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TECHNICAL NOTE

A simple device for multiplex ELISA made from melt-extruded plastic microcapillary film†

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We present a simple device for multiplex quantitative enzyme-linked immunosorbant assays (ELISA) made from a novel melt-extruded microcapillary film (MCF) containing a parallel array of 200μm capillaries along its length. To make ELISA devices different protein antigens or antibodies were immobilised inside individual microcapillaries within long reels of MCF extruded from fluorinated ethylene propylene (FEP). Short pieces of coated film were cut and interfaced with a pipette, allowing sequential uptake of samples and detection solutions into all capillaries from a reagent well. As well as being simple to produce, these FEP MCF devices have excellent light transmittance allowing direct optical interrogation of the capillaries for simple signal quantification. Proof of concept experiments demonstrate both quantitative and multiplex assays in FEP MCF devices using a standard direct ELISA procedure and read using a flatbed scanner. This new multiplex immunoassay platform should find applications ranging from lab detection to point-of-care and field diagnostics.

Background

Immunoassay is a core biochemical technique for the detection and quantification of molecules in biological samples. As the number of important analytes grows, and the number of measurements required expands, there is an increasing drive to simultaneously measure multiple analytes within a single sample, a method referred to as multiplex immunoassays. The measurement of a panel of biomarkers is increasingly required in diagnostics and there is a growing need to conduct multiplex immunoassays outside the laboratory either in the field or at the point of care.[1–3] Assay miniaturisation is therefore required to reduce volumes and achieve faster assay times, ease of use and portability. A plethora of microfluidic technologies have been explored ranging from the use of individual capillaries[4–6] through to fully integrated systems containing complex microchannels.[1,7–10] In all cases, significant challenges include reducing device manufacturing costs, incorporating antibodies/antigens onto a detection surface, and signal detection.

Immunoassays have been conducted in individual capillaries for several decades,[11] but recent developments have focussed on multiplexing, integrating fluidics, and improving signal detection within capillaries.[6] To conduct multiplex immunoassays, multiple individual capillaries coated with different antibodies or antigens can be connected to a single sample feed[4,5] or multiple bands can be coated within a single capillary by photolithographic or micro-syringe methods.[12,13] Optical detection is particularly challenging because individual capillaries represent cylindrical lenses when viewed from the side. To overcome this, the capillary body can be used as an optical waveguide analogous to an optical fibre,[13] or converted substrate can be eluted into a flow-through optical sensor.[4]

Many different microfluidic immunoassay devices have also been developed using microchannels formed *in situ* ranging from portable fully integrated microfluidic devices driven by syringes[3] or capillary action[2] to discs in which fluid movement is controlled by centrifugal force.[14–16] Although lab methods allow rapid prototyping of microfluidic devices, mass fabrication is more challenging leading to interest in simpler materials such as paper[17] and development of advanced manufacturing methods.[3,10] Furthermore, even after fabrication, labile and costly antibodies or antigens must somehow be immobilised onto the detection surface within the device.[7–9] Methods to achieve this include depositing antibodies onto microchannels prior to assembly,[2,7] introducing microparticles coated with detection reagents into the device,[14,18,19] and chemical methods such as DNA-directed assembly.[20] Finally, low-cost, sensitive and portable detection methods are also vital for signal measurement in microfluidic devices;[21] approaches include using solid state components (*e.g.* LEDs, CCD arrays),[21] lasers,[16] colour amplification for visual readout[3] or non-optical methods such as electrochemical[8] or magnetic[22] detection.

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In this paper we propose for the first time using plastic microcapillary film (MCF), a novel cheap microengineered material, for conducting multiplex immunoassays, as an alternative to individual capillaries or *in situ* formed microchannels. MCF are long melt extruded plastic films containing a parallel array of microcapillaries with controlled size and shape, introduced during extrusion by air injection[23,24] (Fig. 1A). We present proof-of-concept data demonstrating quantitative and multiplex ELISA in MCF, and highlight possible advantages of this approach.

