GTG ATA GAC CTA TAC GTG GCC TAT TTG AAG CGG GAC G, Mw 51 488</td>
</tr>
<tr>
<td>HSVTar-iCy5</td>
<td>CAG CTG GCT GAA CGT CTT/ iCy5/TTC GCA CTC GAG TTT GAT G (Mw 44 864.1)</td>
</tr>
<tr>
<td>CMV Tar (HHV5)</td>
<td>NC_006273.2:121510-121546, TGG GAC ACA ACA CGG TAA AGC CTT TGC GCT CGG G</td>
</tr>
</tbody>
</table>

Accession number and position of target sequence in virus genome.

**Capture and reporter probe density**

Estimation of the capture and reporter DNA probe density was made on batch couplings for the magnetic and fluorescent nanoparticles using a flow cytometry saturation method. Cy5 labelled target DNA (HSVTar-iCy5) was hybridised to a fixed number of particles (1 × 10⁶ fluorospheres, 9.56 × 10⁴ magnetic beads) in a 180 min incubation (4× SSC, 0.1% Triton X-100, pH 7.9). A series of reactions were conducted with increasing quantities of fluorescent target with a range of 0 to 3200 femtomoles. The geometric mean of the fluorescence intensity (Red laser, 660/20 filter) of each bead as measured on a BD FACSCantoII was plotted to determine the fluorescent saturation quantity. The resultant data are presented in the ESI† yielding estimates for probes per particles of 2760/magnetic bead and 753/fluorescent particle with a surface coverage of 3.5 × 10¹¹ probes per cm² and 6.0 × 10¹⁰ probes per cm² for the magnetic and fluorescent particles, respectively.

**Hybridisation assays**

Hybridisation assays were conducted on a Biotek PrecisionXS robotic system using BD Falcon™ assay plate storage 96 well V-bottom polypropylene 96 well plates (cat. 353263). All bead and particle optimisation assays were conducted in a two step manner where the target DNA was first bound to magnetic beads (10⁻¹) in a 90 μL reaction, for 90 min, with robotic pipetting every five minutes to mix the samples and keep the particles suspended. 10 μL of fluorescent reporter particles (7.26 × 10⁸) was subsequently added, followed by another 90 minute hybridisation with robotic mixing every 5 minutes. Reactions were conducted, unless otherwise stated, in 4× SSC (600 mM NaCl, 60 mM trisodium citrate, 0.1% Triton X-100, pH 8, 25 μg mL⁻¹ single stranded calf thymus DNA (Sigma D8899-5 mg)). Following hybridisation, the particles were washed with five cycles of magnetic capture on a 96 position magnet rack (Agencourt Bioscience Corporation, AGN #32782 SPRIPlate) and resuspended into a final volume of 80 μL post-hybridisation buffer (0.4× SSC (60 mM, 6 mM trisodium citrate, 0.1% Triton X-100, pH 8, 25 μg mL⁻¹ calf thymus DNA) for aggregate plate reading analysis or 20 μL for injection into the microfluidic chamber. The buffer ionic strength experiment was conducted in 4×, 2.67×, 1.33×, 0.67×, 0.33×, 0.17× SSC in 0.1% Triton X-100 with washing conducted in 0.4× SSC 0.1% Triton X-100. The target DNA was typically serially diluted for final reaction concentrations of 500, 50, 5, 0.5, 0.05 pM and zero target control.

Surfactant selection was performed using 2 mM concentration of Tween20, Triton X-100, n-dodecyl-β-D-maltoside (DDM), cetyl trimethylammonium bromide (CTAB) and 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) with 4× SSC for the hybridisation reaction. Post-hybridisation wash steps were conducted in 0.4× SSC (60 mM NaCl, 6 mM trisodium citrate, pH 8).

**Microplate aggregate fluorescence assays**

Aggregate fluorescent measurements were made on a BMG Labtech PolarSTAR Omega with a 485 nm/10 nm excitation filter and 510 nm/10 nm emission filter. Samples were loaded into
Black 96-well Microtest polystyrene assay plate from BD (353241) for aggregate measurements.

