Electronic Supplementary Material (ESI) for Analyst.

Novel strategies for covalent immobilisation of antibodies in Telfon-FEP microfluidic devices

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Electronic Supplementary Information

1 Supplementary methods

1.1 Measurement of hydrophilic PVOH coating. To determine if PVOH coatings were removed by repeated washing, we measured the presence of a hydrophilic coating within the otherwise highly hydrophobic microcapillaries using a simple capillary rise method. Test strips were held vertically with the lower end submerged in ultrapure water, and the maximum capillary rise recorded in mm.

1.2 Quantitation of immobilized capture antibody. To measure the density of capture antibody immobilised by passive adsorption or different covalent bioconjugation methods, a solution of goat anti-mouse IgG antibody HRP conjugated were prepared in the custom multi-well plate (150 µl per sample) at a concentration of 0.6 µg / mL and aspirated into MCF test strips attached to a multi-syringe aspirator. After 10 min of incubation, the MCF test strips were washed 3 times by aspirating 150 µl of PBS-Tween20 0.05%. The OPD enzymatic substrate (1 mg/mL in 0.125 M phosphate-citrate buffer (pH 5.0) with 1 mg/mL H_2O_2) was then aspirated into the MCF and the whole multi-syringe aspirator device was then placed in a flatbed scanner and the strips were scanned in intervals of 5 min over 15 minutes of incubation. The RGB images were analysed using ImageJ software, from which the absorbance on each individual capillary was determined from the grey scale peak height of the blue channel.

1.2 Quantitation of immobilised biotin and immobilised streptavidin. To evaluate levels of biotin immobilization, biotinylated MCF strips were trimmed into 30 mm long test strips and attached to a push-fit seals onto a 8-channel semi-disposable multi-syringe aspirator that enables the parallel and sequential aspiration of reagents pre-loaded on a custom multi-well plate as previously described [1-3]. Briefly, 150 µL of each reagent or wash buffer was loaded in the custom multi-well plate, and 80uL of this aspirated upwards into the MCF test strips using the disposable 1mL syringes, thereby ensuring complete replacement of the previous reagent or wash solution. After 30 min blocking with Superblock blocking buffer, strips were tested with high sensitivity streptavidin conjugated to horseradish peroxidase (HSS-HRP). The concentration of enzyme conjugate had to be adjusted depending on the levels of biotin immobilised. For photoactivated biotin coated microcapillaries, 4 µg/mL HSS-HRP was used. However, when NHS-biotin was immobilised onto PVOH with or without APTES-treatment, enzyme levels were too high to quantify with 4 µg/mL HSS-HRP as the OPD substrate converted completely within seconds and rapidly precipitated. For this reason, the concentration of HSS-HRP was reduced to 0.04µg/mL to quantify biotin levels achieved with NHS-biotin. After 10 min incubation, the test strips were washed three times with PBS-Tween 0.05% (PBS-T). Finally, a HRP substrate consisting of OPD at 4 mg/mL in 0.125 M phosphate-citrate buffer (pH 5.0) with 1 mg/mL H_2O_2 was aspirated into MCF test strips, and the entire multi-syringe aspirator device was placed in the flatbed scanner to image substrate colour within MCF test strips at 5 min intervals up to a

total of 15 minutes incubation. ImageJ software was used to calculate the converted substrate absorbance for each individual capillary from the grey scale peak intensity in the blue channel, as previously described [1,3]. Each data point represents the mean absorbance or fluorescence intensity of 10 individual capillaries within each MCF test strip, and error bars indicate standard deviation of these 10 replicate assays.

To determine biotin-binding capacity of streptavidin coated biotinylated PVOH-coated MCF, biotin immobilized MCF strips were trimmed into 30 mm long test strips, attached to the multi-syringe aspirator device and incubated 30 min in Superblock blocking buffer. Six 1:2 dilution series of streptavidin (from 500 to 15.625 µg/mL) diluted in Superblock blocking buffer were then pre-loaded in the custom multi-well plate (150 µL per sample) and simultaneously aspirated in the capillaries. Strips were then incubated for 10 min in a biotin-HRP conjugated enzyme solution diluted in Superblock blocking buffer. Subsequently, 3 washing steps with PBS-Tween 0.05% were performed. Finally, 150 µL of enzymatic substrate consisting of a solution of OPD at 4 mg/mL in 0.1 M phosphate-citrate buffer (pH 5.0) with 1 mg/mL H_2O_2 was aspirated. The multi-syringe aspirator device was then placed in a flatbed scanner and the strips were scanned in intervals of 5 min over 15 minutes of incubation. The RGB images were analysed using ImageJ software, from which the absorbance on each individual capillary was determined from the grey scale peak height in the blue channel. The optimum streptavidin concentration was then determined as the concentration at which the absorbance is maximum.

