An off-the-shelf integrated microfluidic device comprising self-assembled monolayers for protein array experiments

Mirit Hen,¹ Maria Ronen,² Alex Deitch,² Efrat Barbiro-Michaely,² Ziv Oren,³ Chaim N. Sukenik,^{1,a)} and Doron Gerber^{2,a)}

¹*Chemistry Department and Institute of Nanotechnology and Advanced Materials, Bar Ilan University, Ramat Gan 5290002, Israel*

²*Mina and Everard Goodman Life Science Faculty and Institute of Nanotechnology and Advanced Materials, Bar Ilan University, Ramat Gan 5290002, Israel*

³*Israel Institute for Biological Research, P.O. Box 19, Nes Tziona 7410001, Israel*

(Received 23 July 2015; accepted 31 August 2015; published online 16 September 2015)

Microfluidic-based protein arrays are promising tools for life sciences, with increased sensitivity and specificity. One of the drawbacks of this technology is the need to create fresh surface chemistry for protein immobilization at the beginning of each experiment. In this work, we attempted to include the process of surface functionalization as part of the fabrication of the device, which would substitute the time consuming step of surface functionalization at the beginning of each protein array experiment. To this end, we employed a novel surface modification using self-assembled monolayers (SAMs) to immobilize biomolecules within the channels of a polydimethylsiloxane (PDMS) integrated microfluidic device. As a model, we present a general method for depositing siloxane-anchored SAMs, with 1-undecyl-thioacetate-trichlorosilane ($C_{11}TA$) on the silica surfaces. The process involved developing PDMS-compatible conditions for both SAM deposition and functional group activation. We successfully demonstrated the ability to produce, within an integrated microfluidic channel, a $C_{11}TA$ monolayer with a covalently conjugated antibody. The antibody could then bind its antigen with a high signal to background ratio. We further demonstrated that the antibody was still active after storage of the device for a week. Integration of the surface chemistry into the device as part of its fabrication process has potential to significantly simplify and shorten many experimental procedures involving microfluidic-based protein arrays. In turn, this will allow for broader dissemination of this important technology. © 2015 AIP Publishing LLC.

[<http://dx.doi.org/10.1063/1.4930982>]

INTRODUCTION

Microfluidics is a technological platform that enables scale reduction in sample volume and measurement times,^{1–3} versatility in design, improved sensitivity, and selective reactions for surface patterning.^{4–9} Its advantages opened new possibilities in the study of biomolecules. Polydimethylsiloxane (PDMS), a popular elastomer, is the most widely used material in the construction of microfluidic devices^{10–12} due to its straightforward manufacture,¹³ low fabrication costs, high flexibility, optical transparency, and ease of bonding to silica/glass surfaces. Additionally, PDMS is relatively inert, non-toxic, and fully bio-compatible. These advantages allow for the integration of complex processes into microfluidic lab-on-a-chip devices that serve as multifunctional platforms for a variety of analytical assays.¹⁴ Microfluidic-based protein arrays are one such promising application, with high impact in proteomics. A major bottleneck hindering dissemination of the latter technology is the long

^{a)}Authors to whom correspondence should be addressed. Electronic addresses: chaim.sukenik@biu.ac.il and doron.gerber@biu.ac.il

experimentation time, ranging from 3.5 to 7 h, stemming mostly from the surface chemical modification procedure for immobilizing proteins.^{3,15}

Glass slides or other silica surfaces such as silicon wafers or quartz¹⁶ are typically used as the solid support for the immobilization of biomolecules within PDMS microfluidic devices. Efficient immobilization of biomolecules onto silica surfaces is required for optimal device function. However, in contrast to the large efforts invested in developing new microfluidics devices for biological studies and applications, little attention has been paid for the systematic development of efficient surface treatment methods for facilitating biomolecule immobilization within the microfluidics devices. Maintaining the conformation and controlling the orientation of biomolecules, while minimizing nonspecific binding, are important for successful biomolecule immobilization. Current immobilization methods include non-covalent absorption,⁷ direct covalent linkage to a chemically activated surface,⁷ and indirect binding through a functionalized molecule.^{17,18}

