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# On-chip immunoprecipitation for protein purification†

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Immunoprecipitation (IP) is one of the most widely used and selective techniques for protein purification. Here, a miniaturised, polymer-supported immunoprecipitation ( $\mu$ IP) method for the on-chip purification of proteins from complex mixtures is described. A 4  $\mu$ l PDMS column functionalised with covalently bound antibodies was created and all critical aspects of the  $\mu$ IP protocol (antibody immobilisation, blocking of potential non-specific adsorption sites, sample incubation and washing conditions) were assessed and optimised. The optimised  $\mu$ IP method was used to obtain purified fractions of affinity-tagged protein from a bacterial lysate.

## Introduction

Intracellular communication involves an intricate and highly dynamic system of biochemical reactions that form a network of signal transduction pathways, where external stimuli or internal cues trigger a cascade of protein–protein interactions.<sup>1</sup> Unravelling the intricacies of signalling pathways is of great importance for the understanding of fundamental cellular activities and control mechanisms, as well as for the development of therapies for a number of diseases where inappropriate pathway activation has been implicated.<sup>2</sup> One of the key aims of proteomics is, therefore, to elucidate the protein–protein interactions involved in these pathways.

To achieve this, high fidelity techniques are required to enrich proteins of interest along with their interacting partners. One such technique is immunoprecipitation (IP). IP is a powerful separation technique, extensively used in cell biology research, which involves simplifying complex samples (such as cell lysates) by the antibody capture of proteins followed by washing and elution.<sup>3</sup> It can be employed to obtain a purified solution of a single protein, or to pull down a protein known to be a constituent of a protein complex that is then released along with its interacting partners (often referred to as co-IP).<sup>4</sup> Eluted samples can be analysed by a range of techniques including electrophoresis, western blotting and mass spectrometry. IP is typically carried out in batch mode or in columns using millilitre volumes and agarose beads onto the surface of which capture antibodies have been immobilised.

Miniaturisation of the IP process in a microfluidic format would offer a number of benefits. In particular, it would make IP more amenable to applications where only small numbers of cells are

available (such as needle biopsy samples, aged cells, populations of rare sorted cells and purified stem cells). It would also enable multiplexing of several parallel analysis streams to allow for the simultaneous screening of either multiple samples for the interacting partners of one protein or one sample for many pathway components. Furthermore, a lab-on-a-chip approach to IP would be amenable to integration with upstream sample preparation components, such as cell lysis devices,<sup>5</sup> which would minimise sample handling and therefore loss of proteins due to surface adsorption (a major problem when processing small samples). The analysis of transient interactions, including the characterisation of protein complex subpopulations, would also be improved by the faster processing times offered by such an approach. Therefore, to attain these benefits we have developed a micro-immunoprecipitation ( $\mu$ IP) column and an optimised method for on-chip protein capture and elution for enriching proteins from cell lysates.

There have been a number of reports describing microfluidic protein assays that involve protein capture by bound antibodies. A variety of different formats have been employed,<sup>6–12</sup> the most common being simple physisorption of the capture antibodies onto the surface of a device. Alternative antibody immobilisation strategies include the exploitation of biotin–avidin interactions or the use of covalent attachment procedures. Applications of such protein capture systems have included ELISA assays and the development of high-specificity biosensors, in addition to the separation of rare cell types from a population *via* antibody interactions with cell surface proteins.<sup>13–16</sup> All of these studies have involved the detection of the captured protein whilst it is bound to the surface. By far the most common detection strategies employed are optical, using either fluorescent or chemiluminescent approaches.

A smaller number of devices for selective protein enrichment have also been reported.<sup>17–20</sup> These include bead-based affinity methods, where beads with an appropriate surface functionality are packed into a microfluidic channel, and the partitioning of proteins using two-phase flow extraction methods. In nearly all reports to date, model mixtures have been used to demonstrate protein enrichment and very little has been reported on the separation of complex biological samples. An exception is the work by Meagher *et al.*,<sup>20</sup> where recombinant proteins from an *Escherichia coli* lysate were enriched within a PEG-rich phase through use of a hydrophobic partition tag.

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† Electronic supplementary information (ESI) available: Additional Materials and methods section (microfabrication and soft lithography protocols; protein expression and cell lysis protocols; conventional IP methods), contact angle time courses of solvent-soaked PDMS (Fig. S1), influence of washing and incubation times on  $\mu$ IP (Fig. S2) and  $\mu$ IP using a stored column (Fig. S3). See DOI: 10.1039/c005295g

Immunoaffinity approaches such as IP allow for a level of selectivity that is not generally possible with other approaches. However, very little has been reported on their miniaturisation. There have been two reports<sup>21,22</sup> describing chromatin IP (ChIP) in a microfluidic format. (ChIP is used to investigate protein–DNA interactions and involves crosslinking protein–DNA complexes, purifying *via* antibodies to a DNA interacting protein and subsequent PCR analysis). Both of these employed a bead-based format. Wu *et al.*<sup>21</sup> described a four-channel, microfluidic platform where protein–DNA capture by antibody coated beads and subsequent washing were performed on-chip, before collecting the beads for off-chip elution and PCR analysis. A single channel device was employed by Oh *et al.*<sup>22</sup> to capture interacting protein–DNA from a pre-cleared sample, previously incubated with an appropriate antibody, onto protein A coated beads.