2 Materials and methods

2.1 Reagents and materials

The MCF studied here produced from fluorinated ethylene propylene (FEP MCF), and containing 10 capillaries with a mean diameter of 206 ± 12.2μm and external dimensions of 4.5 ± 0.1mm wide by 0.6 ± 0.05mm thick (Fig. 1A), was produced by Laminar Dielectrics Ltd (Billingshurst, West Sussex, UK). Purified mouse IgG, mouse and goat serum, horseradish peroxidase conjugated anti-mouse IgG- (HRP-anti-mIgG), Alkaline Phosphatase conjugated anti-mouse IgG (AP-anti-mIgG), FLAG peptide, and mouse monoclonal anti-FLAG peptide (anti-FLAG), Tween 20, phosphate buffer saline pH7.4 (PBS), protease-free bovine serum albumin (BSA), SigmaFast OPD substrate tablets, and Nunc Maxisorp ELISA plates were from Sigma-Aldrich (Dorset, UK). Recombinant Hepatitis B Core Antigen (rHB-CAg), and mouse monoclonal anti-HB CAg (anti-HB CAg) were from Abcam (Cambridge, UK). HRP-conjugated anti-mouse IgG1 detection antibody was from Invitrogen (Paisley, UK). Fluorescein diphosphate (FDP) substrate (Cambridge Biosciences, Cambridge UK) was used at 20μM dissolved in 50mM Tris pH 9.0, 10mM Glycine, 10mM $MgCl_2$. Washing solution was 0.05% v/v Tween 20 in PBS (PBS-T) and blocking solution was 1% w/v BSA in PBS or 1% w/v BSA plus 1% v/v goat serum in PBS.