Binding to a surface via a functionalized self-assembled monolayer (SAM) is a simple way of changing surface properties and installing specific functional groups on a surface. SAMs can control, for example, hydrophobicity/hydrophilicity, electrochemical properties,¹⁹ chemical resistance,²⁰ biocompatibility,²¹ and sensitization.²² SAM forming molecules include a surface reactive anchored group, an alkyl chain or aromatic ring that promotes self-assembly into a densely packed, organized molecular layer, and a terminal functional group to be displayed on the exposed SAM surface for conjugation to biomolecules.^{23,24}

Immobilization of biomolecules on surfaces using SAMs has the advantages of defined orientation, biocompatibility, enhanced sensitivity due to the high density of the functional terminal groups, reproducibility, and durability. However, the use of SAMs in PDMS microfluidic chips is limited by the fact that the PDMS will, in general, not adhere to the support if SAMs have been deposited on the silica surfaces prior to PDMS bonding. In one case, (3-Glycidioxypropyl)methyltriethoxysilane (GPTMS) SAMs could be deposited on glass prior to PDMS bonding^{15,25} and then used for biomolecule immobilization. However, GPTMS SAMs provided incomplete surface coverage, leaving room for nonspecific binding of biomolecules.

Hydrolyzable silanes are the most common class of molecules for the functionalization of silica-based surfaces. Immobilization of biomolecules via silanization of silica-based surfaces is a known strategy.²⁶ Previous studies reported that biomolecule microarrays manufactured using SAM surface functionalization provide homogeneous and reproducible microarrays.²⁷ Well-ordered, robust, SAMs on glass were first made by Netzer and Sagiv²⁸ using trichlorosilanes as an anchoring group within a molecule containing a long alkyl chain. This combination provided significantly improved surface coverage relative to GPTMS SAMs. However, combining trichlorosilane anchoring chemistry with a PDMS microfluidic device surface suffers from the incompatibility of the PDMS with the solvents typically used for trichlorosilane SAM deposition.²⁹ Mostly, trichlorosilane SAMs are deposited from hydrophobic solvents like dicyclohexyl (DCH)^{17,29} and these cause PDMS to swell.³⁰ Thus, the goal of making such SAMs within a preformed PDMS microfluidic device requires a substantial improvement in deposition conditions.

SAMs based on 1-undecyl-thioacetate-trichlorosilane (C₁₁TA) were first reported by Whitesides³¹ as an adhesive layer for gold on a silica surface after thiol deprotection. Thiol and disulfide decorated interfaces have been used for immobilizing biomolecules,^{18,32} *in situ* surface chemistries such as click chemistry,³³ and metal adsorption. Artel *et al.*³² established a method for the controlled formation of thiols and disulfides on silicon or quartz surfaces and demonstrated its use as an efficient basis for the anchoring of either thiol- or disulfide-bearing molecules onto SAM surfaces with excellent packing and surface coverage.

In this paper, we present a method for depositing C₁₁TA SAMs, as a model, on silica surfaces within a PDMS microfluidic device. C₁₁TA group was chosen as it has been previously demonstrated to efficiently form SAMs on silanol-bearing surfaces.^{31,32} This involved developing both PDMS compatible deposition conditions and a method to remove the thioacetate group and form thiol or disulfide groups within the microfluidic channels. This chemistry is verified by reacting the SAM-coated silica surface with a fluorescent biomolecule. We demonstrate that

we can produce, within integrated microfluidic channels, C₁₁TA monolayers to which a protein can be directly conjugated, thus allowing for direct bioactivation. In addition, this opens the possibility for carrying out a variety of different reactions and surface modifications within the device. The creation of the functionalized SAM after device fabrication eliminates the problems inherent in PDMS adhesion to SAM functionalized glass surfaces and thus increases the range of possible working pressures with the device. We then showed that this surface chemistry remains active after a week of storage. This, in turn, enabled the transfer of the surface chemistry process from the experimental procedure to the device fabrication stage, significantly shortening the typical microfluidic-based protein arrays.

