Simple Microfluidic Tool for Facile Assessment of Biomolecule Adsorption on Diagnostic Device Substrates

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ABSTRACT

Herein we present a simple analytical tool for the measurement of biomolecule adsorption on substrates used in detection devices. Typically, the substrates are made of disposable polymer/plastic material, which does not possess native functional groups for specific reactions with biomolecules. Therefore, they have to be functionalized by surface chemistry methods. We evaluated the adsorption of antibodies and oligonucleotides on three types of surfaces; positively charged, negatively charged and neutral, hydrogel-like film with polyethylene glycol (PEG) characteristics. We have used a goat anti-human IgG and a 20-mer DNA as a model molecules. These simple experiments enabled us to build a fundamental understanding of the interaction forces in real systems, and how these relate on the one hand to the surface composition and chemistry, and on the other hand to the sensitivity and resistance to non-specific binding. In addition, the surface area of the channels in this universal microfluidic chip was increased by etching/micromilling of microscale trenches. Such modified surface was then coated with 3-aminopropyltriethoxysilane (APTES) and successfully tested for its capacity to serve as a unique protein dilution feature.

KEYWORDS: antibody adsorption, DNA adsorption, microfluidics, polymer substrates, Zeonor®

INTRODUCTION

Current requirements for biomedical diagnostic devices and microfluidic diagnostic devices in particular include the need for inexpensive, disposable substrates, the ability to take measurements of complex biological samples with volumes ranging from microliters to milliliters, and the need for a minimum of user intervention. As a result, the substrate utilised for microfluidic diagnostic devices is very often a disposable polymer/plastic material.

Cyclo olefin polymers (COP) or co-polymers (COC) are prime examples of a new class of materials suitable as disposable substrates for diagnostic devices[1, 2]. These materials have been engineered to meet key criteria such as low autofluorescence, ease of fabrication and manipulation, excellent mouldability and good machinability to form complex microfluidic features such as channels and valves, and low

non-specific adsorption of biomolecules. The two latter parameters are of particular importance. Specific to single-use bioassay chips, there is a need for easy fabrication to incorporate various features as follows such that the samples can be pre-processed with relative ease: dilution or concentration in an appropriate buffered solution, filtration, purification, and the interaction with other bulk or surface-confined reagents. Morever, the design may include more than one measurement area and a control experiment to validate the test. This can been achieved by incorporation of surface-immobilised biomolecules, microfluidic features, and other functional components onto or into the substrate/chip.

The second requirement, low non-specific biomolecule adsorption, is due to the fact that the analyte of interest is usually present in the sample in extremely low quantities (e.g., 10 molecules per 1 mL), especially as compared to other, non-analyte constituents. It is therefore critical to maintain the concentration of the target molecule(s) while delivering the analyte-containing fluid through the microfluidic pre-processing features to the active assay area of the chip substrate. Antibodies and oligonucleotides are common examples of analytes in diagnostic devices, and can randomly adsorb onto the surface of the plastic substrate[3]. Therefore, their final concentration over the detection area can be quite different from the original crude sample, which can contribute to false negatives or inaccurate quantitation. To overcome this problem, the pristine plastic material is very often functionalised using a number of various surface chemistries to prevent such non-specific adsorption[4,5]. However, to the best of our knowledge, there is no simple, readily available analytical tool that allows for characterisation of biomolecule adsorption on chemically modified substrates.

In this work, a simple microfluidic device is presented for novel yet facile characterisation of conformal surface chemistries and their interactions with biomolecules. In particular, the biomolecule (viz., antibody or DNA) adsorption properties of surface coatings deposited using plasma-enhanced chemical vapor deposition (PECVD) are examined using the microfluidic chips as tools towards the development of diagnostic systems.

While in this study we have chosen to functionalise polymer substrates by PECVD, the analytical tool presented is by no means limited to this particular surface chemistry technique or surface/substrate type.