1.3 Sandwich immunoassays in the multi-syringe aspirator. Immunoassays in the multi-syringe aspirator device were performed using a microcapillary ELISA method reported previously in detail [1,3]. Briefly, MCF strips coated with capture antibody using the different methods described above were trimmed into 30 mm long test strips and attached to the multi-syringe aspirator device. All reagents including 1:3 dilution series of target analyte (i.e. human recombinant IL-1ß, purified human cTnI) or 1:2 dilution series of recombinant human BNP, biotinylated detection antibody (detAb) (20 µg/mL) and high-sensitivity SA-HRP enzyme conjugate (4 µg/mL) were pre-loaded in a custom multi-well plate (150 µL per sample). All reagents were diluted in Superblock Blocking buffer. For each ELISA, the purified or recombinant analyte samples (e.g. recombinant human IL-1ß for IL-1 ß assay; purified cTnI for cTnI assay) were first aspirated with the multi-syringe aspirator and incubated for 30 min. After washing with PBS-Tween 0.05%, biotinylated detection antibody (detAb) was aspirated (e.g. biotinylated anti-human IL-1ß for IL-1ß assay), incubated for 3 min and washed with further PBS-Tween 0.05%. Subsequently, HSS-HRP conjugated enzyme was aspirated and incubated for 5 min in the capillaries, followed by three washing steps with PBS-Tween 0.05%. The enzymatic substrate (OPD, 4 mg/mL) was then aspirated into the MCF and the whole multi-syringe aspirator device was then placed in a flatbed scanner and the strips were scanned in intervals of 2 min over 15 minutes of incubation. The RGB images were analysed using ImageJ software, from which the absorbance on each individual capillary was determined from the grey scale peak height in the split blue channel. In some cases, colorimetric HRP detection was substituted by fluorimetric enzyme detection using streptavidin conjugated alkaline phosphatase (SA-AP) plus Attophos substrate, as follows. Instead of incubation with SA-HRP, test strips were filled with SA-AP diluted to 4 µg/mL in Superblock blocking buffer and incubated for 5 min, followed by three washing steps with 150µL of PBS-Tween 0.05%. Subsequently, the alkaline phosphatase substrate (AttoPhos®, 125mM) was loaded into the MCF. Fluorescence pictures were taken in a dark box, illuminated by a blue LED light box (IO Rodeo, San Diego, USA), through an amber

acrylic emission filter (IO Rodeo, San Diego, USA) with a Canon Powershot S120 camera (aperture f/2.2; exposure 0.6s) in intervals of 2 min up to 30 min incubation. RGB images were analysed using ImageJ software (NIH, USA), with fluorescence for each individual capillary was determined from the grey scale peak height in the split green channel. To compare between assays and to control for any fluctuations in illumination intensity, all test strip images were taken alongside a 200mm long reference strip of MCF containing a fixed concentration of Attophos converted in solution using alkaline phosphatase conjugate; relative fluorescence values for each capillary were calculated by normalisation to the signal from this reference strip.

Where indicated in the text, limits of detection for assays were calculated as previously described [1-3]; briefly, a 4-parameter model was fitted to the assay data and limit of detection calculated as the analyte concentration where absorbance or fluorescence exceeded the mean value of the blank plus three times the standard deviation of the blank.

2 Supplementary results

2.1 Permanent hydrophilic coating using high molecular weight polyvinylalcohol

In our previous report , we developed permanently hydrophilic FEP capillaries using a glutaraldehyde crosslinked polyvinylalcohol coating [4]. When we discovered that glutaraldehyde crosslinked PVOH frequently gave high background in ELISA, we needed to develop an alternative coating method without glutaraldehyde. We tested a range of different polyvinylalchol preparations with differing degrees of deacetylation and varying molecular weight, and found that in contrast to lower molecular weight preparations where covalent crosslinking was essential to retain a permanent hydrophilic coating, with high molecular weight PVOH (>120,000 g/mol) the coatings were not removed by washing, and a low contact angle detected by high capillary rise was maintained even after 4 successive and extensive washes with 3 mL wash volume (Fig. S1).