MATERIALS AND METHODS

Materials

All reagents and solvents were obtained from Sigma-Aldrich, Acros Organics, Fluka, Bio-Lab Ltd. or Merck. The 1-undecylthioacetate (C₁₁TA) used herein was prepared using published procedures,³⁴ and its spectral properties were identical with those in the literature. Commercial extra dry acetonitrile was further dried by running the solvent through the acetonitrile cartridge of a Vacuum Atmospheres Solvent Purifier from VAC—Vacuum Atmospheres Company. Biotinylated anti-penta-His antibody (Qiagen, #1019225) was obtained from Qiagen. GFP was expressed using a S30 *in-vitro* high yield protein expression system by Promega. Anti-GFP from was obtained from Abcam, USA. PDMS SYLGARD 184 was obtained from Dow Corning, USA. Water was deionized and then distilled in an all-glass apparatus. Silicon wafers were obtained from Virginia Semiconductor (N-Type; undoped, $\langle 100 \rangle$, $>1000 \Omega \text{ cm}$). Quartz substrates were obtained from Quarzschmelze Ilmenau, Germany.

Surface preparation

Silicon/quartz wafers were cleaned with chloroform, acetone, and ethanol and blown dry in a filtered nitrogen stream. This was followed by treatment of the wafers with piranha solution (H₂SO₄ (97%):H₂O₂ (30%), 70:30 v:v, 80 °C) for 20 min to remove organic contaminants. The treatment was followed by 3 rinses with deionized, distilled water and by drying under a filtered nitrogen stream.

C₁₁TA in acetonitrile deposition

The substrates were immersed in a solution of extra dry acetonitrile (10 ml) and 25 μl of C₁₁TA and allowed to stand overnight. Then, the wafers were cleaned by rinsing in acetonitrile and then by sonication in acetone.

Cleavage of surface thioacetate groups

Cleavage by 1,8-diazabicycloundec-7-ene (DBU)

The thioacetate decorated surfaces were immersed in a solution of 10 ml methanol and 87 μl of DBU.³⁵ After 2 h at room temperature, the wafers were removed from the solution, cleaned by rinsing in methanol, and then by sonication in ethanol. Then, they were dried under a nitrogen stream.

HCl 10% acidic hydrolysis

The thioacetate coated wafers were immersed in 10% aqueous HCl and heated to 40 °C for 3 h. The wafers were then removed from the solution and rinsed 3 times in water. This was followed by an ethanol rinse and drying under a nitrogen stream.

Disulfide or thiol group identification

Lithium aluminum hydride (LAH) reduction of disulfides to thiols

The C₁₁TASAM-coated wafers were immersed in a solution of LAH (1M in THF). After 2 min, the wafers were withdrawn from the solution and cleaned for 30 min in 10% aqueous HCl. They were then cleaned with ethanol and dried under a nitrogen stream.

Dithiothreitol (DTT) reduction of disulfides to thiols

The disulfide SAM-coated wafers were immersed in a solution of DTT (0.1M) in TRIS buffer (pH 8.5). After 1.5 h, the wafers were withdrawn from the solution and cleaned for 30 min in 10% aqueous HCl. They were then further cleaned with ethanol and dried under a nitrogen stream.

1-Fluoro-2,4-dinitrofluorobenzene (DNFB) reaction

The SAM-coated wafers were immersed in a solution of KOH in ethanol at pH 8 with 1% V/V DNFB.³² The reaction was allowed to proceed for 5 min at room temperature. The wafers were then withdrawn from the solution, cleaned with ethanol, and dried under a nitrogen stream.

Characterization methods

Attenuated total reflectance Fourier transform infrared spectroscopy

ATR-FTIR spectroscopy was performed using a Tensor 27 (Bruker) spectrometer. Spectral parameters were as previously reported.^{34–37}

UV-vis spectroscopy

UV spectra were measured using a Cary Model 100 spectrometer (in double beam transmission mode) as previously reported.³⁷ All samples were measured in the 200–600 nm wavelength range.