The microfluidic chip, based on a standard slide-based format (75 mm x 25 mm), was created using simple and inexpensive fabrication materials and methods accessible to any research group. Adhesive tapes cut using a computer-controlled knife and polymer sheets cut using a standard laser-cutter were laminated together to form the microfluidic cell (Figure 1). PECVD surface chemistries were easily incorporated into the device by coating of the polymer layers before assembly. While microfluidic pumping on the chip is centrifugally-enabled using a simple motor for spinning, the device presented can easily be actuated using standard pressure-driven syringe pumps.

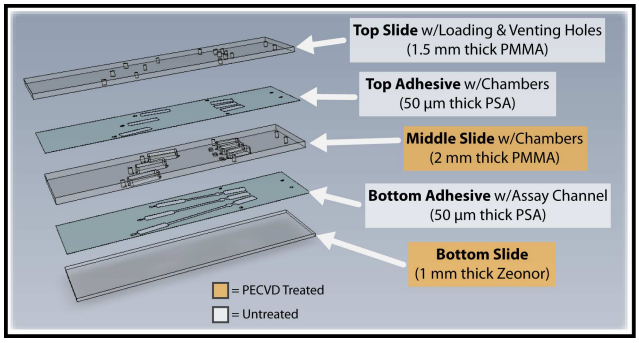

Figure 1. Schematic showing the 5 layers of the slide-sized microfluidic device. The microfluidic channel is located in the bottom pressure-sensitive adhesive (PSA). The system provides modularity as the bottom slide can consist of any material desired (e.g., glass) and the geometry design of the chip can be easily altered.

In this paper, the facile microfluidic fabrication methods are highlighted and an example device to study protein (IgG) and DNA adsorption is presented. The effects of aminopropyltriethoxysilane (APTES) coatings and diethylene glycol dimethyl ether (PEG) coatings on biomolecule adsorption are compared to the native surface of a model COP substrate, Zeonor®, a polymer commonly used in microfluidic diagnostic devices. The surface-coating characterisation system presented has a broad range of applications, and the easy fabrication methods ensure changes can be made to suit a variety of studies.

EXPERIMENTAL

Microfluidic Chip Design & Fabrication

Cyclo olefin polymer (COP) slides (Zeonor® 1060R) (25 mm × 75 mm, 1 mm thick) were injection-moulded and supplied by Sigolis (Uppsala, Sweden). Phosphate buffered saline (PBS, pH 7.4, 0.01 M), bovine serum albumin (BSA, 98%), 3-aminopropyltriethoxysilane (APTES), diethylene glycol dimethyl ether (DEGDME, $CH_3O(CH_2CH_2O)_2CH_3$), and Cy5-IgG were all purchased from Sigma Aldrich (Dublin, Ireland). ssDNA (15-mer) with a 3'-C_6-NH_2 modifier and a 5'-Cy5 label was purchased from MWG Biotech (Ebersberg, Germany). All chemicals were used as received without further purification. The microfluidic chip was designed using AutoCAD (AutoDesk, US) and consisted of a total of 5 laminated layers of laser-cut polymethylmethacrylate (PMMA) (#824-632 & #824-480, Radionics, IE), knife-cut, double-sided, pressure-sensitive adhesive (PSA) (ARcare 8890, Adhesives Research, IE), and Zeonor® substrates. (Figure 1). The PMMA layers were laser-cut using a relatively inexpensive benchtop laser-cutter (Epilog Zing, Epilog, US) and the PSA layers were cutting using a benchtop, craft knife-cutter (Craft Robo-Pro, Graphtec, UK). In all cases, the AutoCAD drawing layers were easily imported into the software for the respective pieces of equipment.

All polymer chip components (including the bottom Zeonor® substrates) were ultrasonically cleaned before assembly and/or PECVD coating. After PECVD-coating of the middle (PMMA) and bottom (Zeonor®) parts, which consisted of the roof and floor of the microfluidic channels, respectively, the layers were manually assembled using alignment pins. Finally, the completed parts were rolled under pressure to ensure excellent adhesion between the polymer and adhesive layers. This is similar to methods already reported in the literature for fabrication of polymer microfluidic devices (Siegrist et al. 2010).