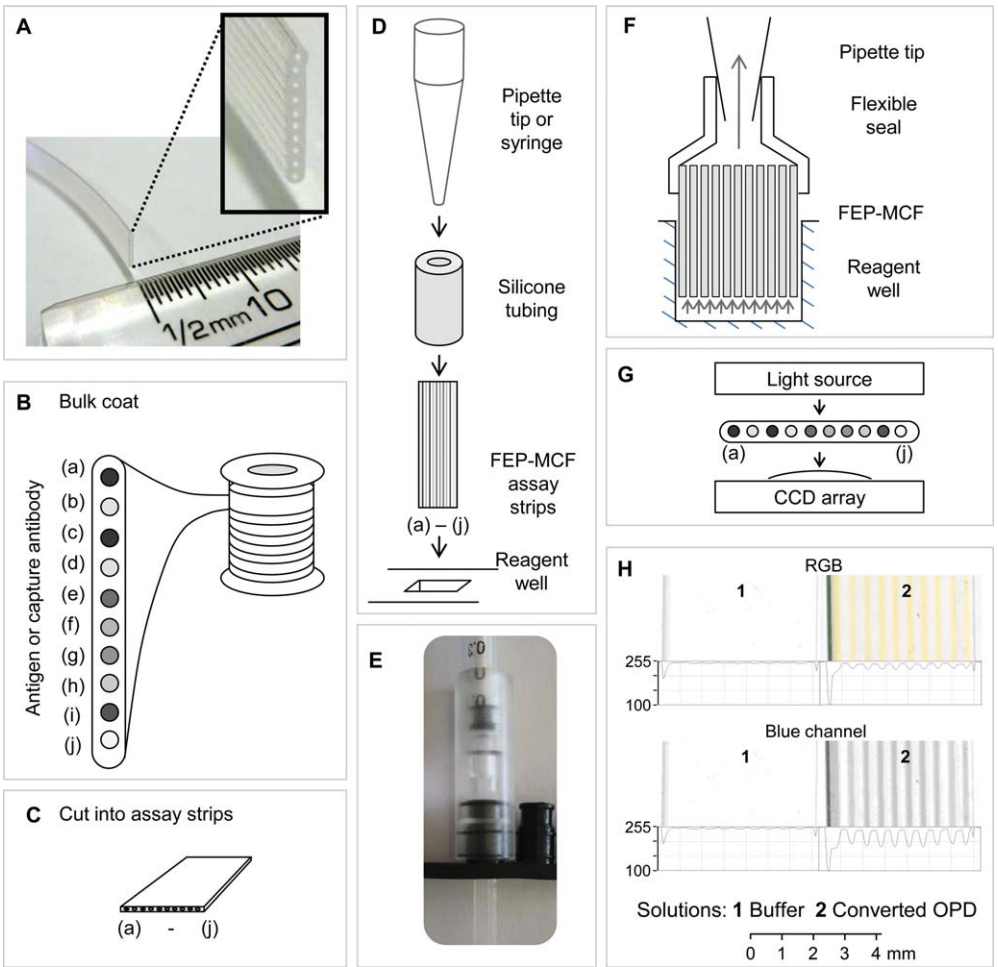

Fig. 1 Making multiplex microfluidic ELISA devices from melt-extruded fluropolymer microcapillary film. **A** Microcapillary film melt extruded from fluorinated-ethylene-propylene copolymer (FEP MCF). **B–D** Indicate the process used to make multiplex ELISA devices from FEP MCF; **E–G** illustrate the device fluidics and signal detection. **B** Coating the internal surfaces of individual capillaries within a long reel of FEP MCF with different antibodies or antigens (labelled a to j). **C** Cutting individual sample analysis strips from reel. **D** Interfacing individual sample strips with a pipettor or syringe, using silicone tubing, plus a simple reagent well to complete the microfluidic device. **E** Customised push-fit seal to interface MCF to syringe. **F** Device cross-section in use with sample strip in reagent well showing fluid flow. **G** Colourimetric signal detection using simple optical interrogation. **H** Example transmission flatbed scanner images illustrating optical clarity of FEP MCF filled either with buffer or converted substrate. RGB colour image (top) and blue channel alone (bottom) demonstrate the strong absorbance of OPD in the scanner blue channel.

2.2 ELISA procedures

To coat with antigen, 1μg ml^{-1} of recombinant HB CAg in PBS was either filled into all capillaries within 1m of FEP MCF, or 50μl/well was added to microtitre ELISA plates (MTP) and incubated overnight at 4 °C. MCF and MTP were then washed and blocked for at least 2h at room temperature with 1% BSA plus 1% goat serum in PBS. To simulate seropositive sera with defined amounts of reactivity to HB CAg, normal mouse serum was spiked with known concentrations of a mouse IgG1 monoclonal antibody against HB CAg. A high-titre serum sample contained 50μg ml^{-1} monoclonal anti-HB CAg antibody, and a low-titre sample contained 2μg ml^{-1} monoclonal anti-HB CAg. Positive samples and a control sample were diluted 1 : 20 followed by 4-fold serial dilutions, and either aspirated into 30mm long MCF test strips or 80μl per well added to MTP in duplicate. After at least 1h incubation at room temperature, MCF strips and MTP were washed 3 times and HRP anti-mouse IgG1 detection antibody at 1 : 3000 in PBS containing 1% BSA + 1% goat serum added. Between solutions the FEP MCF test strips were not emptied. After at least 45 min incubation with detection antibody at room temperature, MCF strips and MTP were washed extensively and OPD substrate added. Measurement of absolute anti-HB CAg concentration was compared in FEP MCF *vs.* MTP using a similar procedure, except antigen was coated at 10μg ml^{-1}, block buffer was 1%BSA in PBS without goat serum, samples were diluted in 1% BSA in PBS without serum, and the detection antibody was HRP-anti-mIgG at 1 : 5000.

For signal detection, after 20–40 min of incubation with OPD, FEP MCF devices were scanned with a HP ScanJet 4050 Photo Scanner in transmittance mode (1,200–2,400 dpi resolution). Images were post-processed with ImageJ software[25] to determine the absorbance in each capillary for the blue channel that gave strongest absorbance for the converted OPD substrate; image processing is described in supplementary methods. MTP were measured using a plate reader after 1h incubation, either at 450nm without stop solution or at 490nm after adding 3M HCl. Absorbance values expressed as cm^{-1} were calculated relative to pathlengths of 0.02cm for MCF and 0.3cm for MTP to allow direct signal comparison.

To demonstrate multiplex immunoassays with fluorescent signal detection, the 10 capillaries within 5m of FEP MCF were each individually coated by injection with PBS (negative control), 10μg ml^{-1} mouse-IgG (positive control), 10μg ml^{-1} Hepatitis B core antigen protein or 10μg ml^{-1} FLAG peptide in PBS in the indicated pattern followed by blocking of all capillaries with PBS/BSA. Individual 50-mm long pieces were cut and loaded with one of three test samples containing PBS alone, anti-HB CAg, or anti-FLAG peptide monoclonal antibodies and incubated for 1h. After washing, the pieces were incubated with AP-anti-mIgG followed by washing and addition of the fluorescent substrate FDP, and then scanned by confocal microscopy after 15 min incubation and scored as positive or negative; examples of images are shown in Fig. S3D, ESI.†