**PDMS device fabrication**

The devices used to define a fixed volume of particles were designed in L-EDIT (Tanner Research, USA) and fabricated using standard SU8 and PDMS processing. Each device had eight chambers (5 × 1 mm) with a pitch of 1.125 mm and height of ~0.2 mm. SU-2100 (Microchem, USA) was used to make the mould for PDMS fabrication. Photosistor was spun (1; 15 s, 500 rpm, 133 rpm s⁻¹, 2; 45 s, 1500 rpm, 400 rpm s⁻¹) and soft baked (1; 65 °C, 6 minutes, 2; 95 °C 20 minutes). The wafer was then exposed through a photomask printed on a HY2 high precision photoplate (Konica-Minolta, Japan), with 314 mJ cm⁻² using an OAI blanket exposure system. The mask was printed with a high resolution Mivatec photoplotter (MIVA Technologies GmbH, Germany). Post exposure, wafers were baked for 5 min at 65 °C and 12 min at 95 °C and then developed in propylene glycol monoethyl ether acetate (PGMEA) until features were fully resolved. Sylgard 184 was mixed with cross-linker 10% w/w, vacuum degassed and poured over the mould for PDMS fabrication. Photosistor was spun (1; 65 °C, 6 minutes, 2; 95 °C 20 minutes). The wafer was then exposed through a photomask printed on a HY2 high precision photoplate (Konica-Minolta, Japan), with 314 mJ cm⁻² using an OAI blanket exposure system. The mask was printed with a high resolution Mivatec photoplotter (MIVA Technologies GmbH, Germany). Post exposure, wafers were baked for 5 min at 65 °C and 12 min at 95 °C and then developed in propylene glycol monoethyl ether acetate (PGMEA) until features were fully resolved. Sylgard 184 was mixed with cross-linker 10% w/w, vacuum degassed and poured over the mould and heated at 65 °C for 120 min. Devices were manually diced with fluid inlet and outlet holes punched prior to placing the device onto a glass cover slip for injection. Devices were washed for reuse in running water with gentle manual agitation.

**Particle and aggregate counting**

Particle and aggregate counting was conducted on an inverted laser scanning confocal microscope (Carl Zeiss, LSM710). Each chamber with the PDMS device was injected with particles and then placed on a high disk magnet (AussieMagnets, #3243; 50.8 mm diameter and 12.7 mm height) for 30 seconds. Particles were imaged with the pinhole set at 3.0 Airy Units using a 20 × 0.4 numerical aperture objective lens with particles excited at 5% laser power from a 488 nm laser with emission collected between 510 nm and 530 nm. The multi-time-series automation macro was utilised to image within each chamber and each of the eight chambers within a device. Images were processed with ImageJ, counting objects greater than 4 square pixels.

**CSF collection**

Swine cerebrospinal fluid was collected from the lumbar region of a sacrificed animal at the Herston Medical Research Centre, Brisbane. The sample was placed immediately on dry ice and frozen at −20 °C. When used, samples were defrosted and DNA was spiked and serially diluted to create the dose response. Ethics approval for CSF collection was obtained (see notes).

**Results and discussion**

Before the assay was conducted in the microfluidic chambers, the reaction conditions for capture and reporter DNA coupling to the nanoparticles were optimized. This optimization was done by variation of buffer conditions, including ionic strength and surfactant concentrations, and selecting conditions for efficient hybridisation and minimum background. The assay was then conducted in the microfluidic chambers, to further optimize the volume analysis and methods.

**Reporter probe coupling to fluorescent nanoparticles**

One of the key factors to achieve a high sensitivity and a linear dose response, is the concentration of the DNA probe on the nanoparticle. Hybridisation efficiency is influenced by the surface probe density, which for flat surfaces is typically in the range of 10¹²–10¹³ probes per cm². With increasing probe surface density, target diffusion and entry decreases, mainly due to electrostatic repulsion and steric hindrances, thereby decreasing the hybridisation efficiency. With this in mind it was important to optimise the probe concentrations in the coupling reactions. Carboxylic acid functionalized polystyrene particles, similar to those utilised in this report, were first coupled with single stranded DNA by Kremsky and Wolf in 1987, with a number of subsequent reports appearing in both the diagnostic and the DNA self-assembly literature.

In this study the coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was used to conjugate amine-terminated oligonucleotide probes to the carboxylic acid functionalised polystyrene fluorescent nanoparticles. The optimisation of the coupling reaction was developed with a range of probe concentrations corresponding to 6 × 10¹², 8 × 10¹², 1 × 10¹³ and 1.2 × 10¹³ probes per cm² of particle surface area. This was conducted in low salt concentration, to minimise particle aggregation, and at moderately low pH of 5.5, to prolong the lifetime of the EDC coupling reagent. The supplier of the nanoparticle reports 0.1–2 milliequivalents (millimoles per gram) of carboxylic acid per gram of particles, which corresponds to 2.2 × 10⁻¹⁹–4.4 × 10⁻¹⁸ moles of acid per bead or 1.1 × 10¹² and 2.1 × 10¹² acid groups per cm². For the coupling reaction we used 1.1 × 10¹¹ particles, corresponding to 25–500 nanomoles of carboxylic acid. For the coupling reaction we also used a 5 mM EDC solution corresponding thereby to a molar excess of 1–20.

