ACS APPLIED BIO MATERIALS

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ACS Appl. Bio Mater., **Just Accepted Manuscript** • DOI: 10.1021/acsabm.9b00214 • Publication Date (Web): 22 May 2019

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Transparent, hydrophobic FEP Teflon offers rapid, robust and irreversible passive adsorption of diagnostic antibodies for sensitive optical biosensing

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ABSTRACT

Current literature data is scarce and somehow contradictory in respect to the suitability of 'non-stick' fluoropolymer surfaces for immobilisation of biomolecules. We have previously shown empirically that transparent FEP Teflon offers rapid and sensitive optical biosensing of clinically relevant biomarkers. This study shows for the first time a comprehensive experimental analysis of passive adsorption of diagnostics IgG antibodies on actual FEP Teflon microfluidic strips. Full equilibrium isotherms and kinetics for passive adsorption were studied and modelled using protein titration method using hundreds of multi-bore microfluidic strips for a range of temperatures, pH, ionic strengths and inner diameters, using both polyclonal and monoclonal antibody systems. Results were benchmarked against other plastic hydrophobic and glass hydrophilic surfaces. For the first time, it was shown quantitatively that the hydrophobicity of fluoropolymer surfaces encourages the passive adsorption of diagnostics antibodies for biosensing, being insensitive the temperature of incubation and ionic buffer strength. The mass of captured antigen increased with increasing antibody surface coverage up to ~400 ng/cm^2, with an optimal adsorbed antibody activity for 45-69% of full monolayer coverage, matching results for other biosensing surfaces. The equilibrium was reached fast, within 5-10 min and surprisingly both the kinetics and equilibrium of antibody adsorption were dependent of the inner diameter of microcapillaries. This is a novel and relevant result that will generally impact on the design of miniaturised microfluidic biosensing devices. The antibody surface densities obtained with hydrophobic plastic surfaces were 2 to 4-fold lower than for a hydrophilic (glass) surface, however the former presented a multi-layered adsorption with a higher level of irreversibility as shown by the adsorption and desorption rates around one order of magnitude smaller than for glass, which is highly desirable for biosensing with surface-coated biomolecules.

Keywords: antibody adsorption, Teflon® FEP, adsorption kinetics, microcapillary film, biosensing, microfluidics

1. INTRODUCTION

Immobilisation of proteins by passive adsorption certainly remains the simplest and most scalable method for manufacturing plastic immunosensing surfaces, however several limitations of this protein immobilisation technique have been identified in literature, such as specificity to surface chemistry, limited surface area available for binding, surface geometry, pH, temperature and buffer ionic strength.[1] Consequently, manufacturing of diagnostic tests tends to avoid adsorption as immobilisation strategy, preferring complex methods that imply surface modification, covalent binding and affinity binding techniques.[2–6] However, for successful commercialization, antibodies need to be immobilised in bulk quantities, which needs to be achieved through a simple, reproducible and cost-effective method.[2] Independently of which immobilisation technique used diagnostics performance needs an antibody monolayer with controlled density, uniformity, stability and orientation for the development of sensitive and robust immunoassays.[7,8]

Hydrophobic substrates, such as plastics and PDMS (polydimethylsiloxane, with contact angle with water ~115º)[2,9,10] are usually chosen for antibody adsorption, since hydrophobic interactions are strong enough to effectively bind an antibody to a surface.[1,2,11] PDMS device fabrication is, however, mainly performed by photolithography and other prototyping methods, which are difficult to upscale, since these manufacture methods do not allow mass production. Nevertheless, thermoplastic resins devices are easily mass produced by melt-extrusion or inject molding.[2,8] Fluoropolymers such as FEP Teflon ® present excellent optical transparency that would make this substrate ideal for optical immunoassays, in addition to excellent chemical and thermal resistance, with non-reactive surfaces for a variety of chemicals and solvents. However previous adsorption studies on fluoropolymer surfaces including Teflon®[12] showed low levels of binding compared to e.g. gold and glass surfaces,[13] consistent with the 'non sticky' commercial status of fluoropolymer surfaces.

Nevertheless, our research group has recently reported unmatched levels of detection of clinically relevant biomarkers in a novel mass-manufactured fluoropolymer microfluidic material.[14–17] The microcapillary film (MCF) is a long ribbon made of Teflon® FEP with variable number and diameter of embedded capillaries, with contact angle with water of 123º.[18] Our previous empirical assay development studies suggested antibody adsorption into FEP Teflon® is stable, reliable, rapid and cost-effective.[19–21] And, although several methods for antibody covalent immobilization onto Teflon® FEP MCF have been reported, passive adsorption remains the optimal method for sensitive assays in this microfluidic platform.

which implies that antibody adsorption onto Teflon® FEP provides an uniform, strongly bound antibody monolayer with active antibodies, oriented for antigen binding.[20] Consequently there is a need to fully understand and characterise passive adsorption of diagnostics antibodies into FEP Teflon® microfluidic devices, something not reported to date.

This study shows for the first time a detailed, systematic experimental study of adsorption equilibrium and kinetics of passive antibody adsorption in actual FEP microfluidic surfaces, and the impact of FEP adsorbed antibodies on optical, enzymatic immunoassays. We explored the effect of pH, temperature and buffer concentration on antibody adsorption, also established quantitatively the effect of inner diameter of microcapillary and the link between antibody adsorption and antigen binding when the coated biosensing surface is used for heterogeneous immunoassay. Results were benchmarked against a MCF manufactured from hydrophobic LLDPE (linear low-density polyethylene) and individual glass capillaries. The low cost of MCF material means adsorption and immunoassay studies could be carried out in this study using hundreds of actual FEP microfluidic devices instead of reusing or coating a surface with a fluoropolymer layer.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents. Mouse-IgG (whole antibody) was purchased from Life Technologies (Paisley, UK), rabbit anti-mouse IgG (whole molecule) conjugated with peroxidase and SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride) tablets were supplied by Sigma-Aldrich (Dorset, UK). The BCA Protein Assay Reagent (bicinchoninic acid) was sourced from Thermo Scientific (Lutterworth, UK). The IL-1β recombinant protein, Anti-Human IL-1β biotin and Anti-Human IL-1β purified were supplied from eBiosciences (Hatfield, UK). High sensitivity streptavidin-HRP was supplied by Thermo Scientific (Lutterworth, UK). The Anti-troponin IgG, clone MF4 was purchased from Hytest (Turku, Finland) and the Fab specific IgG conjugated with FITC was supplied by Sigma-Aldrich (Dorset, UK).

Phosphate buffered solution (PBS) and Bovine Serum Albumin (BSA) were sourced from Sigma Aldrich, Dorset, UK. PBS pH 7.4, 10mM was used as the main experimental buffer. Anhydrous Sodium Carbonate was supplied from Fisher Scientific and HEPES from Sigma-Aldrich (Dorset, UK). The blocking solution consisted of 3% w/v protease-free BSA diluted

in PBS buffer, except for IL-1β assays, which used a superblocking solution supplied by ThermoScientific (Lutterworth, UK). For washings, PBS with 0.05% v/v of Tween-20 (Sigma-Aldrich, Dorset, UK) was used.

The 10-bore MCF material was fabricated from Teflon® FEP using a melt-extrusion process by Lamina Dielectrics Ltd. (Billinghurst, West Sussex, UK). The MCF used for most experiments showed a mean hydraulic diameter, d_h of 212±16.3 μm, however we also tested MCFs having mean d_h of 109±12.2 μm and 375±28.6 μm. A 19-bore MCF materials was fabricated at Cambridge University[18] from LLDPE and showed a mean d_h ~200 μm. Singe-bore glass capillaries, 152 mm in length and internal diameter of 0.58 mm were sourced from World Precision Instruments, Inc. (Hitchin, Hertfordshire, UK).

2.2. Quantitation of antibody mass adsorbed. The antibody mass adsorbed was quantified based on mass balance between an initial antibody solution, in a concentration range 0, 6.25, 12.5, 25, 50, 100, 200 and 400 μg/ml, and a final antibody solution obtained after 2 hour incubation in the capillaries. BCA protein assay was used for quantifying the antibody concentration in the aliquots based on solution depletion technique. Further details are provided in the supplementary information (SI) file.