3 Results and discussion

3.1 Producing simple microfluidic devices from melt-extruded microcapillary film

MCF was extruded from a highly transparent fluoropolymer, fluorinated ethylene propylene (FEP), with dimensions, surface characteristics, and optical properties suited to producing immunoassay devices (Fig. 1A). In order to adapt this material for conducting multiplex ELISA, whilst maintaining the benefits of the extruded manufacturing process and the simplicity of a plastic film, a two stage process was devised (Fig. 1). Firstly, antigens (for direct ELISA) or capture antibodies (for sandwich ELISA) were immobilised in bulk, with each capillary coated with different antigens/antibodies, within the capillaries of a long piece of MCF (*e.g.* 5 metres), conveniently wound on a reel (Fig. 1B). Blocking was also completed in bulk. Secondly individual assay pieces (*e.g.* 20–50mm in length) were cut from the reel of bulk coated MCF (Fig. 1C). In this way, hundreds of multiplex test strips can be produced in a single batch. Individual assay pieces were each fitted with a single feed for uptake of sample and detection solutions into all capillaries (Fig. 1D). A cross-sectional diagram of the resultant device is illustrated in Fig. 1F.

Given the hydrophobic surface properties of FEP, protein antigens and antibodies were simply immobilised by direct adsorption onto the inner surface of capillaries. Crucially, coating was found to be homogeneous along the length of a long MCF reel, demonstrated by direct detection of immobilised antibody in a test strip taken from the inlet (distance 0m) and outlet (distance 5m) of a 5m long piece of FEP MCF (Fig. S1, ESI†). Thus any convenient length of MCF can be coated in one batch, depending on the desired number of assay strips and the length of strip used. No difference was seen when coating lengths varying from 10cm to 5m (data not shown).

Two different simple fluid handling techniques were used to fill capillaries within MCF. To fill different solutions in different capillaries- for example to make multiplex assay strips (Fig. 1B) - individual capillaries were filled by simply injecting individual capillaries using a fine needle (31G) and syringe. Alternatively, all capillaries in a given piece of MCF were filled simultaneously with the same fluid by connecting one end of the test strip to a pipette or syringe using a 20–30mm piece of 3mm internal diameter silicone tubing pushed 5–10mm over the end of the MCF test strip and sealed by clamping with a tubing clamp or simply pinching between finger and thumb. The other end was immersed in the appropriate liquid in a reagent well (Fig. 1D, F). By aspirating measured volumes using a pipette or syringe, solutions were drawn up into all capillaries from the reagent well. A solution can be aspirated into a set of 8 FEP MCF assay strips manually using a single pipette within 2–3 min; more samples can be processed using multichannel pipettes (data not shown). Although silicone tubing is a simple and low-cost method for interfacing with pipettes, we also prototyped simple moulded push-fit seals customised to interface with other fluid handling devices such as syringes (Fig. 1E). Preliminary studies with dye solutions indicated a minimum volume of 30μl was required to equally fill the 10 capillaries in a 50mm piece of MCF from a single microtitre plate well using silicone tubing and a pipette (data not shown); customised reagent wells and moulded push-fit seals would reduce volumes further.

Although cheap to produce (see ESI† for cost estimates) and simpler to manufacture than *in situ* formed microchannels, using extruded material does not allow the integration of multiple steps possible with complex microfluidic devices.[1,2,7–9] However, this disadvantage has been overcome with individual capillary assays

by integrating with other fluid handling components.[4,5] Similarly, microparticles are useful for immunoassays but difficult to process, a difficulty that has been overcome by incorporation into microfluidic devices.[14,18,19] We propose that short MCF strips could be integrated into more complex fluid handling devices in a similar manner.