On-chip IP of individual proteins or protein complexes (more challenging in certain respects, as there is no possibility of amplification of the eluted fraction) has, however, not been reported. We therefore address this here by developing a  $\mu$ IP method based upon a disposable, antibody-functionalized polydimethylsiloxane (PDMS) microcolumn, using a silane-based protocol where antibodies were crosslinked onto the internal surface of the device. All key stages in the method have been optimised, to improve silanisation conditions, to ensure no physisorbed antibodies are present, to determine suitable blockers for preventing non-specific adsorption (NSA) of proteins and to assess the influence of washing and incubation times. The optimised  $\mu$ IP method has been employed to perform protein enrichment from a bacterial lysate containing recombinant affinity-tagged proteins, one of the most common methods used in cell biology.

## Experimental methods

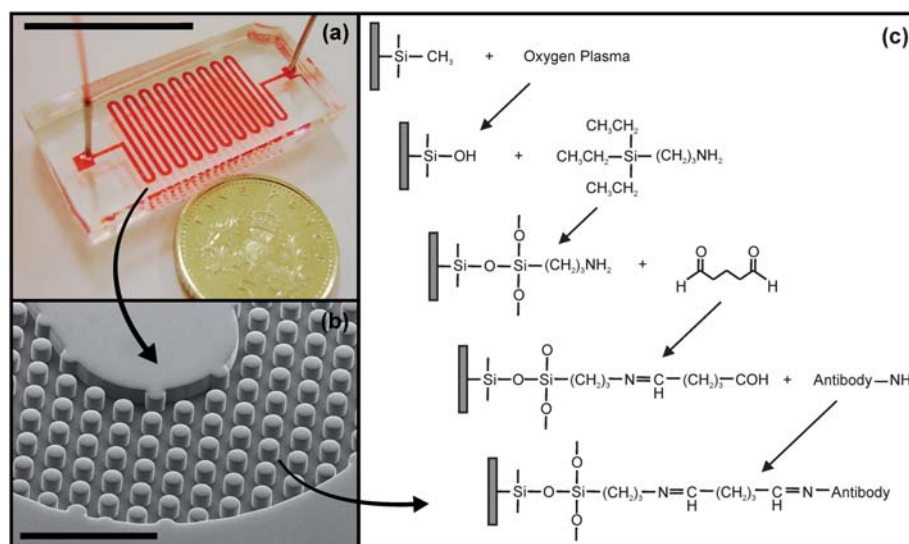
### Materials

The PDMS employed was Sylgard® 184 Silicon Elastomer Kit from Dow Corning (VWR, Leicestershire, UK). All chemicals

were purchased from Sigma-Aldrich (Dorset, UK), except for ColorPlus prestained protein markers, which were from New England Biolabs (Hertfordshire, UK), and Pluronic-F68, AlexaFluor® 488 rabbit anti-glutathione S-transferase (GST) and AlexaFluor® 647 streptavidin conjugate, which were from Invitrogen (Paisley, UK). Bovine serum albumin (BSA) was labelled using the AlexaFluor® 647 protein labelling kit from Invitrogen and silver staining was performed using GE Healthcare's PlusOne™ kit. Upchurch PEEK capillaries and injection port adaptors, used to connect the columns to Hamilton Microliter syringes (VWR, Leicestershire, UK), were purchased from Presearch (Basingstoke, UK) and 0.25 mm internal diameter silicone tubing was from Kinesis (Cambridgeshire, UK).

### Preparation of PDMS columns

PDMS microcolumns were fabricated by standard soft lithography techniques (as detailed in ESI†, Materials and methods) using a silicon master patterned by deep reactive-ion etching and coated with a perfluorooctyltrichlorosilane release layer. The columns consisted of a 50  $\mu$ m deep serpentine channel filled with an array of 50  $\mu$ m diameter pillars that increase the internal surface area to give greater antibody coverage (Fig. 1), resulting in a 4.4  $\mu$ l internal volume. Prior to sealing the patterned column with a PDMS cover plate by oxygen plasma bonding, both the column and cover plate were soaked in a series of acetone, ethanol and methanol (as discussed below). The parts were then blow dried and placed in an oven at 90 °C for 30 min to drive off any residual solvent. Inlet and outlet fluidic ports were attached to the patterned column by coring a hole through the PDMS and inserting the cut-off end of a 25G needle, before sealing the edges with a silicon RTV compound (cured overnight at room temperature). 1.5 cm sections of silicone tubing were employed to connect PEEK capillaries to the needle ports, and the latter were attached prior to plasma bonding so that the freshly bonded device could be connected to a syringe pump straight away.



**3** Fig. 1 (a) A  $\mu$ IP column filled with a red dye solution (scale bar is 13 mm). (b) An SEM showing the internal micropillars (scale bar is 400  $\mu$ m). (c) A schematic illustrating the idealised antibody immobilisation process.

1 Immediately before bonding the columns were cleaned by brief  
sonication in methanol and blow drying with nitrogen.

### 5 Silanisation protocols

APTES functionalisation of oxygen plasma treated PDMS  
(cleaned prior to plasma treatment by sonication in methanol for  
15 min) was tested for a range of APTES solutions. These were:  
5% APTES in 95% ethanol/5% dH<sub>2</sub>O (sample immersed for 20  
10 min and rinsed with ethanol); 2% APTES in 95% ethanol/5%  
dH<sub>2</sub>O (immersed for 20 min and rinsed with ethanol); 2% APTES  
in acetone (immersed for 10 min and rinsed with acetone); 1%  
APTES in dH<sub>2</sub>O (immersed for 20 min and rinsed with dH<sub>2</sub>O);  
and 1% in toluene (immersed for 2 min and rinsed with toluene).  
15 The sample immersion times were shorter for acetone and  
toluene so as to minimise swelling of the PDMS. All samples  
were baked at 80 °C for 30 min following silanisation.