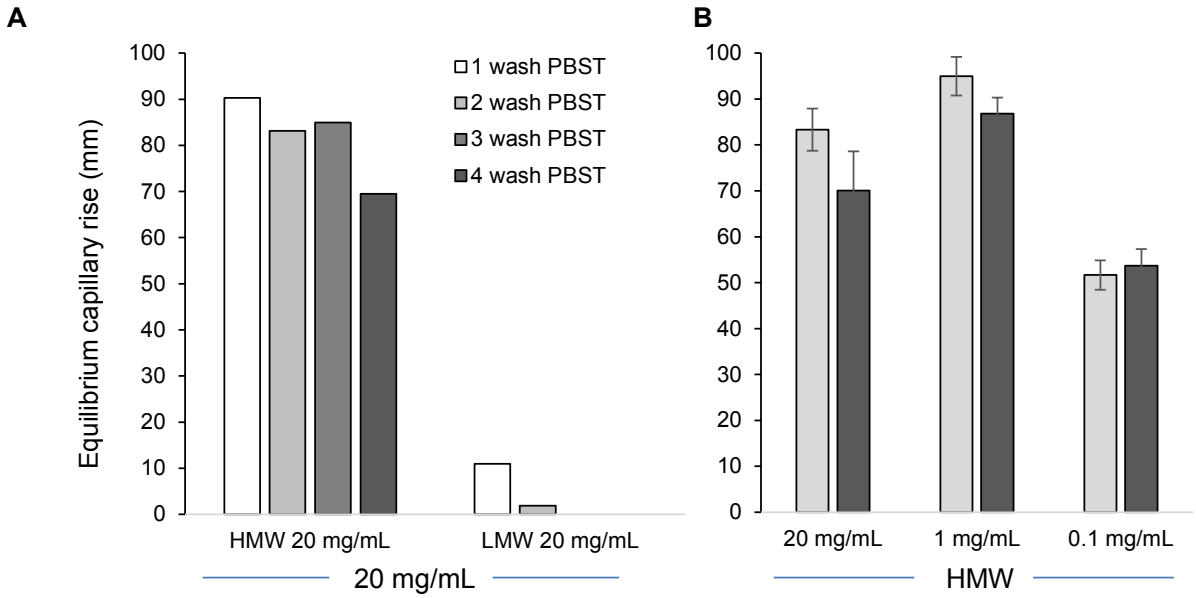

Figure S1.
High molecular weight PVOH coating is not removed by multiple wash steps.

(A) FEP MCF was coated by incubation for 2h with 20 mg/mL high molecular weight (146,000-186,000 g/mol; HMW) and low molecular weight (13,000-23,000 g/mol LMW) PVOH. Surface coating and contact angle was evaluated using the capillary rise method. Test strips were then washed up to 4 times by aspiration of a total of 3 mL of PBST using a syringe, and then air dried and capillary rise measured after each wash. (B) FEP MCF was coated by incubation for 2h with the indicated concentrations of HMW PVOH, and capillary rise measured either after two or 4 repeat washes. Bars indicate mean of 10 capillaries in a single test strip, with error bars indicating 1 standard deviation. Results are representative of at least 3 replicate coatings.

2.2 Limitations of glutaraldehyde immobilised capture antibody

We found that for some assays glutaraldehyde immobilisation led to high assay background. When high assay background was systematically evaluated, it was found to be largely attributable to binding of the biotinylated detection antibody directly to the coated microcapillaries (Figure S2). The level of background was highly dependent on the concentration of glutaraldehyde used to immobilise the capture antibody (Figure 2). The background could be lowered by reducing detection antibody concentration or reducing glutaraldehyde concentration, however the latter resulted in reduced density of immobilised antibody (Figure 2). With stepwise analysis of the IL-1 system, the biggest drop in background seen was observed when the biotinylated detection antibody was removed (Figure S2). For the cTnI assay, lowering glutaraldehyde concentration eliminated the high background, but the assay signal in the presence of analyte was also very low (Figs 2 and S2). Altogether, these data suggest that the high and variable background using this immobilisation chemistry is most likely caused by the glutaraldehyde treated PVOH surface having a significant capacity to directly bind some – but not all – detection antibodies.