Centrifugal Pumping & Imaging

To run the centrifugal microfluidic experiments, a servomotor coupled to a stroboscopic visualization system similar to that already described in the literature[6] was used for fluid-flow tracking during rotation. A servomotor (4490 series, Faulhaber, DE) was mounted to a framed support, and a chuck was machined for securely attaching a centrifugal slide-holder template to the motor's shaft. A CCD camera

(Sensicam series, PCO, DE) was placed directly above the motor, and a combination of optical components (Navitar, NY, USA) were attached to the camera to obtain an image with motorized zoom and focus controls for flow visualization; various optical configurations allowed for microscopic and macroscopic imaging of features on the microfluidic devices. A linear drive was used to radially position the camera along the centrifugal slide-holder.

The camera was triggered to capture one frame per rotation, such that a movie composed of a sequence of still images taken at the same location on the device(s) could be acquired (see supplementary material movie ESI 1). A custom control box was fabricated to handle triggering between the motor, camera, and stroboscopic illumination system; the trigger box also served to control the circumferential location along the centrifugal slide-holder for image acquisition. The combined action of the linear camera drive and the trigger box provided full control to select the desired sector to be investigated and imaged. The stroboscopic system (Drelloscop 3244, Drello, DE) utilised a liquid light-conductor for illumination and was mounted to the side of the camera. A desktop PC (Dell, US) was used to control the spin-speed and sequences of the motor as well as for monitoring and acquisition of the images. The custom spin-stand instrument allowed for real-time movement and magnification of the image acquisition such that flow through the microfluidic devices could be tracked. The optical clarity of the acrylic and adhesive device components provided adequate contrast for visualization.

Flow Characterisation

To characterize the microfluidic device flow, contrast agent (< 1% v/v) was added to phosphate buffered saline (PBS, pH 7.2) and loaded onto the devices using a standard pipette. Mock samples 40 μL in volume where then centrifugally pumped through the system at various spin-speeds (in revolutions-per-minute, RPM) and timed to determine approximate flow-rates.

Surface Chemistry Characterisation

To test the coated surface chemistries on the microfluidic devices, 4 chips were placed at one time onto the centrifugal slide-holder. Samples 40 μL in volume were prepared in PBS buffer. The samples consisted of 4.0 μg/mL of Cy5-labeled goat anti-human IgG and 5.0 nM of Cy5-labeled ssDNA.

Surface Etching

The surface was modified by milling, using a 3-axis micromilling machine (CAT3D M6, Datron AG, Germany). The channel surface was machined with a 100 μm tool at 16,000 RPM using a 0.9 mm/sec feed-rate. The trace was drawn using Excalibur CAD/CAM package (Excalibur XCAD 4.201, Progressive Software Corporation, USA). The trace produced a 100 μm wide x 80 μm deep channel, with each channel separated by a 50 μm wall. Across the channel width of 1.45 mm, the machining produced nine 80 μm troughs.

The liquid samples were loaded onto the chips, the extraction holes sealed using adhesive tape, and the samples centrifugally-pumped through the system at spin-speeds ranging from 300-400 RPM. The samples were then removed using a pipette, loaded in a standard 96-well plate and the fluorescence intensities were analysed on a Safire (Tecan) microplate reader. For the Cy5-labeled materials, the excitation and emission wavelengths were set at 646 nm and 662 nm, respectively.

RESULTS AND DISCUSSION

Microfluidic Operation, Ease-Of-Use, and Advantages

Fabrication and assembly of the devices was simple, as the modular, layer-based format of the system provides easy customization. The standard slide-format size of the system makes it compatible with many established lab protocols and systems, and, moreover, the bottom COP slide can easily be swapped out with any other material. For example, many standard surface chemistries have been developed on glass; using the system presented, the bottom slide can be replaced with glass or any other commercially-available material. This makes the presented system a powerful tool for the analysis of a myriad of standard and customised surface chemistries.

The design consisted of deep chambers that can accommodate realistic sample volumes, from 1 µL up to 40 µL. The microfluidic channel itself is only 50 µm deep, as dictated by the thickness of the PSA, to enable analysis of the surfaces under microfluidic conditions. The thickness of the microfluidic channel can easily be changed by simply using a PSA with a different thickness.