### 20 Antibody immobilisation

The antibody immobilization procedure was as follows: within  
5–10 min of oxygen plasma bonding (18 s, 50 W, 0.2 mbar, 250  
sccm using a Gala Instruments PlasmaPrep 5 barrel asher), a 5%  
aminopropyltriethoxysilane (APTES) solution (in 95% ethanol,  
5% dH<sub>2</sub>O) was flowed through the  $\mu$ IP column for 10 min.  
(Unless otherwise noted, all solutions were flowed through the  
device at 4  $\mu$ l min<sup>-1</sup> using a KDS 210 syringe pump, flowing for  
15 min in the case of washes.) After stopping the flow and  
pausing for 15 min, the device was washed with ethanol and  
flushed with air, prior to baking at 80 °C for 30 minutes. The  
surface amino groups were subsequently reacted with the cross-  
linker glutaraldehyde (1% in dH<sub>2</sub>O, pH 9.2), with a 15 min  
incubation after 10 min flow, using a basic pH to promote Schiff  
base formation. The device was then washed with 0.1 M sodium  
carbonate buffer, pH 9.2, before an antibody solution (0.2 mg  
ml<sup>-1</sup> antibody in 0.1 M sodium carbonate buffer, pH 9.2, con-  
taining 0.05% Tween-20) was flowed through the column for 10  
min. The total number of antibodies was calculated to be  
equivalent to approximately 3–11 times the maximum antibody  
packing density, depending on whether an end-on or flat-on  
antibody orientation is adopted, estimated from the device  
surface area and assuming hexagonal packing and antibody  
dimensions of 14.2  $\times$  8.5  $\times$  3.8 nm.<sup>23</sup> After a 20 minute incu-  
bation, any unreacted glutaraldehyde was quenched by washing  
with 0.5 M Tris (pH 9) containing 0.05% Tween.

When blocking a column to minimise non-specific protein  
adsorption, one of the following solutions (all in 10 mM PBS, pH  
7.4) was then flowed through the device: 1% bovine serum  
albumin (BSA); 1% non-fat milk powder; 0.1% *n*-dodecyl- $\beta$ -*D*-  
maltoside (DDM); or 0.1% Pluronic-F68. Following a 20 min  
incubation, columns were washed with either 10 mM PBS or 10  
mM PBS containing 0.05% Tween-20 (PBS-Tween). Blocking  
efficiency was assessed by flowing through a 0.2 mg ml<sup>-1</sup> solution  
of AlexaFluor® 647 streptavidin in 10 mM PBS, incubating for  
20 min after 10 min of flow, and then washing with PBS-Tween.  
To store an antibody functionalised device, the column was filled  
with 10 mM PBS containing 0.05% sodium azide (PBS-azide)  
and the device immersed in a beaker containing the same solution  
before storing at 4 °C.

### 1 Fluorescently monitoring antibody immobilisation and blocking

Images were acquired using an Andor Luca<sup>EM</sup>-R EMCCD  
camera mounted on a Zeiss Axio Observer inverted microscope  
with a  $\times$ 20, 0.4 NA objective. In all cases, the base of the channel  
was in focus and EM gain and exposure times were held constant  
for each series of experiments. The mean fluorescence level was  
measured between the micropillars (selecting a region of  
approximately 10  $\times$  10  $\mu$ m) at a minimum of four locations  
across the chip, with at least six regions measured at each loca-  
10 tion (errors are given as one standard deviation).

### Contact angle measurements

Contact angle measurements were performed using an EasyDrop  
instrument and software from Krüss (Bristol, UK). Static sessile  
drop measurements, using 1  $\mu$ l dH<sub>2</sub>O, were either performed  
within 10 min of completing a surface functionalisation process  
or at a series of later time points. A minimum of 5 measurements  
were taken across the surface of each sample (error bars are given  
as one standard deviation).

### Protein capture and elution

Immunoprecipitation was performed using clarified bacterial (*E.*  
*coli* BL-21) lysate containing the expressed affinity tagged  
protein GST-FRB (GST-labelled FKBP-Rapamycin Binding  
domain). (For details on the plasmid employed and the trans-  
formation and lysis protocols used, refer to ESI†, Materials and  
methods.) This lysate (or PBS dilutions) was passed through  
a functionalised column, with the total volume (typically 10–25  
 $\mu$ l), flow rate (1–2  $\mu$ l min<sup>-1</sup>) and incubation time (15–60 min)  
depending upon the sample used, as discussed below. The  
column was then washed with PBS-Tween (3.4–4  $\mu$ l min<sup>-1</sup> for  
15–25 min) before competitively eluting the captured protein  
using 10 mM glutathione, pH 7.4. After filling the column with  
the elution solution, the flow was paused for a 20 min incubation  
and then restarted (1.5  $\mu$ l min<sup>-1</sup>) to elute the protein off the  
column.

Protein capture and elution were assessed by collecting wash  
and elution fractions and analysing them by SDS-PAGE and  
silver staining. Wash and elution fractions were collected in  
aliquots of approximately 15  $\mu$ l to which 4  $\mu$ l of protein loading  
buffer (containing 20% glycerol, 2% SDS, 5%  $\beta$ -mercaptoetha-  
nol, 0.5% bromophenol blue and 63 mM Tris) were added. The  
fractions were then heated to 99 °C for 5 min and run on a 10%  
SDS-PAGE gel (along with pre-stained molecular weight  
markers) before silver staining. Gels were imaged using a Syn-  
gene G:Box system.