In order to understand the effect of temperature on antibody adsorption, the temperature was kept constant during IgG adsorption incubations at either 4º, 20º or 37 ºC. For studying the pH effect on IgG adsorption, we prepared a IgG serial dilution in sodium carbonate buffer 10 mM at pH 10.7, in Phosphate buffer (PBS) 10 mM at pH 7.4 and HEPES 10 mM at pH 4.8. The IgG solutions were incubated inside the Teflon® FEP capillaries for 2 hours at room temperature. The effect of surface chemistry on IgG adsorption was studied by comparing antibody adsorption in Teflon® FEP (Fluorinated ethylene propylene, contact angle 123±1.6º with water)[22] with LLDPE (CH3 plastic polymer, contact angle with water ~120º)[23] and glass capillaries (borosilicate glass, with contact angle with water 25º)[24] at pH 7.4, for 2 hours at room temperature.

A 200 μg/ml IgG solution dissolved in 10 mM PBS buffer pH 7.4, was incubated for 2h, at 20ºC in three FEP MCF with three different inner diameters: 109, 212 and 375 μm. Protein content of the initial and final solutions was quantified by BCA method and a IgG mass adsorbed given by the protein mass balance.

The adsorbed concentration, obtained from the protein mass balance before and after incubation in the capillaries, was converted to adsorbed surface density (ng/cm^2) by diving it

by the surface area to volume ratio (SAV, cm^{-1}), which for a circular capillary is linked to the mean d_h, (cm) of the capillary:

$$SAV = \frac{4}{d_h} \quad (1)$$

Antibody adsorption on Teflon® FEP was modelled as a Langmuir isotherm based on equation (2), best-fitted to experimental data using the minimum squares difference in Excel' solver:

$$\tau = \tau_{max} \frac{K \cdot [\mathrm{IgG}]}{1 + K \cdot [\mathrm{IgG}]} \quad (2)$$

where τ is the surface coverage in equilibrium (ng/cm^2), τ_{max} is the number of adsorption sites available given by a maximum adsorbed concentration (ng/cm^2), K is the adsorption constant (ml/µg) and [IgG] is the antibody concentration in solution (µg/ml).

2.3. Kinetics of antibody adsorption onto different microcapillary surfaces. Kinetic studies were also performed using the solution depletion technique and calculating the mass balance between initial antibody concentration and after variable incubation times in the capillaries; for details are provided in SI file.

The kinetics of antibody adsorption kinetics were assumed to follow equation 3, fitted to experimental data based on minimum squares difference. This equation is the algebraic solution of a differential equation given by the difference between the adsorption and desorption processes of the reactant to free binding sites:

$$\tau(t) = \frac{K_{on}[IgG]}{k_{on}[IgG] + K_{off}} \left[1 - \exp\left[-\left(K_{on}[IgG] + K_{off}\right) \cdot t\right]\right] \quad (3)$$

where τ(t) is the surface coverage (ng/cm^2) at a given experimental time, t (min), $[IgG]$ is the antibody bulk concentration (M), K_{on} is the adsorption rate (M^{-1} min^{-1}) and K_{off} is the desorption rate (min^{-1}).

For simplicity, kinetic adsorption data has been presented as percentage surface coverage, ϕ computed by normalising τ with the theoretical antibody monolayer assuming vertical antibody orientation based on a reference dimensions for an antibody molecule (14.2nm×8.5nm×3.8nm)[25]. In order to account for different sizes of microcapillaries and enable direct comparison of different capillary systems, the percentage ϕ was further normalised by SAV described in equation 1.

2.4. Effect of buffer ionic strength in antibody adsorption onto Teflon® FEP by confocal microscopy. In this study 25 and 50 μg/ml solutions of anti-TnI were prepared in different concentrations of PBS buffer at pH 7.4: 0.625, 1.25, 2.5, 5 and 10 mM. The 10 solutions were aspirated into 8 MCF strips, each 8 cm in length, and incubated for 2 hours. After a blocking step, Fab specific IgG conjugated with FITC was incubated, followed by a final washing. The strips were inserted into a MCF holder[17] and imaged with a confocal microscope (LSCM, Nikon inverted Microscope ECLIPSE TE300 with Bio-Rad RAD200, scan head 60X-1.20NA objective lenses, excitation peak wavelength of 488 nm and emission peak wavelength of 530 nm, operating Laser Sharp 2000 software). Fluorescence images were then analysed with ImageJ software (NIH, USA), splitting the RGB image, and using the green channel to produce a greyscale plot, where the height of fluorescent peak was considered the fluorescent intensity.

2.5. Quantitation of antibody adsorbed onto Teflon® FEP using polyclonal mouse-IgG/anti-mouse IgG ELISA. To study the effect of immobilised antibody density in antibody binding in an assay (i.e. capacity of a coated solid phase to specifically capture molecules), a total of 8 Teflon® FEP MCF strips were incubated for 2 hours at room temperature with 0, 6.25, 12.5, 25, 50, 100, 200 and 400 μg/ml of mouse IgG in PBS 10 mM, pH 7.4. The strips were then washed and non-specific surface sites blocked. Three different concentrations of anti-IgG polyclonal conjugated to peroxidase (60, 600, 6000 μg/ml) were added and incubated for 10 minutes. After a washing step the OPD enzymatic substrate was added and images were taken with a flatbed scanner. More details are provided in SI file.

In order to understand the effect of surface area available, this experiment was repeated using 600 ng/ml of anti-IgG conjugated with peroxidase in different bore MCFs of 109 μm and 375 μm. Note that in this method of quantitation the ELISA returned an optical signal that cannot be converted to surface coverage, therefore the relative antibody adsorption was modelled

based on absorbance values and a modified Langmuir isotherm detailed in supporting material.

The effect of immobilised IgG incubation time in antibody binding was studied by incubating 40 μg/ml of IgG in PBS in Teflon® FEP MCF strips from 0 to 120 minutes, before washing the strips with 1 ml PBS-Tween. A solution of 600 ng/ml of anti-IgG, peroxidase conjugated was then added and incubated for 10 minutes. After another washing step, OPD enzymatic substrate was added at the concentration of 1 mg/ml. The MCF strips were then imaged after 5 minutes incubation of OPD in transmittance mode.

2.6. IL-1β sandwich ELISA using monoclonal antibodies. In order to determine the optimum antibody surface coverage for a monoclonal antibody system, 6 cm long Teflon® FEP MCF strips were filled with IL-1β monoclonal antibody solutions of 20, 40, 100 and 140 μg/ml and incubated for 2 hours. Further steps involving surface blocking, washing, incubation of 0.5 ng/ml of recombinant IL-1β, addition of IL-1β biotinylated antibody and High Sensitivity Streptavidin-HRP with OPD enzymatic substrate were followed with final MCF strips images taken with a Flatbed Scanner at 2400 dpi. Further details are provided in SI.

For the IL-1β response curves, three 30 cm long Teflon® FEP MCF strips were filled with 40 μg/ml of IL-1β capture antibody (capAb). One of the strips was incubated for 30 minutes and the other two for 2 hours. The strips underwent blocking, washing, incubation of eight solutions of IL-1β from 0 to 1 ng/ml, addition of IL-1β biotinylated antibody followed by a wash step and high sensitivity streptavidin-HRP and OPD enzymatic substrate incubation. The MCF strips were imaged by a Flatbed Scanner.

2.7. Image Analysis of MCF ELISA strips. RGB digital images were split into 3 separated channels in Image J (NIH, USA). The blue channel images were used to calculate absorbance values, based on the grey scale peak height of each individual capillary of Teflon® FEP MCF as described elsewhere.[16,21] Further details about image analysis procedure are provided in SI.
2.3. Kinetics of antibody adsorption onto different microcapillary surfaces.

3. RESULTS AND DISCUSSION

3.1. Effect of temperature, pH and buffer ionic strength on adsorption equilibrium. Conventionally, the driving force for protein adsorption is regarded as an entropy gain arising from the release of surface adsorbed water molecules and salt ions and from structural rearrangements inside the protein,[26] therefore the first set of experiments aimed at exploring

the effect of temperature, pH and buffer ionic strength on antibody adsorption equilibrium for the 10-bore, Teflon® FEP MCF strips containing microcapillaries with mean d_h of 212 μm, using mouse IgG as a model.