The particular design presented here consisted of three separate channels, A, B and C, each of a different length (A being the shortest) as seen in Figure 2. This enabled an analysis of how the surface area affects biomolecule adsorption.

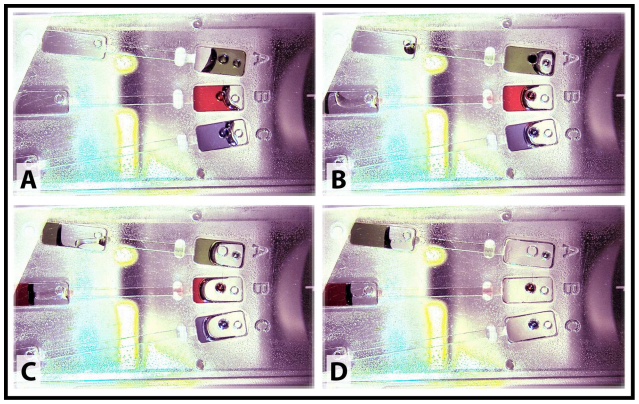

Figure 2. Photos showing the various stages of microfluidic pumping (device loaded with contrast dyes). (**A**) Samples loaded, (**B**) Samples begin flowing as the device spins, (**C**) Sample continue flowing, and (**D**) Samples have finished flowing across the coated surfaces and are ready to be removed for analysis.

With such a simple design, the channels could easily have all been the same length to allow high-throughput replicates. Moreover, the design can easily be changed to include narrower channels and other varied geometrical designs. Finally, whether using centrifugal- or pressure-driven flow, the flow rate can be varied to enable additional analysis parameters. In the development of microfluidic diagnostic devices, the interaction of surface chemistries under active, laminar flow (as opposed to stagnant, well-plate or test-tube conditions) is very important and often significantly changes when moving from standard benchtop assays. The system presented allows for facile analysis of surface

chemistries under microfluidically-relevant conditions as is required during the development of microfluidic diagnostic devices.

Flow Analysis

The spin-speeds used to test the native COP, APTES, PEG, and APTES-etched surfaces ranged from 300-400 RPM. The PEG surfaces, being more hydrophilic, required a lower spin-speed to achieve flow while the pristine COP surfaces, being more hydrophobic, required a higher spin-speed to achieve flow (for water contact angles and thickness of the films, see Figure 3). In all cases, the flow-rates achieved were approximately 0.3 µL/s. Some variability was observed, likely due to anomalies in the surface coatings and chip fabrication quality. Figure 2 shows a sequence of movie frames from sample loading to completion of centrifugal flow-through.

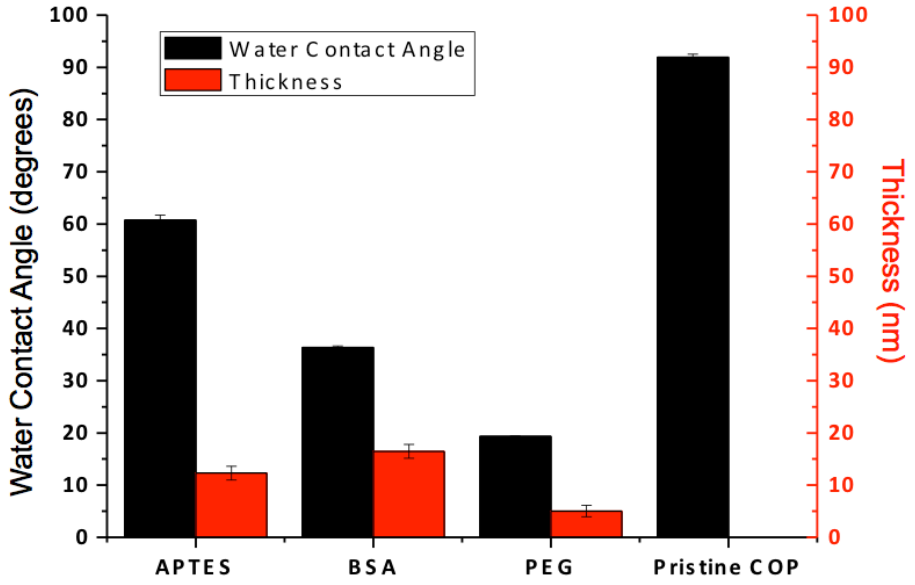

Figure 3. Thickness of films prepared by PECVD and their corresponding water contact angles (n=3, error bars are ±1 standard deviation).