3.2 Unique optical properties of FEP MCF

A major barrier to the use of individual fused silica or plastic microcapillaries for immunoassays has been the difficulty of optically interrogating the contents due to the cylindrical capillary geometry and the refractive index of the capillary wall. The capillary represents two concentric cylindrical lenses with distortion caused by refraction at both the air to capillary surface interface, and also usually at the inner surface to water interface. In contrast, the FEP MCF used in the current study has two unique features that are ideal for optical signal detection by simple transmittance imaging (Fig. 1G). Firstly the flat parallel faces of the MCF provide a short path length through the film wall, with no curvature to refract the light path. Secondly, FEP has a refractive index of 1.338 which is very close to water (1.333), resulting in minimal optical refraction at the water: capillary wall interface and thus no distortion is caused by the capillaries when filled with aqueous solutions. Thus although capillaries are clearly visible when FEP MCF is air filled (Fig. 1A), they completely disappear when filled with aqueous solutions (Fig. 1H). Refractive index matching using FEP for undistorted optical detection has been previously exploited to image microbial growth on packed beds.[26] To demonstrate the importance of its unique flat-sided geometry for simple optical detection of coloured substrate, FEP MCF was shown to have superior transparency to different sized individual, cylindrical FEP capillaries produced from the same fluoropolymer (Fig. S2A, ESI† *vs.* B,C). To demonstrate the importance of refractive index matching that of water, FEP MCF was shown to have superior transparency to other MCF with similar geometry but melt extruded from a range of different plastics with differing refractive indexes (Fig. S2A, ESI† *vs.* D–H).

In the present proof-of-principle study, two different optical detection methods were used. As an example of the simplest detection method needed, some experiments were conducted using a flatbed scanner in transmission mode to detect a colorimetric ELISA substrate (Fig. 1H, 2, S3B, ESI†). The optical clarity could be exploited with other detection devices, for example CCD cameras, photodiode arrays, CMOS sensors, or even - with suitable background illumination and holder - a smartphone camera. Other optical immunoassay signals could be measured such as chemiluminescence and fluorescence, and a fluorescent ELISA substrate was detected using confocal microscopy (Fig. S1, S3D, ESI†). The use of an off-the-shelf consumer flatbed scanner offers advantages over the measurement of converted substrate in a flow cell following elution from individual assay capillaries,[4] and over the customised optical detectors needed to measure light emission from capillary ends utilising waveguide effects[13] or to analyse multi-capillary cassettes.[5] Furthermore, colour changes within MCF are clearly visible to the naked eye, allowing visual readout without the signal amplification described recently for field clinic microfluidic devices.[3]

3.3 Quantitative ELISA in FEP MCF devices

Having developed a method for making devices from FEP MCF and established its favourable optical properties, it was essential to demonstrate that quantitative, sensitive and multiplex immunoassays could be conducted in these devices. Three proof-of-principle direct ELISA assays were completed to demonstrate quantitative assays in real biological fluids (serum), to compare sensitivity with microtitre plates (MTP), and to illustrate multiplexed detection.

To test if relevant biological fluids could be quantitatively analysed using FEP MCF, a simple direct ELISA (Fig S3A) was conducted to detect mouse anti-HB CAg antibodies in parallel in both FEP MCF devices and MTPs. For simplicity and to determine inter-capillary variation, singleplex assay strips were made with the same antigen adsorbed in all capillaries. High or low amounts of monoclonal anti-HB CAg were spiked into serum samples, and compared with a control serum with no added anti-HB CAg. Seroreactivity against HB CAg was then measured using a conventional endpoint titre protocol, whereby these three serum samples were serially diluted and tested for reactivity to adsorbed antigen. An intense yellow colour indicating OPD substrate conversion was seen as increased amounts of anti-HB CAg were present in samples (Fig.S3B, ESI†). The strongest absorbance by OPD was seen in the blue channel (Fig. S3B, ESI†), and thus to quantify the signal the maximum blue light absorbance was determined for each capillary. As expected, absorbance increased when more anti-HB CAg was present and decreased with dilution confirming that quantitative direct ELISA is feasible for analysis of real biological fluids using FEP MCF, with conventional assay chemistry and read using a flatbed scanner (Fig. 2A, B). The pattern of absorbance seen with the three titrated mouse sera was broadly similar with FEP MCF and MTP, suggesting similar assay performance to conventional MTP ELISA (Fig. 2A, B). Due to the high concentrations of IgG in serum, at low dilutions control sera containing no specific antibodies gave a background signal; this was higher in microtitre plates than in FEP MCF devices (Fig. 2A, B), demonstrating that FEP MCF gives no additional background compared to conventional MTP. However, when antibody against HB CAg was present in the serum samples, far higher signals were detected at higher dilutions. Furthermore the absolute absorbance values measured in FEP MCF were significantly higher than those observed in MTP (Fig. 2A, B), but in contrast the endpoint titre at which signal dropped below a threshold for the two positive sera was somewhat lower for FEP MCF compared to MTP, indicated some reduction in sensitivity in this assay. In this experiment, identical reagent conditions – optimised for MTP – were used in MCF; however, increased assay sensitivity should be possible in MCF by optimising reagent concentrations and improving scanner sensitivity.