On-chip surface chemistry and operation

Integrated microfluidic device fabrication

The microfluidic devices were fabricated using PDMS as previously reported.²⁵ The double-layer microfluidic device consists of two PDMS layers that were aligned one on top of the other. The flow layer (0.03 mm thick) consists of the device's inputs and channels, while the control layer (5 mm thick) consists of the micromechanical "address" valves that control the experimental procedure ("control valves" and "button valves"). The address valves were filled with DuPont Krytox perfluorinated oil. MTP device contains 16 inputs and 4 experimental channels with 4 outputs (Fig. S1 in the supplementary material).^{24,45} The average unit height of the channels is 15 μ m and width is 400 μ m. DNA array to protein array (D2P) contains 8 inputs, one output, and 1000 experimental chambers diameter of 80 μ m, as previously described.²⁴ Each chamber contains a button valve.²

The PDMS device was bonded to a plasma-activated glass surface by heating on a hot plate at 80 °C for 2 h.

C₁₁TA deposition within the microfluidic device

C₁₁TA in dry CH₃CN solution was flowed through the device at ~10 Psi. The outputs of the experimental channels valves are closed, trapping the solution in the experimental channels while the channels remain pressurized. Then, the input channels are filled with the acetonitrile-C₁₁TA solution. The solution in the experimental channels was allowed to incubate in the

device overnight. Then, the device was washed with acetonitrile for 5 min and then with acetone for 5 min at a 10 psi flow pressure.

Cleavage of thioacetate group

Cleavage by DBU

Cleavage was achieved by slowly flowing (~ 2.5 PSI) the DBU in methanol solution described above through the device channels for 2 h at room temperature. Then, the device was rinsed with flowing methanol and then ethanol for 5 min each at 10 psi flow pressure.

Cleavage using 10% aqueous HCl

Cleavage was done by slowly flowing (~ 2.5 PSI) 10% aqueous HCl through the device channels for 3 h at 40 °C. The device was then washed with water and then ethanol for 5 min each at 10 psi flow pressure.

Disulfide reduction

DTT 0.1M in a TRIS buffer solution at pH 8.5 was flowed slowly (~ 2.5 PSI) through the device channels for 1.5 h at room temperature. Then, the device was washed, first with water and then with ethanol, for 5 min each at 10 psi flow pressure.

Detection and analysis

The chip was washed with HEPES buffer pH 7.3 for 5 min. Next 3 μ l of biotinylated anti-penta-His antibody (Qiagen, #1019225) in 27 μ l HEPES solution was flowed for 5 min and then incubated for 1 h. Afterwards, the device was washed with HEPES buffer for 5 min. His-tagged GFP was expressed by S30 T7 High-Yield Protein Expression System (Promega). It was flowed through the device for 20 min to enable it to react with surface bound antibodies. Then, the device was washed with HEPES for 5 min (Fig. S2 in the supplementary material).⁴⁵ The fluorescence intensity of GFP was measured with a microarray scanner (LS 166 Reloaded, Tecan) with fluorescent excitation at 488 nm (for GFP) and emission at 532 nm.

Images were analysed using GenePix pro program. 40 spots at even distances were sampled throughout the middle of the microfluidic channel. The median fluorescence level of each spot was determined. The average fluorescence level of each channel was determined by calculating the average and standard deviation of the extracted median values of each channel. The signal to noise ratios were determined by calculating the ratio between each spot median fluorescence level from the experimental channel to a comparable spot median fluorescence level from the control channel. The average ratios and their standard deviations were calculated. The P values were analyzed using the Mann-Whitney U test.

Selective protein attachment within D2P and long term storage

The entire chip surface was activated with disulfides. We then passivated the activated disulfide surface around the buttons by closing the button valves in each chamber and flowing for 30 min an alexa 647 antibody, which does not bind GFP. Then, we introduced anti-GFP for 30 min with open buttons. Next, we tested the surface chemistry by flowing GFP for 20 min into the device. Between each step, there was 5 min rinsing step with HEPES buffer. The fluorescence intensity of GFP was measured with a microarray scanner (LS 400 Reloaded, Tecan) with fluorescent excitation at 488 nm (for GFP) and emission at 532 nm. The fluorescence intensity of the alexa 647 (background) was measured with fluorescent excitation of 635 nm. For testing long term storage, the device was dried after the surface chemistry and then stored for a week at room temperature. After a week, the device was rehydrated with the washing buffer and GFP was introduced to test the activity of the surface.

Images were analyzed using GenePix pro program. The median fluorescence level of each spot was determined. The average fluorescence level of each channel was determined by

calculating the average and standard deviation of the extracted median values of each channel. The signal to noise ratios were determined by calculating the ratio between each spot median fluorescence level in each button to its surrounding median fluorescence level. The average ratios and their standard deviations were calculated.