Adsorption of Proteins and DNA on Microchannel Substrates

In this study, we chose to study the antibody and DNA adsorption on four different surface coatings. The first coating was prepared by deposition of APTES, an amine-functionalized siloxane. We have

previously reported on the preparation and characterisation of such films by number of analytical methods[7]. Considering the pKa of APTES is around 10, the surface is mainly positively charged upon contact with the PBS solution (pH=7.2). The second coating was rendered negatively charged by immersing APTES-coated substrates into a 6% w/v solution of BSA[8]. BSA is commonly used in as a biological blocking agent. Due to its low isoelectric point of 4.7, BSA adsorbs in multilayers on positively charged surfaces at pH~7, hence effectively increasing the negative charge at the biomolecule-surface interface. The PEG precursor used in the third type of surface possess no charge. While plasma assisted deposition could have fragmented its native structure and formed a small number of ions and reactive radicals, we consider this surface to be close to neutral, especially when compared to the charged APTES and BSA films. The fourth studied surface was a pristine, unmodified COP (Zeonor®) slide. The differences in chemical groups exposed to the measured samples are illustrated schematically in Figure 4.

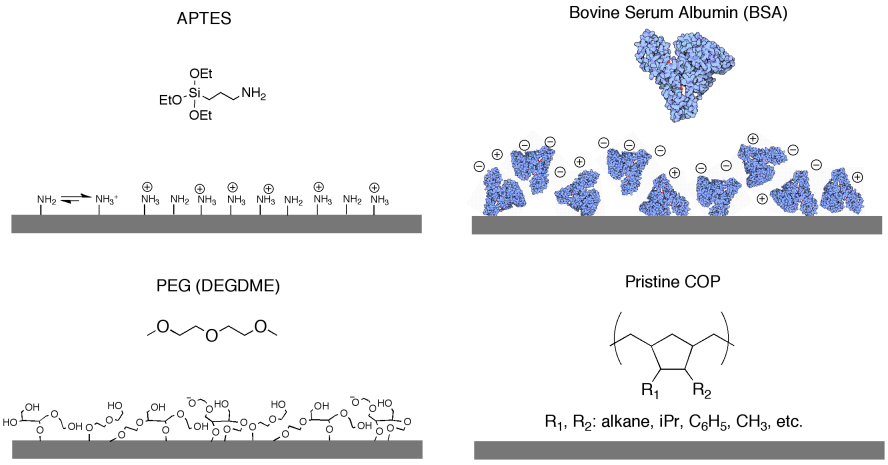

Figure 4. A schematic of functional groups present at the biomolecule-surface interface on the four coatings characterised in this study.

The substrate adsorption characteristics of the Cy5-labeled IgG antibodies and DNA are presented as percentages of capture efficiencies for each corresponding channel length. The values were simply calculated as the fraction of the molecules collected (once they finished flowing across the coated surfaces) to the starting molecule concentration. The results are summarized in Figure 5.