### 50 Results and discussion

For performing  $\mu$ IP, a simple, disposable device would be of  
great benefit. A microfabricated, monolithic column is preferable  
to a bead-based approach, as it avoids the need for additional  
components and barriers, results in a lower back pressure and  
enables more control over the internal surface-to-volume ratio  
55 (microfabrication allowing for versatile packing with internal  
pillars, whose size, shape and spacing can be freely varied). The  
latter is important in terms of tailoring column dimensions to the

1 available sample volume and the concentration of the desired  
protein it contains. This is to minimise NSA of contaminating  
sample proteins and to maximise the recovery of the desired  
protein, by ensuring there are sufficient binding sites to capture  
5 all the protein of interest without having a greater surface area  
than necessary—for a fixed surface chemistry, the greater the  
total surface area, the greater the number of sites available for the  
non-specific adsorption of unwanted proteins. Covalently bound  
antibodies are also preferable, as physisorbed antibodies are  
10 likely to be desorbed during the many washing and incubation  
steps required, particularly when solutions containing detergents  
are used (typical in cell lysates), leading to irreproducibility in  
results and antibody contamination of the eluted fractions.

15 Therefore, our  $\mu$ IP approach is based upon a PDMS micro-  
column packed with an array of micropillars, the internal surface  
of which is functionalised with covalently bound antibodies.  
PDMS was selected as the substrate in preference to glass or  
silicon, the most common supports for silane coupling methods,  
as the simplicity and low cost of soft lithography fabrication  
20 techniques are more amenable to the production of disposable  
devices. The homobifunctional crosslinker glutaraldehyde was  
employed to immobilise antibodies to amino-silane functional-  
ised, plasma-treated PDMS, as outlined in Fig. 1c. To develop  
a reliable  $\mu$ IP method, the antibody immobilisation process was  
25 first optimised, then NSA of sample proteins was minimised,  
before assessing the performance of the  $\mu$ IP method under  
a variety of processing conditions.

### 30 Antibody immobilisation

To immobilise antibodies onto the PDMS surface, the first step  
following oxygen plasma treatment was to react the newly  
generated surface silanol groups with APTES, performed as soon  
as possible after plasma processing so as to limit the effects of  
35 PDMS hydrophobic recovery.<sup>24</sup> There are a large number of  
protocols in the literature for silanisation with alkoxysilanes,  
including deposition from water, ethanol/water, acetone and  
toluene.<sup>25</sup> Different silanisation methods and different substrates  
result in different surface characteristics. As there is no clear  
40 indication as to which method is the most suitable for creating  
a high density of surface amino groups on oxygen plasma treated  
PDMS, the use of each of the aforementioned solvents was  
assessed by measuring the water contact angle after silanisation.  
A 5% APTES solution in 95% ethanol/5% dH<sub>2</sub>O was found to  
45 produce a contact angle of approximately 67°, with respect to 74°  
for a 2% solution in ethanol/dH<sub>2</sub>O, 77° for a 2% solution in  
acetone, 80° for a 1% solution in dH<sub>2</sub>O, and 94° for a 1% solution  
in toluene. Contact angle measurements reported for amino-  
alkoxysilane functionalised surfaces range from approximately  
50 40–70°<sup>26–28</sup> and it has been inferred that a complete amino-  
terminated surface monolayer would have a contact angle of  
approximately 60°. As the results obtained using 5% APTES  
solution in 95% ethanol/5% dH<sub>2</sub>O were in the closest agreement  
with the literature, this method was employed for silanisation.

55 Glutaraldehyde crosslinking of antibodies to the resulting  
surface amino groups was then monitored fluorescently, using  
AlexaFluor® 488 labelled anti-GST (Fig. 2) to assess the degree  
of antibody immobilisation and the level of antibody NSA (that  
is the level of surface adsorbed antibodies as opposed to

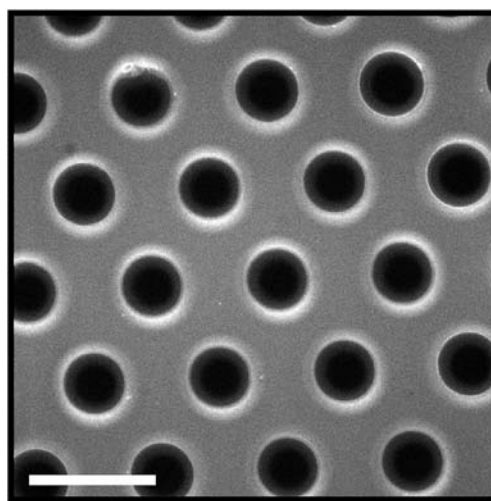
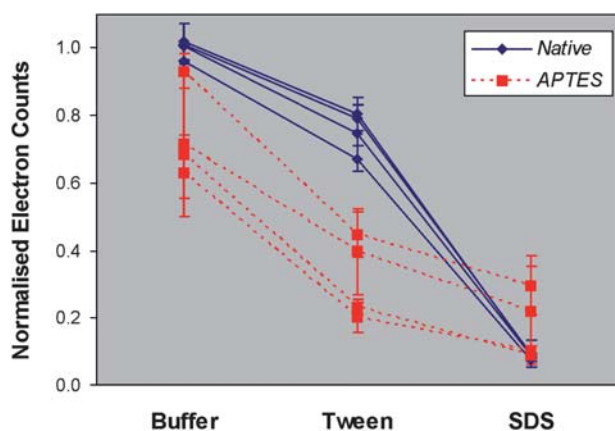