Surprisingly, the effect of temperature was found negligible in respect to the mass of IgG adsorbed as shown by the Langmuir isotherms shown in Figure 1A and best-fitted Langmuir model parameters summarised in Table 1. Values of τ_{max} and K (equation (2), for 10mM PBS and pH 7.4) varied less than 2% and 7%, respectively, for the range of temperatures tested (4, 20 and 37 ^{o}C), with an average value of τ_{max} =476 ng/cm^2, which contradicts the temperature enhancement of protein adsorption reported in literature for other biosensing surfaces,[1,11,27] however in alignment with protein adsorption studies on hydrophobic surfaces. As FEP is substantially hydrophobic[28] (i.e. water repellent) our results suggest passive adsorption of IgG molecules to FEP is connected to the rate of release of water molecules which remained constant for the range of temperatures tested. Chen et al.[29] showed the number of water molecules released from the protein-adsorbent binding process remains approximately constant for temperatures below 40 ^{o}C. Also, the same study showed the binding affinity of protein adsorption to hydrophobic surfaces remained almost unchanged at temperatures below 40 ^{o}C due to proteins maintaining the original protein conformation at such temperatures. Although extensive literature has identified enthalpy and entropy effects in hydrophobic interaction systems, it appears protein adsorption to FEP at these temperatures follows a simple monolayer adsorption behaviour. This represents an advantage in respect to manufacturing of FEP microfluidic biosensing devices, as it removes the need of precise temperature control, lowering the cost of manufacturing and enabling a higher degree of freedom to operators.

Reduction in pH below the isoelectric point (typically 6.3-8.9 for IgG[30]) resulted on an increase to the amount of IgG adsorbed onto Teflon® FEP microcapillaries (Figure 1B), with τ_{max} increasing by 76%, from 484 to 853 ng/cm^2 (Table 1) whereas an increase in pH to 10.7 resulted on a 58% decrease to τ_{max} down to ~200 ng/cm^2. Conventionally, adsorption rates are higher when protein and substrate bear opposite charges, since electrostatic attractions accelerate the migration towards the surface[1,31], consequently the Langmuir isotherm plots in Figure 1B suggested at first sight that FEP is negatively charged (which is the case for some FEP resins available in the market), however the rate of adsorption equilibrium constant, K, in FEP microcapillaries was smaller at lower pH, meaning no charge difference between the surface and the IgG molecules. This increase in mass of antibody adsorbed at lower pH

(Table 1) can instead be explained by protein denaturation, which promotes unfolding and aggregation of antibody molecules, in line with the works of e.g. Wright and co-authors[32] reported about 50% of the antibody denatured in solution at pH 4.95.

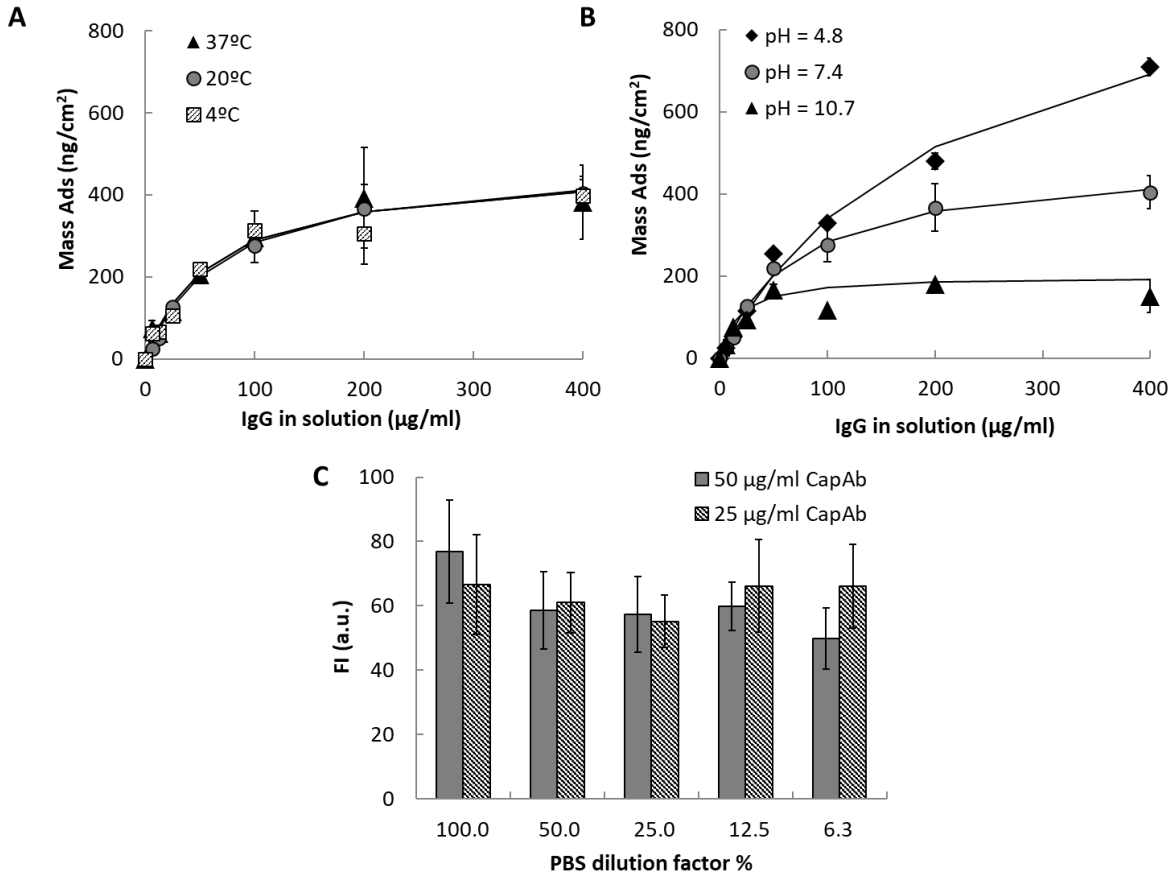

Figure 1. Effect of temperature, pH and buffer concentration on adsorption of mouse IgG on 10-bore, Teflon® FEP MCF strips having mean d_h of 212 µm. **A** Adsorption isotherms at 4, 20 and 37 °C (constant pH 7.4). **B** Antibody adsorption isotherms at pH 4.8, 7.4 and 10.7 (constant temperature 20 ºC). **C** Effect of buffer dilution on antibody adsorption, as recorded with fluorescence confocal microscopy. Note that initial PBS solution contains 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride. The continuous lines in A and B represent the best-fitted Langmuir isotherms with parameters summarised in Table 1.

The Langmuir plots shown in Figure 1B suggested adsorption of IgG in FEP microcapillaries was most effective at a pH closest to the isoelectric point of IgG, where electrostatic protein–

protein repulsions are minimized and higher packing densities possible on the FEP surface. This agrees with our vast experience in immunoassays development in the FEP microcapillaries, with ideal pH for immunoassays always around the isoelectric point, see for example Barbosa *et al.*[14,16] and Castanheira *et al.*[15]

We also noticed the concentration of dissolved ions (also known as ionic strength) in the bulk antibody solution had negligible effect on the amount of immobilized antibody (Figure 1C). There was no significant change in the fluorescent signal used to identify immobilised antibody layers dissolved PBS buffer at different dilution ratios, yielding a range of 0.6-10mM for phosphate, 0.3-2.7 mM potassium chloride and 17.1-137 mM sodium chloride (Figure 1C). It has been reported previously that increased salt concentrations reduce electrostatic repulsion between like-charged material, favouring IgG adsorption, and decreases electrostatic attraction between oppositely charged material, impeding adsorption.[33] Consequently, these new results suggest antibody adsorption onto Teflon® FEP is driven by hydrophobicity not by electrostatic interactions. A previous study of protein adsorption on silicon surfaces by Zhao *et al.*[34] reported a reduction on antibody adsorption only for very high salt concentrations of 150 mM and above, at lower salt concentration antibody adsorption remained very consistent. Our data shows no difference in antibody adsorption to FEP microcapillaries up to 137 mM of sodium chloride, 2.7 of potassium chloride and 10 mM of phosphate buffer, which suggests antibody adsorption to Teflon® FEP is stable even for high salt concentrations (Figure 1C).

Table 1. Best-fitted parameters for Langmuir isotherms (based on equation 2 and plotted in Figure 2) describing antibody adsorption in Teflon® FEP microcapillaries at varying pH and temperatures.