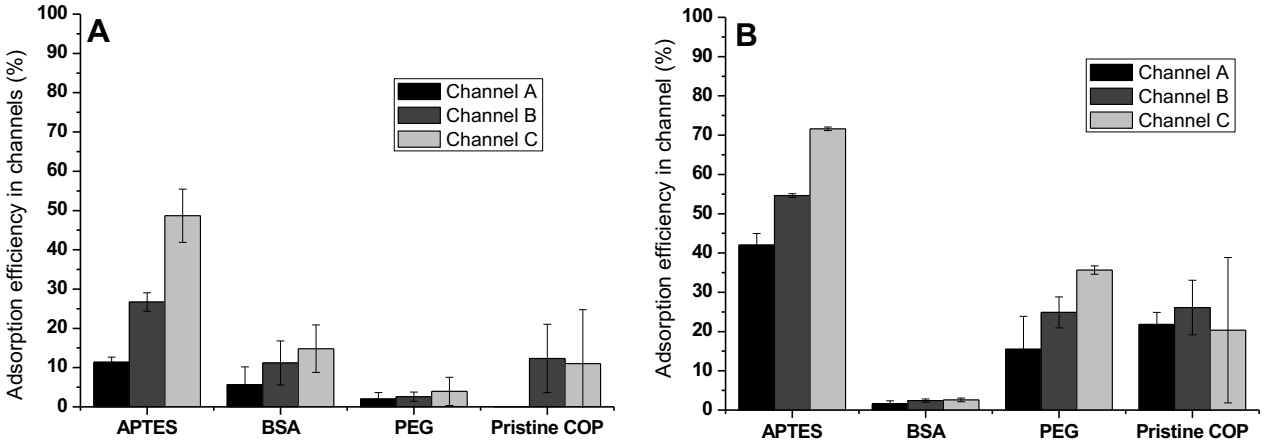

Figure 5. The capture efficiency of IgG antibodies (A) and ssDNA (B) on COP slides treated with various surface chemistries (n=3, error bars are ±1 standard deviation).

IgG antibodies adsorbed well on the positively charged APTES surface, while the PEG and BSA-modified films reduced IgG binding significantly. The most efficient film in terms of non-specific adsorption prevention was PEG. The repulsion of proteins by PEG-like materials observed in this study is in agreement with previous work[9]. We have recently reported that the PECVD-prepared PEG film exhibits roughness increases and significant swelling upon contact with water; such surfaces become very well hydrated and resemble the structure of hydrogels.

The three channels differ in length and hence in surface area available for adsorption. We have calculated the theoretical amount of antibody (assuming its surface footprint is ~100 nm^2) and DNA (~14 nm^2 for DNA 20-mer)[10] that can be realistically adsorbed on the channel surface. We assumed that both anti-IgG and DNA would adsorb as monolayers and would pack on the surface, effectively covering a maximum of 50% of the available area; these theoretical values are summarized in Table 1.

Table 1. Dimensions of the channels with the maximum theoretical amount of IgG and ssDNA adsorbed per channel.

	Channel A	Channel B	Channel C
flat / etched surface area	33.8 mm^2 / 67.4 mm^2	46.8 mm^2 / 93.3 mm^2	59.8 mm^2 / 119.2 mm^2
Theoretical maximum of IgG per channel*	0.3×10^{-12} mol	0.4×10^{-12} mol	0.5×10^{-12} mol
	0.048 μg	0.064 μg	0.080 μg
Theoretical maximum of ssDNA per channel*	2.0×10^{-12} mol	2.8×10^{-12} mol	3.5×10^{-12} mol
	13.2 ng	18.5 ng	23.1 ng

* Assuming a maximum of 50% coverage

The amount of antibody adsorbed in the channels was in all cases proportional to their length, with the exception of the pristine COP. The hydrophobic pristine COP shows large variations in its adsorption capacity for antibodies. We speculate that the antibodies can adsorb on the COP only if they change their native conformation, exposing some of their hydrophobic residues and allowing for effective π-π and Van der Waals interactions with the unmodified COP surface. We reason that the timescale of such events is longer than the exposure time of the antibody solution during the flow, which leads into larger irreproducibility between individual experiments. This is an important result in light of diagnostic device design, as the native properties of the COP cannot be relied upon to satisfactorily inhibit non-specific adsorption. Indeed, a specific surface coating is needed to prevent non-specific biomolecule adsorption.

Similar trends were observed when using ssDNA. The intrinsically negatively-charged oligonucleotide showed very good adsorption on the positively charged APTES and exceptionally low adsorption on the negatively charged BSA surface. The fact that the neutral PEG film was not as effective at reducing non-specific binding of charged DNA molecules confirmed that the dominant forces behind the binding phenomena are the electrostatic interactions.