Fig. 2 Fluorescent image of AlexaFluor® 488 anti-GST bound to an amino-functionalised PDMS  $\mu$ IP column *via* glutaraldehyde crosslinking. Following immobilisation and washing, bound antibodies could clearly be observed, with brighter regions around the perimeter of each pillar due to antibodies binding to the pillar sidewalls. The scale bar is 100  $\mu$ m.

covalently bound antibodies) under a variety of conditions. NSA  
was examined by flowing the anti-GST antibody solution  
through an unmodified PDMS column—returned to its native  
hydrophobic state by baking in an oven at 100 °C for 2 hours and  
waiting at least 24 h after bonding before use—and an APTES  
functionalised column that had not been exposed to glutaralde-  
hyde. The former has a hydrophobic surface that is well known  
25 to strongly adsorb proteins,<sup>29</sup> whilst the latter is hydrophilic and  
so surface adsorption will be largely through electrostatic  
attractions. In both of these columns, any antibodies observed  
will have bound to the surface by non-specific physisorption  
mechanisms. These two different surface chemistries were  
30 examined as it is likely that there will be a range of surface  
chemistries found within a glutaraldehyde functionalised  
column, stemming from initial variation in the surface groups  
produced by plasma treatment, partial hydrophobic recovery,  
incomplete silane coverage, unreacted amino groups and intro-  
duced impurities. All of these factors will contribute to some  
35 extent to variations in the resulting surface chemistry and  
therefore contribute to the error bars in the data presented  
below.

45 After flowing the antibody solution through the native and  
APTES columns and washing with buffer, a considerable  
amount of adsorbed antibodies was observed on the internal  
surfaces of both devices (Fig. 3 and Table 1). Subsequent  
washing with buffer containing Tween-20 (a mild, non-ionic  
detergent) caused the measured fluorescence to drop noticeably,  
50 with a greater decrease obtained for the APTES surface.  
However, in both cases, fluorescence intensity was still substan-  
tially above background level. A further wash with buffer con-  
taining 0.1% SDS, a more powerful detergent than Tween-20  
that effectively disrupts both hydrophobic and electrostatic  
55 interactions, as well as inducing protein conformational changes  
that may break protein–surface interactions,<sup>30</sup> removed the  
majority of the remaining antibody, although still not to back-  
ground in either case. However, whilst it would be reasonably



**Fig. 3** Physisorption of antibodies to native and amino-functionalised PDMS surfaces. Measured electron counts from AlexaFluor® 488 anti-GST, normalised to native PDMS washed with buffer alone, following a series of washes. Measurements from four different locations within a microcolumn are shown for each surface.

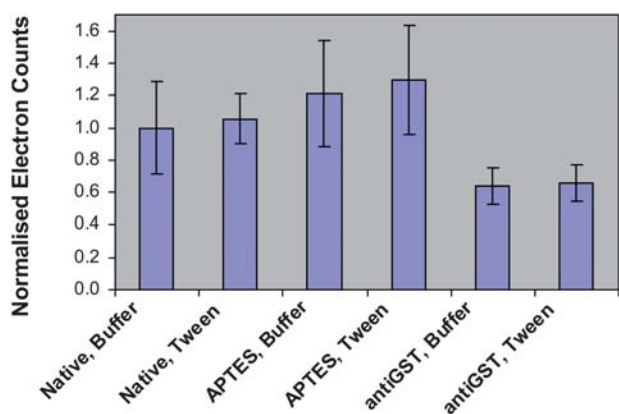
effective at removing any physisorbed antibodies following the glutaraldehyde immobilisation procedure, the presence of SDS is not desirable in an IP column, due to the aforementioned effects.

A more effective approach to reduce antibody NSA was found to be the addition of 0.05% Tween to both the antibody solution and the buffer wash. In this case, NSA was negligible for both the APTES and native PDMS surfaces (Table 1). This result is in contrast to previously reported work where Tween-80 prevented protein adsorption only at the hydrophobic end of a dichlorodimethylsilane–APTES gradient on a silicon substrate.<sup>30</sup> With detergent added to the antibody solution, when the full immobilisation procedure was employed (that is plasma treatment followed by silanisation, glutaraldehyde functionalisation and antibody incubation), a strong signal from immobilised antibodies remained. Although the measured level was lower than in the surface adsorbed cases, these results indicate that antibody binding is covalent.

To gain more information on the level of antibody coverage, fluorescently labelled BSA was employed, BSA being commonly used to block potential sites for non-specific interactions. The level of BSA adsorption to native, APTES and antibody immobilised PDMS microcolumns was measured, so as to estimate the percentage coverage of antibodies from the decrease in BSA adsorption on an antibody coated surface relative to the native/APTES surfaces. As expected, the results (Fig. 4) showed a significant decrease in BSA binding when immobilised antibodies are present, and suggest that in the region of 48% (the average decrease in intensity for the immobilised anti-GST surface with respect to the APTES surface) of the probable surface capacity has been functionalised with antibodies. This is in reasonable agreement with the results presented in Table 1, which showed 38% covalently immobilised antibodies relative to the maximum level of surface adsorbed antibodies. Although antibody surface coverage could, therefore, be further increased, this may be neither desirable nor necessary: the optimum density for an antibody layer remains uncertain, in terms of retaining antibody reactivity and binding affinity,<sup>23,31,32</sup> and as the results below demonstrate satisfactory  $\mu$ IP performance.