RESULTS AND DISCUSSION

SAM characterization

The thiol-functionalized SAM was formed by deposition of trichlorosilane C₁₁TA on a silica-based solid surface (Fig. 1(a)). We established the conditions for deposition using silicon wafer and quartz surfaces, the main adjustment to previously prepared SAMs being the change in the deposition solvent to PDMS-compatible acetonitrile.³⁰ Optimum deposition conditions for creating C₁₁TA trichlorosilane SAMs on a silicon wafer using acetonitrile as a solvent involved an overnight deposition time. This resulted in good monolayer coverage (based on the intensity of the corresponding IR signals observed (Fig. 1(b))). However, the monolayer obtained was poorly ordered, based on the position of the two methylene peaks at 2925 and 2854 cm⁻¹. These spectral results were compared with SAM deposition performed using DCH solvent, which gave methylene peaks at slightly lower frequency (2923 and 2852 cm⁻¹). The methylene stretches at ~2920 and 2850 cm⁻¹ are the standard peaks observed for monolayer packing and order, with the increased order and packing giving lower frequency methylene stretches.³⁸ The somewhat poorer packing and order for SAMs deposited from a polar solvent has been reported by McGovern *et al.*³⁹

Reaction conditions for the cleavage of the thioacetate group, for deprotection and activation of the thiol group, were also limited due to the incompatibility of the PDMS with several of the reported reagents and conditions.^{31,32} The standard method of using aqueous HCl was a good example of this limitation. Wasserman *et al.*³¹ had reported such a cleavage using concentrated HCl. We found that 10% aqueous HCl is the highest concentration compatible with the PDMS devices. However, these conditions gave incomplete cleavage of the thioacetate group. ATR-FTIR was utilized to show the extent of thioacetate cleavage, which was incomplete using 10% aqueous HCl (Fig. 1(b)). A higher concentration damaged the integrated valves of the device.

Singh *et al.*³⁵ reported thioacetate cleavage using DBU in methanol, and these conditions were found to be effective on the SAM surfaces as well. DBU in methanol gave complete thioacetate removal within 2 h. The lack of change in the position of the methylene stretches suggests that there is little or no change in monolayer packing under these conditions.

Thioacetate cleavage can result in either disulfide or thiol formation.^{35,40} To differentiate thiols from disulfides on the surface, we used DNFB, which has been shown³² to react with thiols and not with disulfides. Figure 1(c) shows that after cleavage with DBU, there was no reaction of DNFB with the surface, but after exposing the surface to LAH it did react. This suggests that DBU hydrolysis produces a disulfide decorated SAM surface versus the thiols produced with LAH. However, the LAH procedure was incompatible with the microfluidic device.

To obtain thiol-decorated surfaces under PDMS compatible conditions, the disulfide obtained from the DBU procedure was treated with DTT. This reduction occurs in solutions with a pH above 7,⁴¹ allowing the use of TRIS buffer. After DTT treatment, the surface reacted with DNFB, confirming that a thiol-decorated surface was obtained (Fig. 1(c)).

To examine C₁₁TA deposition within a microfluidic device, we used a PDMS microfluidic device with 16 inputs and 4 experimental channels, all controlled by micromechanical valves. This network allowed us to conduct 4 different experiments on a single device using up to 16 different reagents (Fig. S1 in the supplementary material).⁴⁵ We divided the channels into an “experiment” channel, which was exposed to all surface treatments and “control” channels that were used for detection of non-specific binding of the GFP or the antibody.