The effect of different coupling probe concentrations on the hybridisation reaction was assayed. The target DNA was first hybridised with 10⁷ capture probe coated magnetic beads with 500 pM and zero DNA in a 90 μL reactor. Reporter probe coupled fluospheres were added and, following a 90 minute hybridisation and 5 wash steps, the particles were assayed in a fluorescent plate reader. A probe to surface area of 10¹³ probes per cm² gave the optimum signal to background compared with the three other probe concentrations as shown in Fig. 3. This concentration was taken forward for subsequent batch probe yellow-green fluosphere conjugations. The specificity of the assay was also investigated by using a non-specific DNA molecule derived from the genome for cytomegalovirus (Table 2). Aggregate fluorescent data (Fig. 3) show no significant difference between 500 pM and no DNA controls.

In any sensitive biomolecule assay the importance of inhibiting non-specific interactions cannot be overstated. This is true for signal amplification techniques and in particular when using hydrophobic polystyrene nanoparticle labels, which have a tendency to give a high background. Although these particles are functionalized with carboxylic acid groups and coupled with oligonucleotides, the fluospheres retain some level of surface hydrophobicity and therefore can non-specifically
In the current work an amine PEG with an average of 14 monomer repeats was covalently coupled to the bead surface, using the same EDC coupling chemistry following probe immobilisation. In order to determine its effectiveness in reducing non-specific binding, an experiment was conducted in which the non-complementary DNA derived from cytomegalovirus (CMV) was added to the hybridisation reaction at 5 µM concentration. The results showed a significant effect in reducing non-specific binding with the addition of the amine PEG. The particles with PEG treatment gave an average signal of 14 000 ± 2500, while the non-PEG treated particles gave a higher signal of 23 400 ± 2500 (n = 3). All subsequent coupling reactions were conducted with the addition of the amine PEG at 3 x 10³ molar equivalents to particles and 1 x 10¹³ probes per cm².

**Capture probe optimisation on magnetic beads**

While the current assay was established and optimised in a monoplex format, the capture probe immobilisation...
architecture was designed to enable multiplexing. In this manner a 166 bp linker was ligated with a target specific 39 basepair capture probe with a common ligation sequence and unique probe sequence. The double stranded linker had a number of functions: (a) the negatively charged phosphodiester backbone helped reduce non-specific interactions between the magnetic nanoparticles and the polystyrene reporter particles, (b) the linker also served to increase the number of target molecules which can hybridise between the particles due to its extended length.

Two capture probe immobilisation methods were investigated. Ligation was conducted on or off the bead with the linker to bead concentration modified to be 1.2, 1, 0.8, 0.6 times the biotinylated oligobinding capacity of the beads as stipulated by the supplier. Stoichiometric biotin binding capacity yields a theoretical coupling density of 2.4 \times 10^{12} biotins per cm^2. The results (ESI†, Fig. 3) show that ligation prior to coupling to the beads leads to a stronger signal to noise ratio for the 1.2× and 1× molar excess of linker to biotin capacity of the bead. The 1.2× molar excess showed the least background signal. All future magnetic bead coupling reactions were conducted with ligation prior to coupling the linker to the magnetic nanoparticle with a 1.2× molar ratio of linker to biotin binding capacity.

Hybridisation buffer

According to the well established DVLO (Deryaguin, Verwey, Landau and Overbeek)\(^{39,40}\) theory, as the ionic strength of the dispersant increases, the Debye double layer length decreases, thereby enabling the short range van der Waals forces to become influential with resultant aggregation of the particles. Non-specific aggregation could have a detrimental effect on the assay as monodisperse particles are required to maintain a high particle loading. In contrast the cations in the hybridisation buffer are required to shield the negative charges of both the immobilised capture and reporter probes and the target DNA to enable their interaction. Therefore it was important to determine experimentally how ionic strength affected the sensitivity of the assay. The results presented in Fig. 5 show that ionic strength up to 600 mM NaCl improved hybridisation yields for the assay, while causing undetectable non-specific pulldown of fluospheres (blue triangles in Fig. 5). Consequently, 4× SSC (600 mM NaCl, 60 mM trisodium citrate) with 0.1% Triton X-100 was used as the hybridisation buffer for the remainder of the study.

Particle concentration

The number of particles loaded into the reaction was modified to determine the effect of particle concentration on sensitivity. The results (Fig. 6) indicate that increasing the number of magnetic or fluorescent nanoparticles within the reaction doesn’t significantly affect the sensitivity as measured with the aggregate fluorescence technique. A key trend observed during the particle loading experiment was that the background signal increased as more beads were added to the reaction. While the results show that sensitivity was in fact dependent on particle loading (Fig. 6), the remainder of the assays reported in this current work was conducted with 1.0 \times 10^7 and 7.3 \times 10^6 magnetic and fluorescent particles, respectively.