**Table 1** Normalised electron counts measured following antibody binding to native, APTES functionalised and glutaraldehyde–APTES functionalised PDMS microcolumns for a variety of experimental conditions

PDMS surface	Antibody solution	Wash conditions	Normalised electron counts
Native	No Tween	Buffer Tween → SDS	1.0 (±0.054) 0.75 (±0.066) → 0.088 (±0.010)
APTES	Tween No Tween	Buffer Tween → SDS	0.005 (±0.004) 0.74 (±0.16) 0.32 (±0.13) → 0.18 (±0.10)
APTES–glutaraldehyde	Tween Tween	Tween Tween	0.018 (±0.005) 0.38 (±0.019)



**Fig. 4** Adsorption of BSA to native, APTES functionalised and covalently bound anti-GST PDMS surfaces, following washing with either PBS or PBS-Tween. Measured electron counts from AlexaFluor® 647 labelled BSA, normalised to native PDMS washed with PBS.

### Stability of APTES surface

It may be convenient to store devices following silanisation with APTES, to allow any antibody to be immobilised at a later date. However, effects due to hydrophobic recovery of the PDMS, as well as ageing of the APTES layer,<sup>33</sup> would result in significant inactivation of the surface with time. Storing the devices in an aqueous solution should limit this, as should a preliminary solvent extraction of the PDMS.<sup>34</sup> Solvent extraction involves soaking the native PDMS in a series of solvents that swell the PDMS and remove residual non-crosslinked PDMS oligomers and crosslinking agent. When carried out before oxygen plasma treatment, this slows post-treatment surface rearrangement, limiting hydrophobic recovery. This also results in an improved surface for subsequent functionalisation.

Therefore, to attempt to produce a more reproducible and stable surface for the silanisation stage used here, a mild solvent extraction process was tested that did not risk causing distortion of the device features (cracking and buckling of PDMS surfaces can result when de-swelling high swollen PDMS). This involved soaking the PDMS parts first in acetone (soaking for 2 h in 250 ml, replacing with fresh acetone, then soaking for a further 2 h), then in ethanol (1 h), then methanol (1 h). This produced an initial contact angle (Table 2) following silanisation that was substantially lower than for PDMS that had been simply cleaned in methanol (15 min in an ultrasonic bath). Petri *et al.* reported similarly low values for a freshly prepared amino-propyltrimethoxysilane monolayer on silicon,<sup>33</sup> which

**Table 2** Initial contact angle measurements on native and functionalised PDMS surfaces compared to measurements made after 9 days. Initial oxygen plasma and APTES samples were measured 10–15 min after treatment. The APTES samples were either soaked in acetone or cleaned in methanol, as described in the text

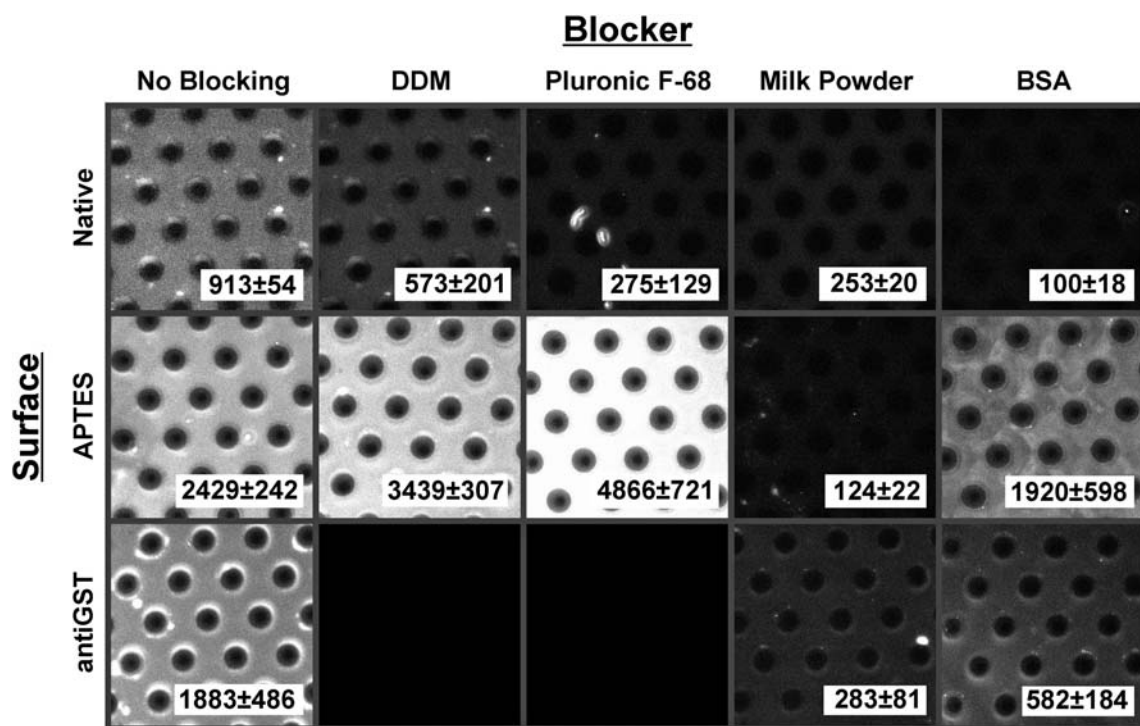
PDMS surface		Initial angle/°	Angle after 9 days/°
Native		116 (±1.8)	
O <sub>2</sub> plasma treated		14 (±2.2)	95 (±6.2)
APTES functionalised	Acetone soak	23 (±9.2)	88 (±12)
	Methanol clean	72 (±2.6)	91 (±3.4)
Crosslinked anti-GST		62 (±1.3)	69 (±3.7)