	Temperature			**pH**		
	4°C	20°C	37 °C	4.8	7.4	10.7
***K* (ml/µg)**	0.015	0.014	0.016	0.007	0.014	0.061
τ_{max} **(ng/cm²)**	472	484	472	853	484	200
R^2	0.9856	0.9963	0.9891	0.9801	0.9963	0.9413

On the overall, the optimal adsorption conditions shown in Figure 1 revealed a surface density of antibody onto Teflon® FEP microcapillaries of ~400 ng/cm^2 when coated with 400 µg/ml of IgG in bulk solution. This yields a "loading density" of ~2,000 ng/cm^2, calculated by dividing the loaded antibody concentration by the SAV (Equation 1). This ~5-fold excess

of antibody required to fully coat the plastic surface is evidence of low affinity for adsorption of IgG to the Teflon® FEP surface, which is also confirmed by the reduced adsorption constant of 0.014 ml/μg (Table 1). This suggests at first sight that antibody immobilisation onto FEP and other fluoropolymer surfaces is very inefficient, nevertheless the mass adsorbed onto FEP microcapillaries was found very similar to values reported to other hydrophobic and fluorinated surfaces at similar physical conditions. For example, a IgG adsorption study on gold electrodes coated with Teflon AF (amorphous fluoropolymers) based on optical waveguide light mode spectroscopy[35] reported a surface density of approximately 200 ng/cm^2, similar to the 220 ng/cm^2 obtained in our Teflon® FEP microcapillaries, both using 40 μg/ml of IgG in bulk solution. Another study by Wiseman and Frank[36] based on quartz crystal microbalance with dissipation in a CH_3-terminated surface (1-dodecanethiol self-assembled monolayer on gold) reported a maximum coverage of 468 ng/cm^2 with 100 μg/ml IgG in solution. This value was ~40% larger than the surface coverage obtained with our 10-bore, 212 μm Teflon® FEP MCF (275 ng/cm^2) using the same IgG concentration. This difference might be due either to the differences in geometry and/or surface chemistry. In spite of a lower 'affinity' to antibody adsorption, Teflon® FEP is not less effective than other surfaces for immobilising proteins and in particular IgG antibody molecules.

3.2. Impact of capillary diameter and surface coverage on antibody-antigen equilibrium. A key parameter able to dictate the fate of an heterogeneous immunoassay, perhaps even more relevant than the total mass of antibody adsorbed, is the amount of antibody than is readily available to bind the antigen or secondary reagents. Consequently we characterised the impact of antibody adsorption and surface coverage on immune-binding of a targeted protein using a labelled secondary antibody.[13] This is an essential feature for successful use of FEP microfluidic surfaces in actual immunoassays, such as colourimetric or fluorescent ELISA. Whenever performing this methodology it is paramount to understand the effect of labelled antibody bulk concentration (that acts as an antigen) in the equilibrium as illustrated in Figure 2A.

As expected, an increase in surface density of adsorbed mouse IgG (computed from the coating antibody concentration and adsorption plots shown in Figure 1) led to an increase on optical signal, meaning a higher extent of binding of adsorbed antibody with the labelled anti-mouse IgG (acting as Ag). We tested 212 μm i.d. Teflon® FEP microcapillaries with concentrations of *Ag* covering three orders of magnitude, being 60, 600 and 6,000 ng/ml of

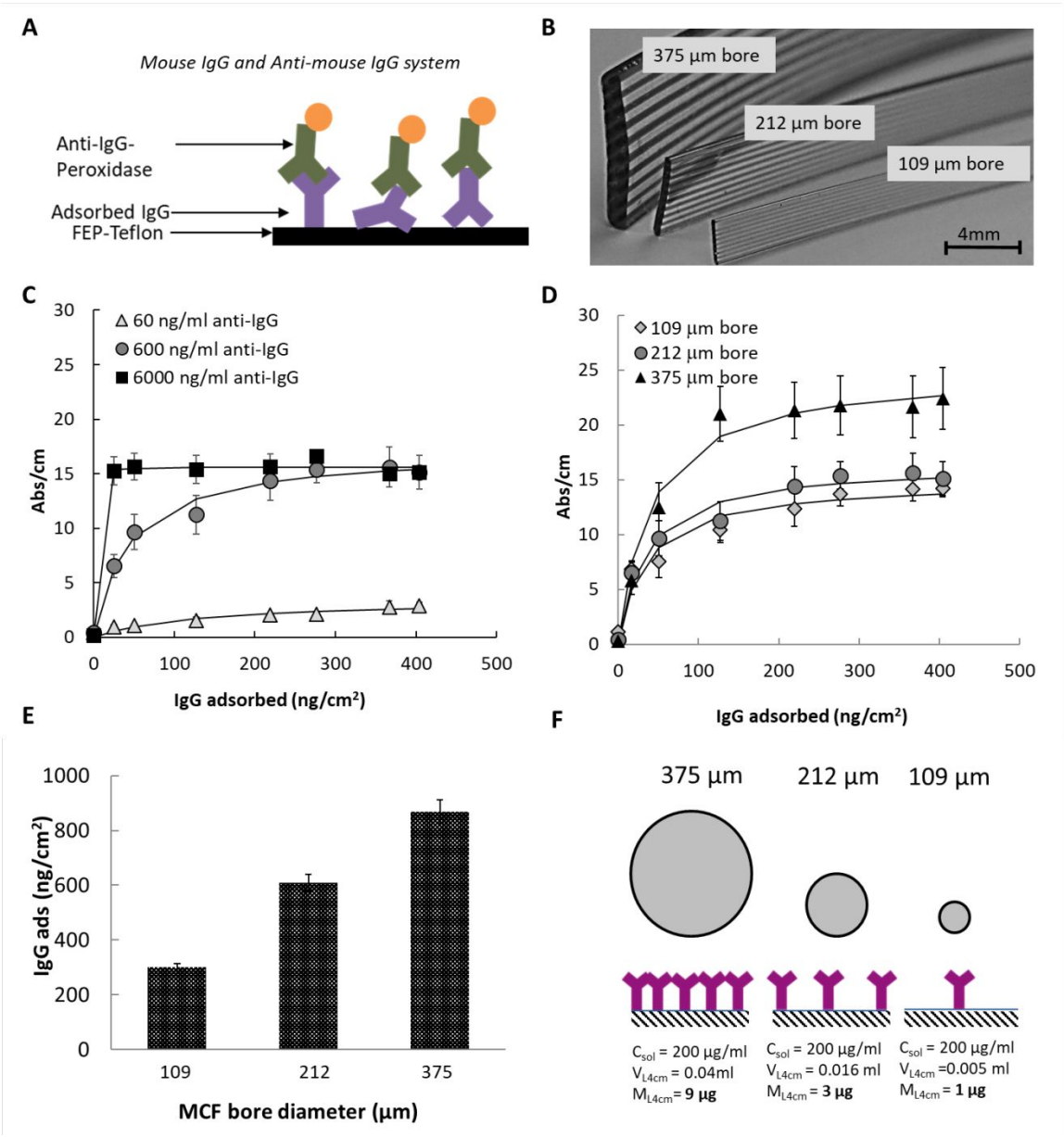

Figure 2. Effect of antibody surface coverage in antibody-antigen binding on Teflon® FEP MCF using a polyclonal, mouse IgG/anti-mouse IgG system. **A** Schematic diagram of direct ELISA with anti-mouse IgG conjugated to peroxidase ("antigen") binding to immobilised mouse-IgG antibody. **B** Microphotograph of 10-bore Teflon® FEP MCFs having different bore sizes: 109±12.2 µm, 212±16.3 µm and 375±28.6 µm. **C** Effect of antibody surface coverage on optical immunoassay signal in the 212 µm mean d_h FEP MCF with different concentrations of anti-mouse IgG. **D** Effect of internal diameter of FEP microcapillary and antibody surface coverage on optical immunoassay signal for a fixed concentration of anti-mouse IgG. The continuous lines in C and D show the best-fitted Langmuir isotherms with parameters summarised in Tables S1 and S2. **E** Antibody mass adsorbed on FEP

microcapillaries with 109, 212 and 375 μm inner bore diameter**. F** Schematic diagram explaining the relation between microcapillary bore diameter and antibody adsorption.

anti-mouse IgG. At the highest concentration of anti-mouse IgG, a small density of IgG adsorbed of 20-40 ng/cm^2 revealed sufficient to saturate the optical signal (Figure 2C). At lower concentrations there was a clear correlation between the surface density of adsorbed IgG and the optical signal (Figure 2C), and followed a typical Langmuir isotherm has shown in Table S1 (see SI file). Note these experiments cannot provide information about antibody orientation as the selected labelled non-specific anti-mouse IgG is able to bind any part of the immobilized IgG antibody, however these provide essential evidence regarding the importance of the labelled antibody (anti-IgG) concentration and immobilized antibody surface coverage in the binding equilibrium.