So far, these simple microfluidic chips proved to be useful in the assessment of adsorption of antibodies and nucleic acids on various surfaces. However, we envision that the same concept can be

used in situations where, prior to detection, sample dilution is required. Common dilution designs rely on mixing of a fraction of the analytical sample with a precise volume of buffered solution. This is quite challenging when considering the available surface area of the substrate and the fact that the buffers must be stored on the biochip, including valves for accurately releasing and dispensing the desired volumes. Moreover, the resulting diluted solution should be homogeneous to ensure maximum reproducibility. Therefore, we decided to test the potential of the microfluidic device presented in this article in a dilution experiment with goat anti-human IgG in channels with an increased surface area coated with APTES. The increase in surface area was achieved by etching/micromilling the normally flat COP slides with trenches that were 100μm wide, 80μm deep, and with 50μm spacing along each channel.

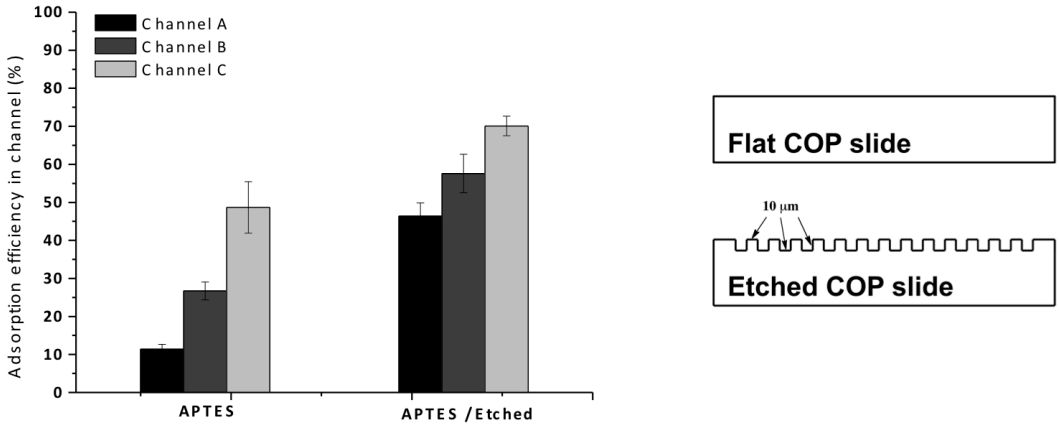

Figure 6. The capture efficiency of the goat anti-human IgG antibodies in flat and pre-etched channels subsequently coated with APTES.

As seen in Figure 6, the capture efficiency increased in the channels that were etched prior to deposition of APTES. Interestingly, the effect was more noticeable in the shorter channel A than in the longer channels B or C. We attribute such discrepancies to two factors. One is related to the poorer control and resolution of the etching/micromilling process on the nanoscale, which is important when considering the surface footprint of an antibody (~100 nm^2). The second factor relates to the uniformity of the deposited layer of APTES. The more uniform the coating, the more homogeneus is the adsorption process. The presented data suggest that improvements in surface chemistry and more precise control

over the surface area by introduction of trenches, pillars[4], or microbeads in the microchannels could become a useful microfluidic feature for sample dilution.

CONCLUSIONS

The presented microfluidic chip represents a simple and inexpensive option for the analysis of interaction forces between the surface composition and biomolecules, typically used in bioassays. The main advantage of this concept is its compatibility with many established lab protocols and systems; and its capacity to analyze many types of commonly used substrates. We also believe that this simple device offers the ability to fundamentaly assess and tailor the interfacial tension of the surface in order to control both the specific and non-specific binding. This will provide an indispensable help in the design of rules and implementation strategies for optimisation of bioassay performance based on engineering of the physico-chemical properties of the substrate surface (COP, glass, etc.), and the biomolecules of interest. The microfluidic device was also used to probe its capacity to identify the appropriate molecular surface composition for enhaced adsorption of antibodies, for the purpose of fabrication of unique protein-dilution features. Further advances in the development of such concept will be presented in due course.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE (ESI): [ESI 1 – a movie showing the operational mode of the microfluidic device]

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