subsequently increased with time. For APTES-PDMS, the reason for the low contact angles obtained following solvent soaking is unclear but could stem from a combination of improved amino functionalisation and the retention of more hydrophilic surface groups following oxygen plasma treatment, which would be exposed through any gaps in the APTES layer. Nevertheless the level of antibody binding obtained appeared to be more reproducible when using this soaking procedure and so the acetone soak was incorporated into the microcolumn preparation protocol. Whilst it was not expected to be nearly as effective for slowing hydrophobic recovery as stronger solvents (acetone has a PDMS swelling ratio of 1.09, compared to 1.58 for previously used triethylamine and 1.18 for ethyl acetate<sup>35</sup>), the APTES-PDMS surface did remain hydrophilic for more than 1 week, as shown in the contact angle time courses in ESI†, Fig. S1.

### Blocking of potential NSA sites

As the microcolumn surface was optimised for the covalent binding of antibodies, following antibody immobilisation it was necessary to block potential sites for NSA of sample proteins. A wide range of substances have been used as blocking agents, with different surfaces being blocked effectively by only certain compounds, depending upon the relative chemical properties of surface and blocker. To establish a suitable blocking protocol for these PDMS microcolumns, a series of potential blockers with varying properties were tested: BSA (a single purified protein) and non-fat milk powder (predominately a mixture of proteins and carbohydrates), both of which are widely used for blocking ELISAs, microarrays and western blots,<sup>36</sup> Pluronic F-68 (a triblock PEO-PPO-PEO copolymer previously reported to suppress protein adsorption<sup>37</sup>) and DDM (a non-ionic detergent recently reported to effectively block native PDMS that was doped with phospholipids<sup>7</sup>).

Fluorescently labelled streptavidin was employed as a test protein to assess the efficiency of these blockers. Initially, each of the blockers was used to treat both native and APTES functionalised PDMS columns, through which a streptavidin solution was then flowed. Following washing with PBS-Tween, each of the microcolumns was imaged to determine the level of non-specific streptavidin binding. The results, summarised in Fig. 5, showed a wide variation in NSA behaviour. For a native, hydrophobic PDMS surface, all potential blockers reduced the level of streptavidin binding with respect to an unblocked column, with BSA reducing NSA the most effectively (blocking efficiency decreased in the order BSA > milk powder > Pluronic > DDM). APTES functionalised surfaces, however, behaved very differently: blocking with both DDM and Pluronic F-68



**Fig. 5** Assessing the efficiency of potential NSA blockers. Variation in the adsorption of AlexaFluor® 647 labelled streptavidin to native, APTES functionalised and immobilised anti-GST PDMS surfaces for a range of blocking conditions. In all images (taken at approximately the same location for each microcolumn), the displayed data range was restricted to 4000 counts above background. The mean electron counts (from regions between pillars measured at a number of locations throughout the columns) and standard deviation are given for each set of conditions.

actually increased the level of streptavidin adsorption with respect to an unblocked column. Presumably the orientation on a hydrophilic surface of DDM and Pluronic F-68, both of which have amphiphilic characteristics, is very different to that on a hydrophobic surface, presenting a more favourable substrate for protein adsorption in the former case. Milk powder was extremely effective at blocking the APTES functionalised surface, whilst the level of streptavidin binding to a BSA-blocked column was less than observed for an unblocked column but significantly greater than for a native PDMS surface.

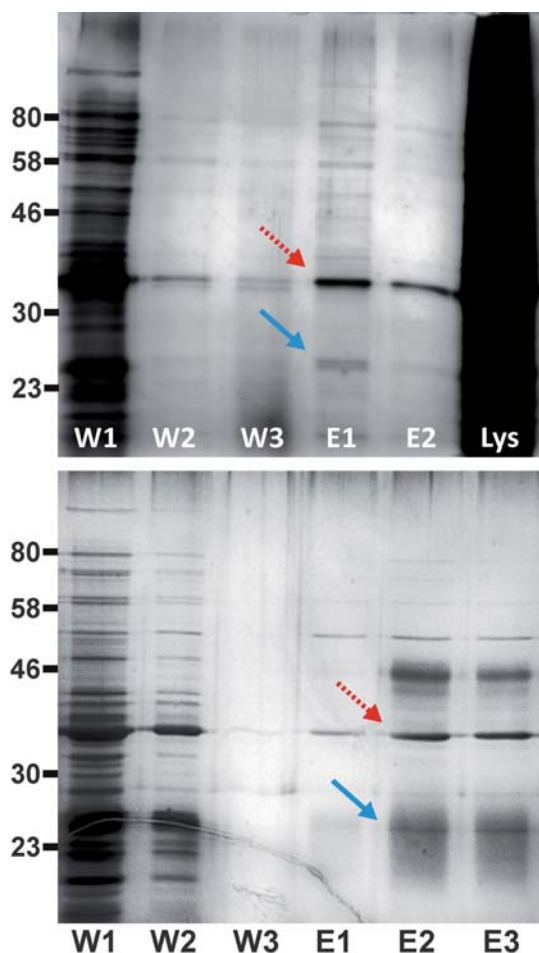
As BSA and milk powder were the most effective on both surfaces they were tested for blocking an anti-GST antibody functionalised microcolumn, which was then probed with labelled streptavidin to measure NSA. Although the results were somewhat different to that obtained with the previous surfaces, possibly because the streptavidin could interact with both the microcolumn surface and the immobilised antibodies, NSA was reduced substantially by both blockers, with milk powder producing the best results. Whilst there will be some experimental variation depending upon sample composition, and bearing in mind that no experimental conditions will entirely eliminate NSA (as discussed by Trinkle-Mulcahy *et al.*<sup>38</sup>), these results suggest that milk powder is likely to be the best blocker for these  $\mu$ IP columns. It was therefore employed in all later  $\mu$ IP experiments.