Additionally, we explored the effect of inner capillary diameter by testing MCF strips with three different inner diameters (Figure 2B) and noticed optical immunoassay signal increased with increasing inner diameters of the FEP microcapillaries. This is also shown by the increasing Abs_{max} values in Table S2 (see SI file). This results from the fact larger capillaries present a larger surface area for adsorbing the capAb, therefore yielding higher antibody surface coverage (Figures 2D-F) which favours antigen binding in an actual immunoassay. The optical signal obtained with 600 ng/ml anti-IgG was >60% larger in the 375 μm i.d. microcapillary compared to the 212 μm i.d. microcapillary, suggesting antibody adsorption is also very dependent on the geometry of the biosensing surface in addition to the surface chemistry (this is further described later in this manuscript). Higher antibody surface coverage for larger inner diameter capillaries was also determined through the IgG mass balance (Figure 2E) confirming the dependency of adsorbed antibody density and inner diameter of FEP microcapillaries. This is to our knowledge the first time this diameter effect has been reported in literature and it might be due to a fact unique to cylindrical microcapillaries, enabling to coat the whole cross section with the antibody/protein, in contrast to conventional microchannel-based devices.

3.3. Effect of antibody surface coverage on antibody activity and orientation. We studied the link between adsorbed antibody surface coverage and antibody activity and orientation in FEP microcapillaries by performing a sandwich ELISA with adsorbed monoclonal

antibodies, capable of uniquely binding to a specific epitope in an antigen molecule (Figure 3A).

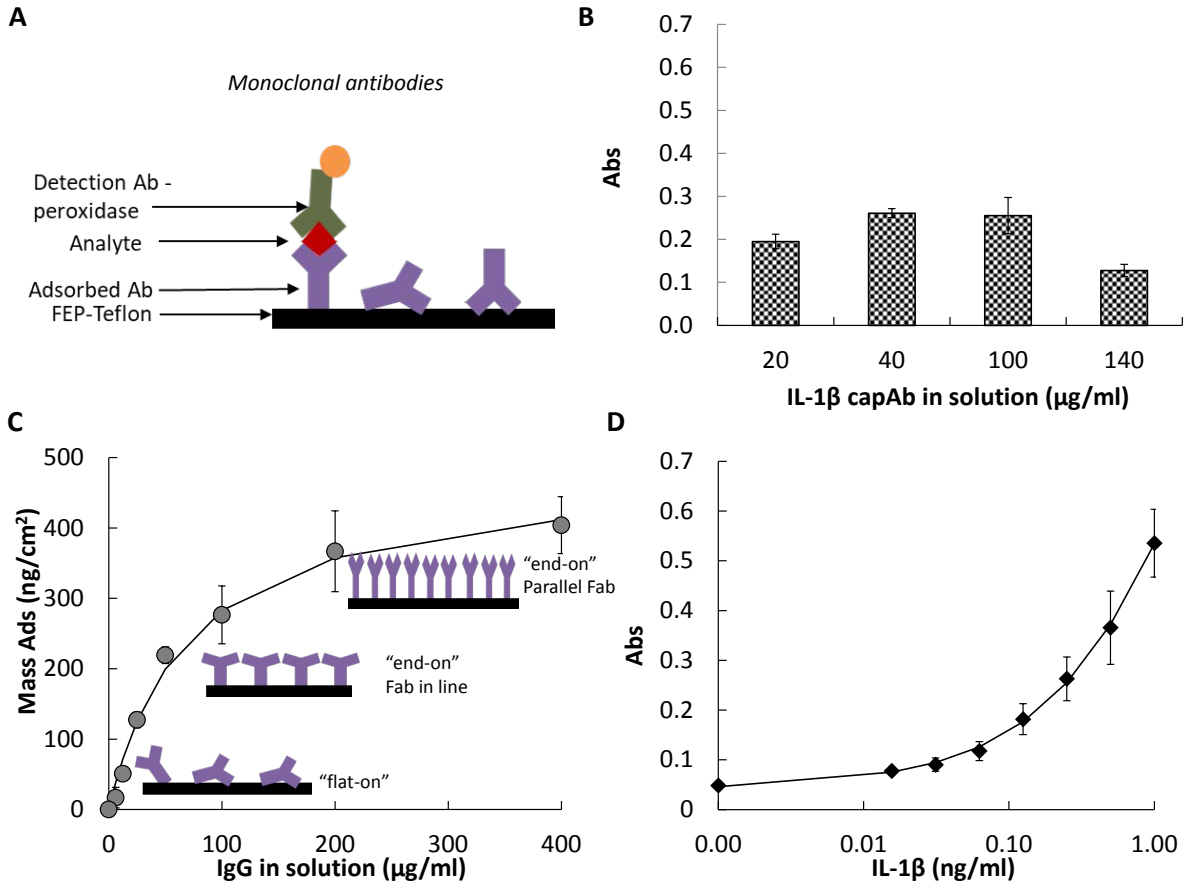

Figure 3. Effect of antibody surface coverage in antibody activity/orientation on 212 µm Teflon® FEP MCF. **A** Schematic diagram of sandwich ELISA based on adsorbed monoclonal capAb and monoclonal detection antibody directly conjugated to peroxidase. **B** Effect of concentration of capAb coating on optical signal, showing antibody surface coverage influences antibody activity/orientation in FEP microcapillaries. **C** Antibody adsorption isotherm at 20ºC, pH 7.4 and 10mM of PBS, with the continuous line representing the best-fitted Langmuir model (Equation 2), with best-fitting parameters summarised in Table 2, along with schematic representation of hypothesized relation between surface coverage and antibody orientation on Teflon@FEP, supported by experimental data and literature. **D** Full response curve for quantitation of IL-1β, showing optical quantitation of clinically relevant biomarkers is feasible based on passive antibody adsorption in fluoropolymer microfluidic devices.

Contrary to the polyclonal system, as the binding region needs to be available within an intact protein structure, a drop on the optical signal in the sandwich immunoassay (schematically summarised in Figure 3A) evidenced denaturation and/or inadequate orientation of capAb during adsorption but also steric hindrance caused by neighbour adsorbed antibodies. Antibody surface coverage and antibody activity are major aspects in consideration for the development of any high-performance immunoassay tests, such as those aimed at cardiovascular diseases and cancer diagnosis, and this has not been studied to date for fluoropolymer surfaces.

In contrast to the polyclonal system discussed previously, we observed a rapid drop in optical signal with the increase in concentration of capAb above the window 40-100 µg/ml (Figure 3B). Note this data was gathered using 0.5 ng/ml of IL-1β antigen, it is likely the threshold will depend on the antibody pair but also on the limit of detection and cut-off aimed for the antigen. The antibody densities estimated with Langmuir model were 175 ng/cm^2 for 40 µg/ml of antibody in solution and 277 ng/cm^2 with 100 µg/ml corresponding, respectively, to 45% and 69% of the maximum total mass adsorbed onto Teflon® FEP capillaries, based on a theoretical antibody monolayer estimated as 440 ng/cm^2, assuming an antibody size of 14.2nm×8.5nm×3.8nm.[25] This suggests the adsorbed antibody achieved the maximum capacity to bind the antigen within less than full monolayer, which is commensurate with data reported in literature for other immunoassay surfaces.[37]