All channels were coated with a C₁₁TA SAM. Successful C₁₁TA deposition within the microfluidic device requires dry conditions since exposure of a trichlorosilane group to moisture results in its polymerization.²⁹ When deposition was attempted in non-dry conditions, both clogging of the microfluidic channel and valve destruction were observed, presumably to

FIG. 1. Thiol-functionalized SAMs on a solid silica-based surface. (a) Schematic diagram of thiol-functionalized SAM formation. C_{11} TA was bound to the surface followed by cleavage of the thioacetate to give disulfide bridges or free thiols. (b) (i) Comparison of ATR-FTIR spectra of the SAM in acetonitrile (pink line) and in DCH (blue line) on a silicon wafer. The methylene peaks at slightly higher frequency 2925 and 2854 cm^{-1} in acetonitrile were indicative of a slightly lower quality SAM in terms of order and packing compared to the SAM deposition with DCH (2923 and 2851 cm^{-1}). (ii) ATR-FTIR spectra showing the extent of thioacetate cleavage, visible by the disappearance of the thioacetate carbonyl peak at 1697 cm^{-1} . Complete cleavage was achieved using DBU in methanol (black line). (c) UV-vis spectra of C_{11} TA on a quartz surface. (i) DBU cleavage results in disappearance of the absorbance peak at 238 nm. After exposing the thiol surface to DNFB, a new absorbance peak appears at 348 nm. (ii) DBU cleavage of the thioacetate resulted in disappearance of the absorbance peak at 237 nm. After exposing the thiol surface to DNFB, an absorbance peak appeared at around 350 nm.

polymerization, and the device was no longer usable (Fig. S2 in the supplementary material). Usually, the address valves in integrated microfluidics are filled with water.^{25,42} In our case, it appeared that the vapor pressure of the water polymerized the trichlorosilane and destroyed the address valves. In order to avoid silane polymerization, we used Krytox perfluorinated oil to fill the address valves. Furthermore, we used the gas permeability of the PDMS to our advantage and the solvent and solutions were flowed at a rate fast enough to allow the trapped air to be pushed out through the PDMS. This provided the necessary environmental isolation inside the experimental channels of the chip.

Thiol deprotection and confirmation of antibody conjugation

To illustrate and detect the deposition and cleavage of $C_{11}TA$ in the experimental channels, we used GFP binding. Penta-His antibody was bound via a reaction with disulfide or thiol groups as previously reported (Fig. 2(a)).³² Four microfluidic channels with four different experimental conditions were utilized to demonstrate successful antibody conjugation: experimental channels A and B and control channels C and D. Experimental channel (A) was exposed to DBU treatment and produced disulfide surfaces that were subsequently reduced to a thiol surface by DTT. After exposing the surface to anti-penta-His, GFP binding, and washing with HEPES (Fig. 2(b)), channel (A) was highly fluorescent, indicating significant presence of the antibody, which had been efficiently conjugated to the thiol surface. Experimental channel (B) that was exposed to DBU treatment and anti-penta-His with no DTT reduction to thiols showed slightly lower fluorescence than channel (A). The fluorescence of the thiol decorated channel was 1.2 times stronger than that of the disulfide channel (with $p < 0.0022$), showing that there was reduced binding of the antibody to a disulfide surface compared to binding to thiol surfaces (Fig. 2(c)). This is consistent with the expectation that the antibody cysteine disulfide reacts more readily with a thiol surface than it would react by simple exchange between two disulfides. It has been shown that thiol-disulfide interchange occurs⁴³ at room temperature and pH 7, while disulfide exchange requires higher pH and temperatures.⁴⁴ The non-specific binding of penta-His antibody to a $C_{11}TA$ SAM control channel (C) exposed only to penta-His antibody and GFP (without removal of the acetyl group) resulted in relatively low fluorescence. The signal to background ratio of 4 (with $p < 1.39 \times 10^{-14}$) resembles the ratio of 2.6 (with $p < 5 \times 10^{-10}$) that was previously reported by Artel *et al.*³² on disulfide decorated quartz surfaces. This demonstrated successful *in-situ* microfluidic SAM deposition leading to an antibody bound to a disulfide reactive surface. Control channel (D) contained bare PDMS without

FIG. 2. GFP fluorescence intensity from binding to a surface conjugated antibody, compared to control experiments. (a) Schematic diagram of covalent conjugation of an antibody via a disulfide disulfide exchange reaction or disulfide/thiol interchange. GFP binding was used as a method of detection of efficiency of the antibody binding. (b) Fluorescent image of the device channels. $C_{11}TA$ SAM was deposited in all four channels. (A) After treatment with DBU, DTT, antibody, and GFP expression. (B) After treatment with DBU, antibody and GFP. (C) Anti penta-His binding to $C_{11}TA$. (D) After treatment with DBU followed by DTT reduction and GFP binding to the thiol surfaces. (c) Average fluorescence levels of the 4 channels of DBU treatments.

antibody for examination of non-specific binding of GFP to the thiol surface. This channel featured a very low baseline fluorescence, consistent with the expectation that protein does not bind to a thiol surface or to a bare PDMS surface.