To further explore the relative sensitivity of FEP MCF *vs.* MTP assays without the complication of background signal caused by high levels of IgG present in whole serum, a direct ELISA was also conducted measuring fixed concentrations of monoclonal anti-HB CAg diluted without serum. Again, the

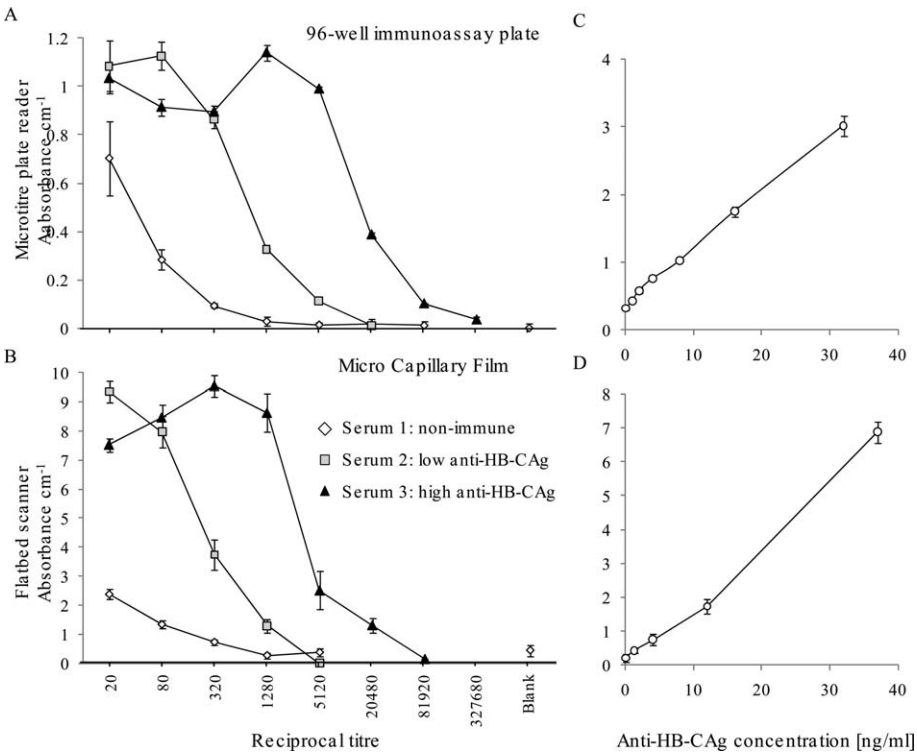

Fig. 2 Hepatitis B antibody detection comparison in FEP MCF devices *vs.* microtitre plates. Measurement of antibodies against HB CAg as endpoint titre in serum (**A, B**) and as absolute concentration (**C,D**) was compared using FEP MCF plus flatbed scanner (**B,D**) *vs.* microtitre ELISA plate plus microtiter reader (**A,C**). Mouse serum samples were made with differing levels of antibodies against HB CAg by spiking control mouse serum with high or low levels of a monoclonal antibody to HB CAg. A control negative mouse serum sample or the spiked positive samples were serially diluted and tested in parallel in microtitre ELISA plates **(A)** or FEP MCF devices (**B**). A sensitivity comparison was completed by completing an ELISA on samples with the same known concentrations of a monoclonal anti-HB CAg antibody in parallel in microtitre ELISA plate (**C**) and FEP MCF devices (**D**). All error bars are shown and indicate ±1 standard deviation for 8 central capillaries within a single FEP MCF device, or triplicate microtitre plate wells. Data are representative of 2–3 sensitivity comparisons with similar results.

intensity of yellow colour increased with incremental anti-HB CAg concentration (Fig. S3B, ESI†). At concentrations over 100ng ml^{-1} anti-HB CAg, the absorbance signal became saturated and non-linear, but below 40ng ml^{-1} a clear linear relationship between concentration and absorbance was seen (Fig. 2C, D, S3B, ESI†). Again, a similar pattern of absorbance with varying concentration was seen with both FEP MCF and MTP, although as before higher absolute absorbance values were seen in FEP MCF than in the MTP assay. The limit of detection of anti-HB CAg with the FEP MCF was approximately 1 ng ml^{-1} which is identical to the sensitivity observed for MTP (Fig. 2C, D).