Single particle and aggregate counting

Following the establishment of coupling conditions for the magnetic and fluorescent particles, the selection of the surfactant and hybridisation buffer ionic strength, the assay was conducted and analysed in the microfluidic chambers on a confocal microscope. The assay employed a wider dynamic range of target concentrations and used the microfluidic chambers and laser scanning confocal microscope for quantification of fluorescent particles. Following the automated washing steps, the particles were eluted into 30 µL and injected into different chambers within the microfluidic device and subsequently placed on a 50 mm diameter magnet to pull the particles onto the surface of the coverslip. The device was then inspected with a confocal microscope.
microscope with images of the yellow green fluorescent nanoparticles analysed using ImageJ (NIH) to count the particles and aggregates which were then plotted (Fig. 7).

In order to establish the limit of detection, defined as three times the standard deviation of the background, the images for the dose response were analysed. An average of 19.6 counted particles within a $421 \times 421$ $\mu$m$^2$ field of view (s.d 6.7, $n = 3$) was established yielding a limit of detection of 0.8 pM, which corresponds to 72 attomoles of target DNA. A limit of quantification, based on ten times the standard deviation of the background, of 4 pM (360 attomoles) for the 90 $\mu$L initial hybridisation volume.

In order to better understand the sensitivity limits for the assay, we calculated the distribution of target molecules across the magnetic beads and then related this distribution to the number of molecules binding the 200 nm polystyrene nanoparticles to the 500 nm magnetic particles. Taking into account the high ionic strength of the buffer, the near continual mixing and the 90 $\mu$L reaction volume, it was assumed that all target molecules hybridised with capture probes within the 90 minute hybridisation reaction. Assuming that 360 attomoles of target DNA were uniformly distributed over the surface of the $10^{10}$ magnetic beads, each particle was coated with $\sim 21$ target molecules, with an average area of $3.7 \times 10^{-10}$ cm$^2$ per target. The following additional assumptions have been made to estimate the number of target molecules linking the magnetic and fluorescent nanoparticles: (a) the 166 bp DNA linker on the magnetic bead surface can stretch to $\frac{3}{4}$ of its full extended length of 0.34 nm per bp$^{11}$ and, therefore, the target DNA immobilised on the linker DNA could hybridise with reporter probes immobilised on the fluosphere approximately 42 nm from the magnetic bead surface; (b) the linker DNA can be compressed to 4.2 nm. This leads to the prediction that the surface area of the two particles which come into contact, allowing DNA molecules to hybridise, equals $1.6 \times 10^{-10}$ cm$^2$. As a result, and with the assumption that 21 target molecules are distributed uniformly over the magnetic beads with an average area of $3.7 \times 10^{-10}$ cm$^2$ there are single molecules binding the fluospheres to the magnetic particles.

The assay was conducted in swine cerebrospinal fluid (CSF) to test its performance in a biological matrix. The assay was conducted identically to that in hybridisation buffer experiment except that the DNA dose response was generated in swine CSF, as opposed to hybridisation buffer. The results were similar to those obtained for the hybridisation buffer with a limit of detection of 1 pM which correlates with 90 attomoles (Fig. 8).

The nucleic acid detection method reported here demonstrates sensitivity in the low pM (fmol) range with a detection method based on counting individual fluorescent particles and aggregates in a microfluidic chamber. While providing sensitivity superior to a fluorescence plate reader, the hybridisation and counting method is significantly less sensitive than amplification-based detection protocols. For instance, Pandori et al.$^6$ demonstrated a 10 copy detection limit for HSV with a quantitative PCR reaction. Therefore, in order to progress towards clinical utility, the technique reported here requires improved sensitivity, sample throughput and capacity to better pre-concentrate analyte and allow for larger sample volume passage within the microfluidic device. The technique would also benefit from automation as a practical method to analyse a larger number of replicates and samples in a timely manner. Efforts to address these issues are currently underway.

**Conclusions**

A set of reagents for a magnetic and fluorescent nanoparticle based assay has been optimised. The optimised coupling quantity for an amine terminated reporter probe to carboxylic acid functionalised fluorescent polystyrene particles was $10^{13}$ reporter probes per cm$^2$ of particle surface. These particles were subsequently co-modified with an amine terminated polyethylene glycol to reduce non-specific binding. The preferred method for immobilising a capture probe via a 166 basepair linker to the streptadivin coated magnetic particle was also investigated. Optimum hybridisation was measured using solution phase ligation of the capture probe followed by coupling using the biotinylated linker. The assay gave a limit of quantification of 4 pM which correlated with single target strands able to bind the fluorescent nanoparticles to the magnetic nanoparticles.
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