The combined analysis of experimental data gathered with distinct methodologies (i.e. protein mass titration summarised in Figure 1, and optical ELISAs using monoclonal and polyclonal antibodies summarised in Figures 2 and 3, suggested surface coverage density is directly linked to antibody orientation and binding capacity of Teflon® FEP capillaries schematically represented in Figure 3C. We hypothesised that lower antibody surface densities favour antibodies adsorbed on "flat on" orientation (both Fc and Fab fragments adsorbed onto the surface) whereas large antibody densities favour "end on" orientation, with Fab region towards the solution (Figure 3C). This hypothesis is supported by current literature. Firstly, based on the dimensions of antibody molecules, Buijs and co-authors[38] suggested a relationship between the mass adsorbed and the orientation of the molecule on the surface, with 200 ng/cm^2 representing a monolayer with antibodies in a "flat-on" orientation, 260 ng/cm^2 in an "end on" orientation with Fab fragments in line, and 550 ng/cm^2 in an "end-on" orientation with Fab fragments close together and parallel, which

explains reduced activity of adsorbed antibody due to the proximity of Fab fragments. This suggests antibody adsorption onto Teflon® FEP at the conditions studied happened through a monolayer formation with the maximum adsorbed amount of approximately 404 ng/cm^2, which suggests a packed antibody monolayer with antibodies oriented "end-on" with Fab fragments in line with Buijs and co-authors[38], based on a theoretical monolayer for Teflon@FEP of ~440 ng/cm^2. Other studies reported antibody denaturation of the Fab region with loss of antibody binding capacity to Teflon surfaces,[39,40] however those findings are not supported by the data presented in this study. The optical sandwich ELISA based on adsorbed antibody yielded a good limit of detection (23 pg/ml or 0.74 pM of IL-1β), for antibody surface coverage of 175 ng/cm^2 (Figure 3D), comparable to gold-standard microtiter plate based ELISA. This confirms again that antibodies adsorbed in Teflon@FEP assume an "end-on" position, available for antigen binding from 45% to 69% of an antibody monolayer, as previously discussed for Figure 3B, which approaches the values proposed by Buijs and co-authors[38]. Xu and co-authors[41] reported a similar relationship with hydrophilic silicon oxide. Zhao and co-authors[34] reported that the binding capacity of an immobilised antibody is greater for surface coverages below 50% of full monolayer, above this threshold the binding sites in the antibody molecules can become inaccessible to the antigen. Also, higher antibody densities decreases the degree of irreversibility of antibodies bond to the surface, with the irreversibly adsorbed amount being a maximum 250 ng/cm^2 on a hydrophilic silicon oxide surface.[41]

Further studies support the relationship between surface coverage and antibody orientation/binding capacity and the hypothesis for antibody adsorption onto Teflon@FEP. The work of Wiseman and co-authors[42] based on quartz crystal microbalance with dissipation, detected a shift in dissipation value of the crystal almost to zero for a mass of antibody adsorbed below 200 ng/cm^2, meaning that this initial mass is strongly attached to the surface and suggesting "flat-on" orientation of the antibody. Neutron reflexion studies by Xu and co-authors[41] revealed a 4 nm thick layer, which is close to the short axial length of an antibody molecule, for a mass adsorbed of 220 ng/cm^2, also suggesting a "flat-on" orientation. Above 200 ng/cm^2 both Wiseman and co-authors[42] and Xu and co-authors[41] showed an increase on dissipation slope, meaning new antibodies are adsorbed onto the surface in a less rigid mechanical coupling, suggesting "end-on" orientation of molecules. Surprisingly, Wiseman and co-authors[42] reported no decrease in antibody binding capacity with surface densities above 50% of the monolayer, suggesting an active antibody monolayer

is achieved at ~468 ng/cm^2. A further increment in bulk IgG concentration resulted in higher dissipation values, suggesting a multilayer formation.[36]

On the overall, the sandwich ELISA with monoclonal antibodies confirmed that antibodies adsorb onto FEP just like other surfaces with good stability and activity, in contrast to what has been suggested by few previous publications in hydrophobic Teflon® surfaces.[39,40] This is probably linked to the fact that we used actual, unmodified fluoropolymer surfaces, whereas previous studies were based on glass surface coated with Teflon or even on latex suspensions as the case of Vermeer and co-authors[39,40] (they used Teflon particles with mean diameter 215 nm), which clearly was insufficient to mimic the real chemistry of fluoropolymer surface.

3.4. Kinetics of adsorption of IgG antibody for FEP. In addition to equilibrium adsorption isotherms, we have also studied the kinetics of IgG adsorption onto fluoropolymer microcapillary surfaces, this is important to inform the on/off rates of immobilised antibodies and the time required to manufacture MCF diagnostic strips, impacting on the throughput and consequently the final cost of the tests. Adsorption kinetics can also inform about the strength of the bond with the plastic substrate (surface), degree of reversible antibody adsorption or antibody denaturation, and modifications in the binding capacity of the adsorbed antibody.

We noticed adsorption of IgG onto Teflon® FEP was surprisingly fast, with equilibrium reached within 5-10 minutes and independent of the concentration of IgG loaded into the microcapillaries. This was unequivocally shown in data collected by both the solution depletion technical (Figure 4A) and ELISA (Figure 4B), the two data sets are further compared in Figure 4C in terms of normalised signal, being 100% the mean value at plateau. We estimated K_{on} in order of 10^5 M^{-1}min^{-1} at small bulk concentrations (20 and 40 μg/ml) and in the order of 10^6 M^{-1}min^{-1} for larger antibody bulk concentrations (200 μg/ml) as summarised in Table 2. The percentage surface coverage ϕ summarised in Figure 4B was computed based on a full theoretical monolayer with all antibodies in the "end on" position. Two full IL-1β response curves made with 30 minutes and 120 minutes of monoclonal antibody adsorption, revealed lower limits of detection of 61 pg/ml (1.97 pM) and 54 pg/ml (1.75 pM) respectively (Figure 4D), which shows that between 30 to 120 minutes of adsorption onto Teflon@FEP, antibody binding capacity is not significantly affected.

3.5. Effect of surface chemistry in antibody surface coverage and adsorption kinetics. Surface chemistry is by far identified in literature as the main factor influencing antibody