Note that in these experiments, the incubation times used for FEP MCF were kept identical to the standard MTP assay. However, in common with other microfluidic devices,[1,7–9,21] reduced diffusion distances should allow reduction in incubation times by 10–20-fold compared to MTP. For example, sandwich immunoassays were completed within 30 min in 320μm internal diameter individual capillaries.[4] Indeed, preliminary tests suggested that in FEP MCF, incubation times of 10 min give similar assay performance to 2h, in contrast to a significant reduction in signal strength observed with 10 min incubations in MTP (data not shown). Similarly, sample and reagent volume requirement are reduced in MCF compared to MTP.

3.4 Multiplex ELISA in FEP MCF devices

For proof-of-concept of multiplex IA, antibodies against two different antigens (HB CAg and the FLAG peptide) were measured using a simple direct ELISA process (Fig. S3C,D, ESI†), each in triplicate within three different capillaries. Duplicate positive and negative control capillaries were also included resulting in assay strips that measure 4 'assay channels'. The antigens, plus positive (coating with mouse IgG) and negative (uncoated) controls, were filled into different capillaries of a 5m reel of FEP MCF in the pattern illustrated in Table 1 and Fig. S3D, ESI.†After coating and blocking, individual 50mm test strips were then filled with three samples containing different antibodies (control, anti-HB CAg, anti-FLAG, Fig. S3D, ESI†), followed by detection with AP-anti-mIgG and the fluorescent substrate FDP. As expected, low fluorescence levels were observed in the two negative control capillaries, and high fluorescence was seen in the positive controls, regardless of the sample used (Table 1 and Fig. S3D, ESI†). In contrast, increased fluorescence was detected with the HB CAg coated capillaries only when the sample contained anti-HB CAg, and similarly the FLAG coated capillaries gave strong positive signal only with the sample containing anti-FLAG antibody (Table 1 and Fig. S3D, ESI†). Some background signal was observed (especially with the detection of antigen (c) in sample (3) in one capillary); this was

Table 1

Capillary number	Coating antigen	Signal seen with: Sample 1 buffer control	Sample 2 anti-HB CAg	Sample 3 anti-FLAG
1	Negative control	—	—	—
2	Positive control- mIgG	+	+	+
3	Negative control	—	—	—
4	Positive control- mIgG	+	+	+
5	Hepatitis B Core Ag	—	+	—
6	FLAG peptide	—	—	+
7	Hepatitis B Core Ag	—	+	—
8	FLAG peptide	—	—	+
9	Hepatitis B Core Ag	—	+	–/+
10	FLAG peptide	—	—	+

due to excess detection reagent concentrations and was eliminated by subsequent optimization (data not shown).

Although some technologies allow the detection of very large panels of analytes, for the majority of diagnostic assays between 2 and 10 analytes are typically measured.[3,5] The current FEP MCF extrusion can detect 8 different agents (disregarding the outer 2 capillaries; Fig. S2, ESI†), which is ideally suited to these requirements; however MCF with 19 capillaries are routinely extruded,[24] giving scope for more analytes or multiple replicate measurements of the same analytes.

4 Conclusions

We present a new concept for a multiplex immunoassay platform based on the multi-channel, microengineered material FEP MCF. With further development and optimisation, this platform should offer the following features and benefits: 1) miniaturised for rapid and low volume assays; 2) simplicity of immobilising a panel of antibodies or antigens onto the detection surface; 3) simple, cheap and potentially portable optical signal detection; and 4) simple and cost-effective manufacturing process. The flat geometry of the plastic film combined with the optical clarity of FEP provides an opportunity for simple optical signal detection, for example using a flatbed scanner or smartphone camera, or using a simple customised detector made from low-cost LED and CCD or CMOS sensor components. Optical detection also allows use of conventional ELISA reagents and existing assay chemistry. We present proof-of-principle data demonstrating that quantitative, multiplex ELISA can be conducted using FEP MCF and quantified using a flatbed scanner, bringing for the first time the benefits of melt-extrusion as a method for manufacturing microfluidic devices, combined with simple optical detection, to the field of immunoassays. Future studies will build on these observations by optimising conditions, determining assay speed and robustness, and developing more sophisticated FEP MCF devices combined with portable signal detection systems. These studies should lead to the development of a range of scalable, cost-effective multiplex immunoassay tools for applications from laboratory research to portable or point-of-care diagnostics.

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