#### Micro-immunoprecipitation ( $\mu$ IP)

The PDMS devices produced had an internal volume of 4.4  $\mu$ l and surface area of approximately 300 mm<sup>2</sup>. Although the

packing density and orientation of the immobilised antibodies is not known, the maximum number of bound antibodies can be estimated by assuming hexagonal packing and an intermediate surface area per antibody of 14.2 nm  $\times$  3.8 nm, resulting in a value of  $5.0 \times 10^{11}$ . However, not all immobilised antibodies will retain their activity: antibody inactivation can result in less than 20% of physisorbed antibodies maintaining their functionality, although greater activity is retained when using methods other than direct surface adsorption.<sup>39,40</sup> It is therefore difficult to estimate the protein binding capacity of these  $\mu$ IP columns. However, even if only 20% of the immobilised antibodies retained their functionality, a surface coverage of 40% would correspond to a protein binding capacity of 0.83 pmoles (*i.e.* 42 ng for a 50 kDa protein). Such protein levels are large enough for off-chip validation by SDS-PAGE and silver staining, which has a detection limit of approximately 0.1 ng. The microcolumn dimensions can of course be simply scaled up or down depending upon the sample available, tailoring the total surface area and volume appropriately.

To demonstrate the  $\mu$ IP method, the capture and elution of GST-FRB (37 kDa) from an *E. coli* lysate was performed. The sample lysate was flowed through a column functionalised with covalently bound anti-GST, following which the lysate-filled column was incubated for 15 min, before washing and eluting the GST-FRB with glutathione. Wash and elution fractions were collected and then run on a SDS-PAGE gel and silver stained. The results (Fig. 6a) demonstrated that GST-FRB was bound by the immobilised anti-GST and successfully and cleanly eluted with glutathione. A conventional IP (Fig. 6b) was performed for



**Fig. 6** Purifying GST-FRB from a bacterial lysate. Silver stained gels from both  $\mu$ IP (upper gel) and conventional IP (lower gel) methods are shown. GST-FRB and free GST (a result of proteolytic cleavage) are marked by red dotted and blue line arrows respectively. Dimerisation of GST can also be seen in the conventional gel.

comparison (details of the protocol employed are found in ESI†, Materials and methods), which showed the  $\mu$ IP method to be as effective despite using substantially less sample (4  $\mu$ g total protein vs. 300  $\mu$ g).

The total time required for an immunoprecipitation stage was 1–2 h depending upon the sample used. As expected, the concentration of the lysate strongly affected the washing and sample incubation steps required. Fig. 6a shows the results obtained when using 25  $\mu$ l of a 1 in 10 dilution of a bacterial lysate (the undiluted lysate concentration was approximately 1.7 mg ml<sup>-1</sup>). If the same sample volume, incubation and washing parameters were employed with a 1 : 1 dilution of the lysate, the final wash was far from clear and the first elution fraction was contaminated with non-specifically adsorbed proteins washing away (ESI†, Fig. S2a). On the other hand, a 1 in 20 dilution (ESI†, Fig. S2c) resulted in a noticeably weaker GST-FRB band. Increasing the sample incubation time for more dilute samples increased the strength of the band obtained. ESI†, Fig. S2c shows two  $\mu$ IP results from 1 in 20 dilution samples, the first using a 25  $\mu$ l sample (4.3  $\mu$ g total protein) and a 15 min incubation, the second using 10  $\mu$ l (1.7  $\mu$ g total protein) and a 60 min total

incubation. It is clear from this that the more concentrated the sample, the greater the washing required, and the weaker a sample, the longer the necessary incubation. This suggests that, not surprisingly, the  $\mu$ IP protocol must be adapted to the sample employed and that the incubation times required will be affected by the binding kinetics of the captured protein. Further tuning of the flow rates employed for washing may also be beneficial, as this could influence washing efficiency.

### Storage of $\mu$ IP columns

In many situations it is desirable to prepare a batch of antibody-immobilised columns in advance of running a series of experiments. To assess their ability to withstand storage, a  $\mu$ IP column filled with PBS-azide was stored following antibody immobilisation at 4 °C for one week. It was then used to capture and elute GST-FRB from an *E. coli* lysate, as described above. Results similar to those obtained previously with freshly prepared devices were achieved (ESI†, Fig. S2) and it is likely that the  $\mu$ IP columns will withstand longer storage. Contact angle measurements were also performed on anti-GST functionalised PDMS surfaces, as detailed in Table 2 (with a contact angle time course provided in ESI†, Fig. S1). The measurements show little change in the contact angle of an anti-GST surface over a 9 day period, indicating that the surface was stable.

### Conclusions

We have successfully optimised a method for functionalising PDMS microcolumns with covalently bound antibodies, whilst minimising protein NSA, and have demonstrated  $\mu$ IP of affinity-tagged proteins from bacterial lysates. The performance of the  $\mu$ IP method is comparable to conventional IP methods, despite using substantially smaller samples. This  $\mu$ IP approach is well suited to performing on-chip protein separations and the microcolumns are amenable to the creation of integrated parallel and serial analysis schemes, opening the door to multiplexed and multistage assays.

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