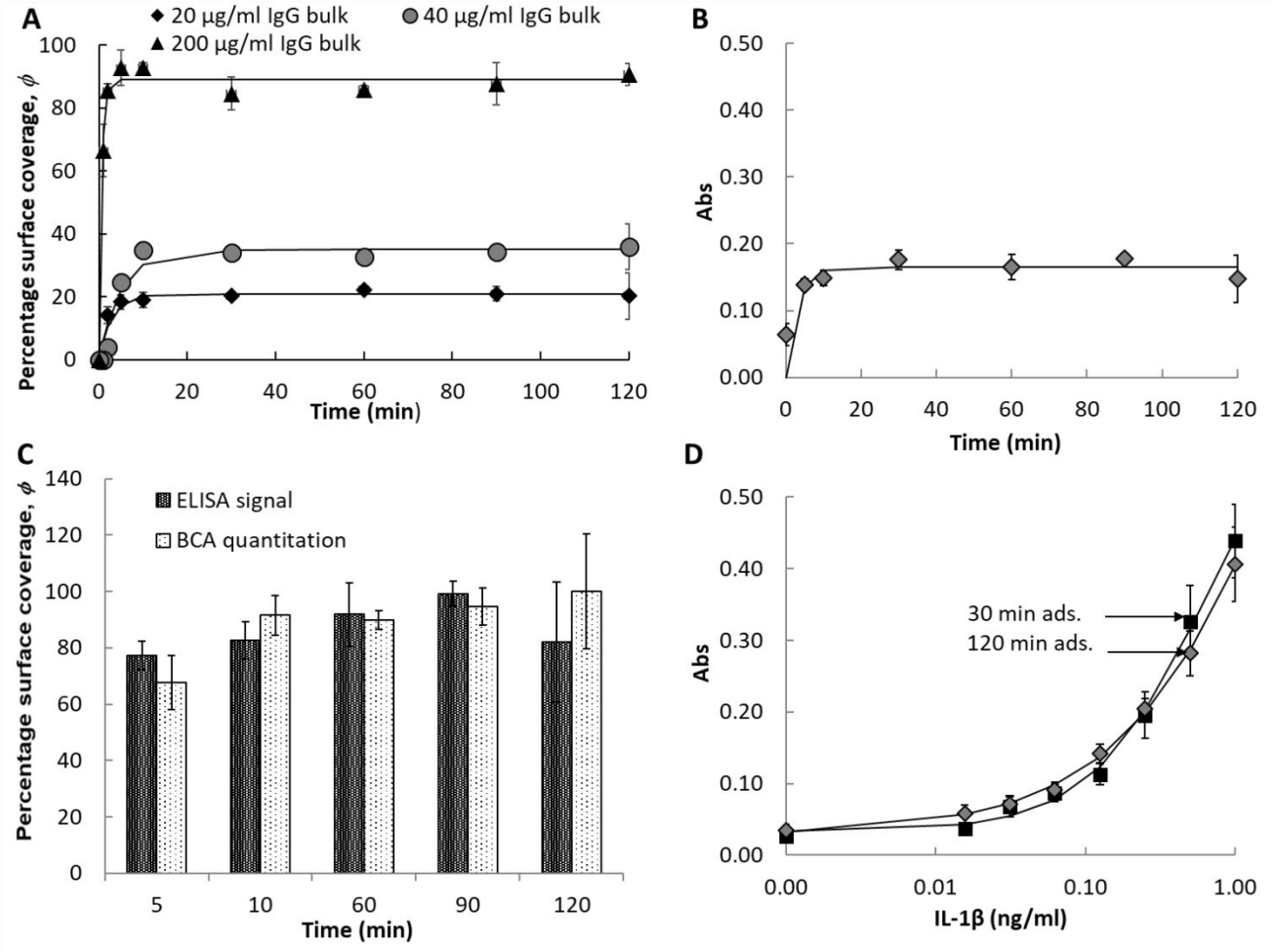

Figure 4. Kinetics of antibody adsorption in Teflon® FEP microcapillaries having mean d_h of 212 µm. **A** Mass of antibody adsorbed expressed in terms of percentage of surface coverage, ϕ using increasing times of incubation of mouse IgG concentrations of 20, 40 and 200 µg/ml (corresponding to 1.3×10^{-7} M, 2.6×10^{-7} M and 1.3×10^{-6} M, respectively), determined by solution depletion technique. **B** Kinetics of generation of optical signal measured by ELISA based on 40 µg/ml of mouse-IgG coating at varying times and 600 ng/ml of anti-mouse IgG conjugated to peroxidase. **C** Direct comparison of mouse-IgG antibody quantitation by BCA assay shown in A with optical ELISA in mouse-IgG/anti-mouse IgG system shown in B signal, showing magnitude of optical ELISA signal is linked to mass of antibody adsorbed. **D** IL-1β full response curves using 40 µg/ml of capAb incubated for 30 and 120 minutes, confirming antibody adsorption is rapid. The continuous lines in A and B represent the kinetic model based on equation 3, with best-fitted parameters summarised in Table 2. The continuous line in D represents the values obtained by the four parameter logistic (4PL) model, commonly used for full responses in immunoassays.

adsorption, therefore we have directly compared antibody adsorption with two other capillary surfaces, being a 19-bore MCF melt-extruded from LLDPE and a single-bore glass capillary (Figure 5A). As the three capillary systems presented different diameters, all adsorption data shown in this section has been normalised in respect to SAV ratio (shown in Equation 1).

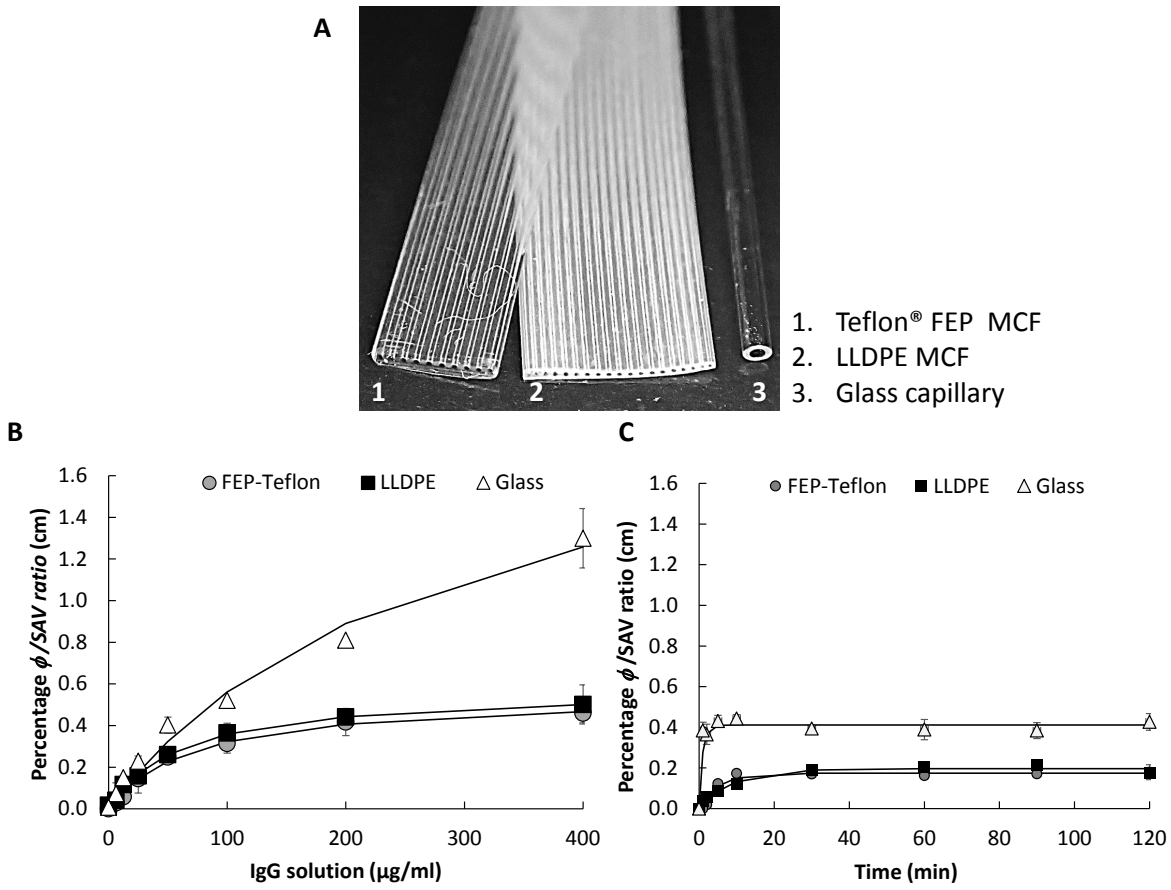

Figure 5. Antibody adsorption onto different capillary surfaces. **A** Microphotograph of capillary systems tested in this study, being: 1 - 10 bore Teflon® FEP MCF, 2 – 19 bore LLDPE MCF, 3 – single-bore glass capillary. **B** Antibody adsorption isotherms expressed as percentage surface coverage, ϕ to SAV ratio (cm^{-1}) of IgG per unit of surface area. **C** Kinetics of antibody adsorption in the different capillary surfaces using 40 µg/ml of mouse IgG in solution; to enable direct comparison of capillaries having different inner diameters, surface coverage has been normalised with SAV ratio. The continuous lines in the B represent the values estimated with Langmuir model (equation 2), with best-fitted parameters summarised in Table 3, whereas in C represents the best-fitted kinetic model (equation 3), with best-fitted parameters summarised in Table 4.

Equilibrium adsorption of IgG onto Teflon® FEP and LLDPE was very similar, as seen from the almost overlapping Langmuir isotherms in Figure 5B and best-fitted Langmuir parameters (Table 3). This is due presumably to similar hydrophobicity of the two polymers, showing a contact angle with water of 123º[22] and 120º,[23] respectively.[21] However, it contrasted with the Langmuir isotherms obtained for the glass capillaries having a contact angle ~25º,[24] which showed 2 to 4-fold larger mass of antibody adsorbed per surface area available based on data shown in both Figure 5B and Figure 5C. Although we have not extensively tested other capillary surfaces in this study, these original results suggest antibody adsorption is very distinct between hydrophobic and hydrophilic surfaces. Some studies reported a higher mass of protein adsorbed onto hydrophilic surfaces, such as bare glass, compared to hydrophobic surfaces such as plastic,[43,44] this is further supported by our data. Note however that the larger surface density obtained with the glass capillaries means antibody adsorption on glass surfaces occurs in multilayers, which in undesirable in affinity biorecognition and in particular heterogeneous immunoassays. Consequently, covalent immobilisation is a preferred strategy for preparation of glass immuno-surfaces.