Similarly, the cleavage of the thioacetate group was performed in 10% HCl rather than with DBU (Fig. 3(a)) and optical microscopy showed that there was no damage to the device. Figure 3(a) shows the fluorescence of channel (A) after exposure to HCl hydrolysis. Channel (B) was used for evaluation of the nonspecific binding of anti-penta-His and exposure to GFP without thiol deprotection C₁₁TA SAM. Low fluorescence indicated very little nonspecific binding. The ratio of A:B was 6 (with $p < 6.45 \times 10^{-7}$). GFP nonspecific binding to the surface after aqueous HCl treatment was examined via channel (C), which showed a very low baseline fluorescence, indicating that GFP does not bind to the partially hydrolyzed surface. Despite partial cleavage of the thioacetate group reported above for a silicon wafer surface, the fluorescence levels after HCl treatment were higher than the fluorescence levels obtained by cleavage with DBU. This could be due to high-density packing, which occurs with deprotection of all thiols and sterically blocks active sites of proteins, interfering with their functional properties.¹⁸ Thus, high-density may actually harm antibody function and result in lower GFP binding. The higher protein signal, despite the partial hydrolysis, suggests that a lower than maximal density is optimal for protein immobilization.

Selective protein mobilization within the D2P microarray

Once we optimized the surface chemistry protocol, we proceeded to test the application of this surface chemistry to microfluidic-based protein arrays. The goal of using the SAM surface chemistry is to eventually include the process of surface functionalization of the device in the fabrication stage of the device, replacing the time consuming step of surface functionalization at the beginning of each protein array experiment. This will significantly shorten and simplify the experimental procedure of these microfluidic arrays. To this end, we activated a D2P device with 1024 chambers and tested GFP immobilization on the surface of the chambers. In order to prevent anti-GFP conjugation outside of the reaction chamber, we blocked the other areas of the device with a non-specific fluorescent alexa 647-labelled antibody. Protein immobilization was performed using the button valve, under which an anti GFP antibody was attached. This enabled us to visualize both the protein immobilization and background at two different wavelengths.

We tested the activity of the surface chemistry using GFP in two circumstances. The first involved immobilization of GFP directly after creating the anti GFP surfaces using the SAMs. The second involved preparation of similar devices, their subsequent drying with argon, and then storage for a week before reactivating them with fresh buffer and immobilizing GFP. The results demonstrated that we can use the SAM surface chemistry to specifically immobilize

FIG. 3. GFP fluorescence after binding to the SAM. (a) Fluorescent image of the device channels containing C₁₁TA SAM. (A) Fluorescence following thioacetate hydrolysis with aqueous 10% HCl followed by exposure to GFP. (B) Fluorescence after non-specific antibody binding to C₁₁TA (without thiol deprotection) and exposure to GFP. (C) After thiol hydrolysis using aqueous HCl 10%, without antibody, followed by exposure of GFP to the hydrolyzed surface. (b) Average fluorescence intensity measurements of the 3 channels of aqueous HCl 10% treatment.

FIG. 4. Fluorescence of GFP binding to freshly-prepared surface-conjugated antibody compared to after dry storage and antibody reactivation. (a) Image from the D2P device after GFP binding, observed as white spots at 488 nm. (b) Background signals of the non-specific alexa 647 antibody at 635 nm (c) GFP binding to 1-week-old antibody, observed as white spots at 488 nm. (d) Background signal bound to non-specific sites after 1 week. (e) Distribution of immobilized GFP signals for the fresh (blue) and one week old (red) devices. The average GFP signal for the fresh surface chemistry was 2739 ± 138 a.u. and for the one week old surface chemistry 3259 ± 139 a.u. The latter was dried with argon and stored on a shelf at room temperature. Each experiment was repeated at least twice. (f) Enlarged image of part of the microfluidic device. The green area shows the experimental chamber, while the red circle shows the area of the button valve used to isolate part of the experimental chamber.