Although this showed for the first time that the versatile and well-studied crosslinker glutaraldehyde can be used for antibody immobilisation in fluoropolymer microfluidic ELISA devices, the method showed significant limitations for two of the three biomarkers. With an ELISA protocol previously optimised to detect IL-1ß with pg/mL sensitivity [3] two problems were encountered. Firstly, even when high concentrations of glutaraldehyde were used to maximise capture antibody immobilisation, the highest capture antibody density achieved remained below 50% of that achieved by passive adsorption onto hydrophobic MCF (Figure 2). Secondly, variable and high background was seen with glutaraldehyde treated PVOH, which could be reduced by lowering either glutaraldehyde concentration (which reduced capture mAb density) or lowering detection antibody concentration (affecting analytical sensitivity) (Figure S2). Similar high background was seen with cTnI ELISA. The level of assay background varied greatly with different capture and detection antibodies, and in contrast to IL-1ß and cTnI, high sensitivity measurement of BNP was achieved using gluteraldehyde immobilised capture antibody (Figure S2). This high background contrasts a previous study where glutaraldehyde was used to activate an APTES-treated PVOH coating on a PDMS surface with reduced non-specific background binding observed [5]. Modified protocols for glutaraldehyde antibody immobilisation onto PVOH coated FEP may therefore be possible that avoid this high non-specific binding.

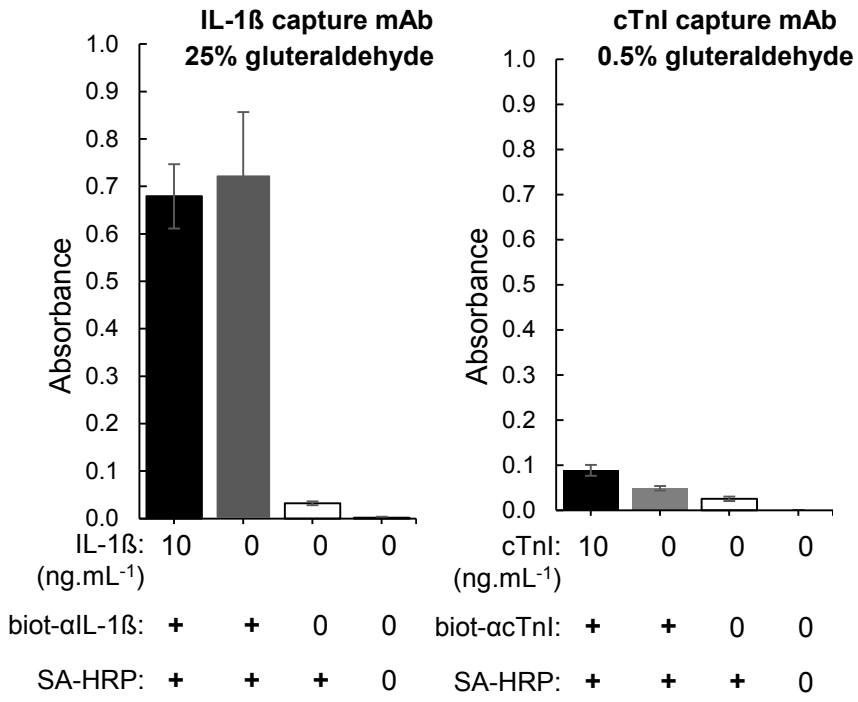

Figure S2. High background with glutaraldehyde-PVOH immobilisation is linked to non-specific binding of capture antibody.

2.3 Determining if polyvinyl alcohol reduces assay background as a blocking reagent for ELISA

We noticed a reduction in ELISA background for some assays when capture antibody was covalently immobilised onto PVOH. To determine if this was due to blocking properties of the PVOH, or differences in capture antibody coating, we tested incubation with PVOH after passive coating capture antibody using direct adsorption. Although we found some reduction in background, when used at higher concentrations significant reduction in signal was also observed (Figure S3), suggesting perhaps that PVOH can hinder analyte and detection reagent diffusion to the capture antibody surface. Coating with PVOH prior to adsorption of capture antibody resulted in very significant reduction in capture antibody density (Figure 1A and 1B and data not shown).