Table 2. IgG adsorption kinetic parameters (based on equation 3 and data plotted in Figure 4A) for Teflon® FEP microcapillaries

IgG bulk (M)	K_{on} (M^{-1} min^{-1})	K_{off} (min^{-1})	R^2
1.3×10^{-7} (20 µg/ml)	5.01×10^{5}	2.76×10^{-1}	0.9556
2.6×10^{-7} (40 µg/ml)	2.69×10^{5}	1.30×10^{-1}	0.9811
1.3×10^{-6} (200 µg/ml)	1.1×10^{6}	1.78×10^{-1}	0.9345

Table 3. Best-fitted parameters for Langmuir isotherms (based on equation 2 and plotted in Figure 5B) describing antibody adsorption in different microcapillary materials. Note in this case τ_{max} in equation 2 was computed as ratio of percentage of surface coverage, Φ to SAV

	Teflon® FEP	**LDPE**	**Glass**
***K* (ml/µg)**	0.014	0.016	0.004
τ_{max} (shown as percentage Φ/SAV, cm)	0.55	0.57	2.15
R^2	0.9963	0.9982	0.9939

The reduced affinity of antibodies for hydrophobic surfaces favours the formation of less dense layers that are actually preferred for sensitive ELISA, as surface coverage affects antibody activity/orientation and smaller amounts of adsorbed antibody encourages a stronger attachment of molecules to the surface.[35,45] In contrast, antibody adsorption onto hydrophilic surfaces yields higher mass adsorbed with the possible formation of antibody multilayers, due to electrostatic interactions between antibodies and hydrophilic surfaces.[46] Also, higher mass of adsorbed antibody promotes easy desorption from the glass surfaces as a result of reduced conformational changes.[39,40] For these reasons hydrophobic surfaces are usually preferred for antibody adsorption in diagnostic test surfaces as immobilised antibodies are more resistant to surfactants and present lower desorption due to irreversible binding between antibody and surface, which is essential for heterogeneous assays.[36,41,47] Note we have extensively washed the capillaries with 0.05% PBS-Tween in all experimental sets shown herein. The irreversibility was related to the conformational changes that part of the antibody undergoes when adsorbed to a hydrophobic surface. Multiple experimental replicas confirmed (data not shown) showed no detectable loss of antibody with the washings. Our data suggested Teflon® FEP microcapillaries present a highly hydrophobic surface, which favours the irreversible nature of antibodies on the surface.[43,45]

In line with previous studies, surface chemistry affected antibody adsorption kinetics, being the adsorption equilibrium reached in less than 5 minutes for glass surfaces, within 10 minutes for Teflon®FEP and take up to 30 minutes for LLDPE (Figure 5C and Table 4).

Table 4. IgG adsorption kinetic parameters (equation 3 and data plotted in Figure 5B) for different capillary surfaces, based on IgG bulk concentration of 40 μg/ml

	K_{on} (M^{-1} min^{-1})	K_{off} (min^{-1})	R^2
Teflon® FEP	2.69×10^{5}	1.30×10^{-1}	0.9761
LDPE	1.14×10^{5}	1.00×10^{-1}	0.9610
Glass	2.94×10^{6}	1.96	0.9634

Antibody adsorbed faster to glass surfaces with an association constant K_{on} around one order of magnitude larger than for the plastic surfaces studied (Table 4), which can be explained by electrostatic interactions between the antibodies and glass surfaces. Note that values for the dissociation constant, K_{off} for antibody adsorption were also one order of magnitude larger in

glass capillaries, which agrees with the reversibility of antibodies adsorbed to hydrophilic surfaces. This is another reason why glass immuno-surfaces tend to imply surface modification strategies and antibody covalent binding for formation of a bio-recognition monolayer.

5. CONCLUSIONS

In spite of the uniqueness of FEP Teflon® (excellent optical transparency, electrostatic and very high contact angle for water) and its "non-sticky" properties, FEP Teflon® microcapillaries revealed to form an optimal bio-recognition monolayer, with antibodies biologically active and irreversible bound to the surface, enabling robust and sensitive optical, quantitative diagnostic testing to be carried out. Passive adsorption of antibodies into FEP microfluidic strips showed insensitive to the temperature of incubation and ionic buffer strength, with a neutral pH favouring robustness in performance. In addition, antibody kinetics onto FEP microcapillaries revealed fast, taking up to 10 minutes to reach equilibrium, with no differences in assays performance. These features are important for the lowering the cost of the diagnostic strips manufacturing process, since it removes the need of precise temperature control, enables a higher degree of freedom to operators and allows high throughput production. Highly sensitive, optical, sandwich assays are possible in FEP Teflon® strips due to the irreversibility of antibodies adsorption to the surface and by the modest antibody surface packing densities, observed as between 45 to 69% for optimal antibody activity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxx.

Supplementary methodology and results, including additional Tables S1 and S2

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ACKNOWLEDGEMENTs

The authors are grateful to Patrick Hester for donating the MCF material and to Loughborough University for funding this study.

ABBREVIATIONS

FEP, Fluorinated ethylene propylene; LLDPE, linear low-density polyethylene, IgG, immunoglobulin G; capAb, capture antibody; POC, point of care; PDMS, Polydimethylsiloxane; PTFE, Polytetrafluoroethylene; MCF, microcapillary film; PSA, prostate specific antigen; IL-1β, interleukin-1 beta; ELISA, Enzyme-Linked Immunosorbent Assay; BSA, bovine serum albumin; PBS, phosphate buffer saline; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BCA, bicinchoninic acid assay; HRP, horseradish peroxidase; OPD, o-phenylenediamine; Abs, absorbance.

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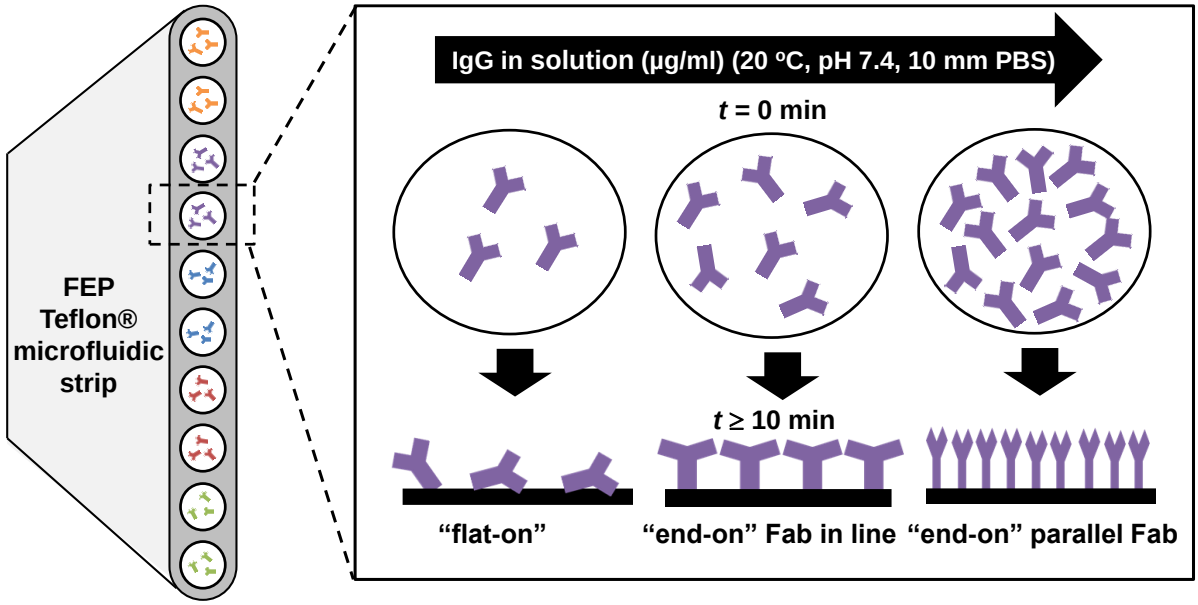
TOC graphic


FEP
Teflon®
microfluidic
strip
IgG in solution (μg/ml) (20 °C, pH 7.4, 10 mm PBS)
t = 0 min
t ≥ 10 min
"flat-on"
"end-on" Fab in line
"end-on" parallel Fab