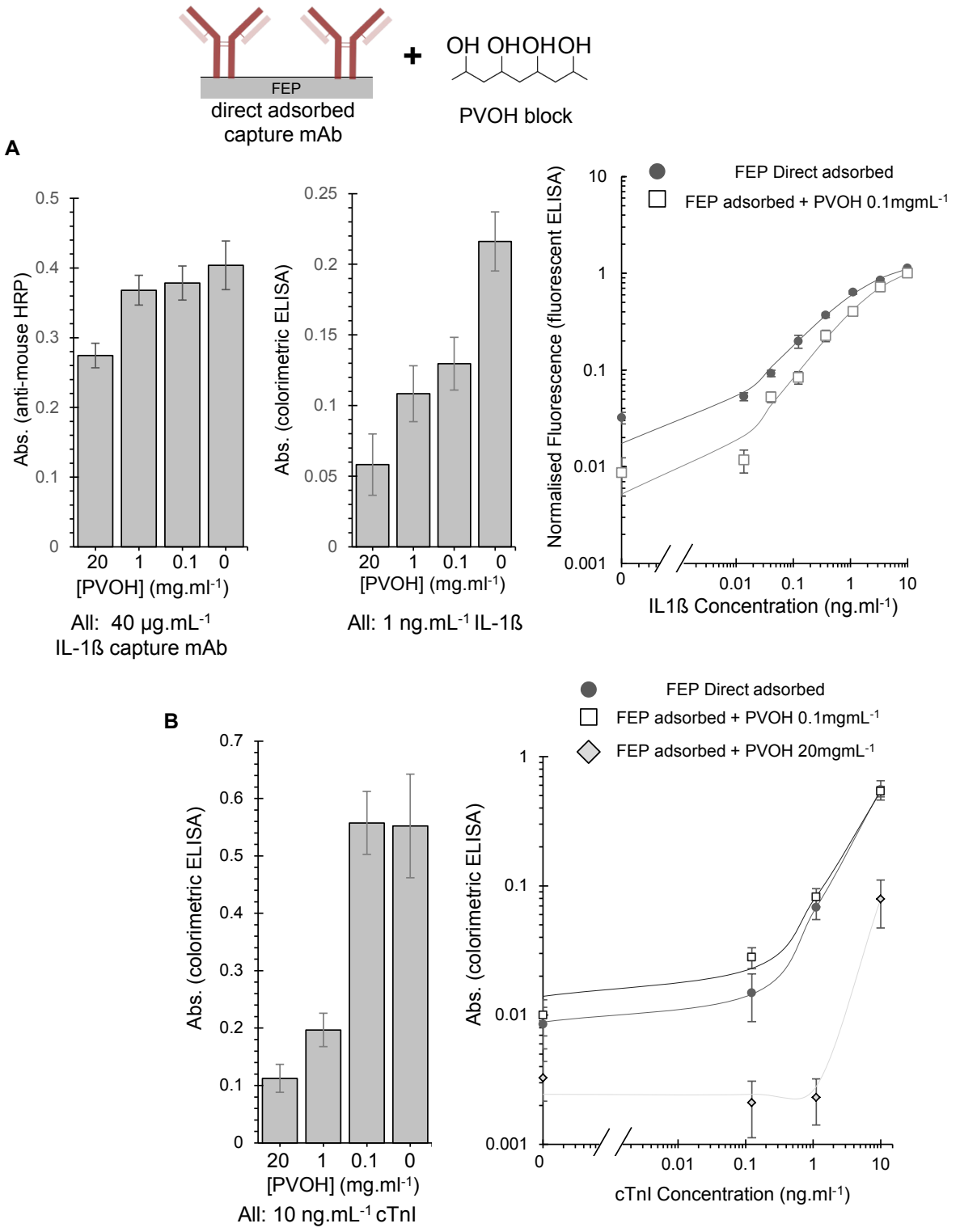

Figure S3.

PVOH can reduce ELISA assay background in FEP microcapillaries.

(A) FEP MCF was coated with IL-1ß capture antibody by passive adsorption, and the effects of varying concentration of PVOH on detectable capture antibody concetration measured using anti-mouse HRP (left) and assay signal (middle) assessed. A full fluorescent ELISA IL-1ß response was completed for 80 µg/mL capture antibody passively adsorbed either without PVOH or with PVOH at 0.1mg/mL (right). B) FEP MCF test strips were coated with

cTnI capture antibody by passive adsorption, and the effects of varying concentration of PVOH on full colorimetric ELISA assay signal assessed with cTnI at 10ng/mL (left). A full colorimetric ELISA response for cTnI was completed for 80 µg/mL capture antibody passively adsorbed either without PVOH or with PVOH at 20 and 0.1mg/mL (right).

Supplementary References

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