GFP proteins in 1024 chambers in parallel. We observed a high GFP signal under the buttons, where the surface was activated with anti-GFP antibody (Figs. 4(a) and 4(c)). Concurrently, we observed a high background (alexa 647) signal around the button and a low background signal under the buttons, indicating that the areas not intended for GFP binding were well blocked (Figs. 4(b) and 4(d)). There was an increase in activity with the one week old surface antibody compared to the freshly immobilized antibody by about 15%, which we attribute to inter experiment variability (Fig. 4(e)). This clearly demonstrated that we can create devices for “off-the-shelf” use and transfer the surface modification procedure to the fabrication stage of the device. This allows for a significant reduction in the time to achieve a protein array, depending only on the protein immobilization step (or in cases of D2P also on the on-chip protein synthesis).

CONCLUSIONS

We have successfully achieved *in-situ* microfluidic device surface modification with a C_{11} TA SAM as a model system. We have also shown that this SAM can be used to create a reactive thiol/disulfide surface. The *in situ* introduction of these surface chemistries within integrated microfluidic devices opens the field of microfluidics to new applications by allowing

researchers to utilize integrated PDMS microfluidics for conducting multiple chemical reactions simultaneously. This also allows for multiple solid-phase chemical reactions to be conducted within a single microfluidic device instead of using multiple substrates. We further demonstrated that the application of this method to microfluidic-based protein arrays allows elimination of the entire surface functionalization process from the protein array experiment and its transfer to the fabrication of the microfluidic chip.

To date, the creation of surface chemistry for binding proteins within microfluidic devices has demanded multi-step processes for the binding of biological molecules (antibodies) so as to build a surface suited for the expression of the target protein.²⁵ Throughout most of the processes involved, the active surface is sensitive and unstable, largely due to interactions among the immobilized proteins.³ The majority of surface chemistries are based on biotin-streptavidin that use non-covalent protein-protein interactions. These are very strong interactions with long off-rates but are unstable under conditions that unfold the protein (i.e., high salt, temperature, or drying). These surface chemistries therefore require fresh preparation before every experiment. The work presented herein demonstrated a protein immobilization assay using SAM modified surfaces. Such assays have several key advantages over the existing Biotin/avidin based assays that use epoxide surface chemistry.^{25,42} In our system, the antibody is bound to the surface covalently, as opposed to the existing biotin/avidin-based assay chemistry. This covalent binding allows for drying the antibody-bearing surface in a way that the antibody can refold and regain its activity for protein conjugation after rehydration. The stability of the covalent binding along with the ability to reactivate the biomolecules (even after extended storage) argues in favor of SAM immobilization being part of the device fabrication process. The integrated SAMs resulted in a proof of concept for devices with off-the-shelf surface chemistry, with functional groups readily available for bioactivation. The prefabricated surface chemistry drastically shortened the actual experimental time, since the most time-consuming step in the current protein array assays is the preliminary creation of the surface chemistry. We demonstrated formation of a SAM with C₁₁TA. However, SAMs offer a broad range of chemistries that can be tailored to different experimental requirements. Finally, and on a broader note, we believe that integrated microfluidic devices with readily available surface chemistry will be a significant step towards disseminating this important technology into the scientific community and exploring other potential applications, such as single molecule experiments that are highly dependent on surface chemistry and are significantly affected by an uneven surface.

ACKNOWLEDGMENTS

C.N.S. gratefully acknowledges the support of the Edward and Judith Steinberg Chair in Nanotechnology. D.G. gratefully acknowledges the support of the European Research Council (ERC) 309600 and Israel Science Foundation (ISF) 715/11. We thank Michal Pellach for critical reading and editing of the manuscript.

¹T. Thorsen, S. J. Maerkl, and S. R. Quake, *Science* **298**, 580–584 (2002).

²P. M. Fordyce, D. Gerber, D. Tran, J. Zheng, H. Li, J. L. DeRisi, and S. R. Quake, *Nat. Biotechnol.* **28**, 970–975 (2010).

³D. Gerber, S. J. Maerkl, and S. R. Quake, *Nat. Methods* **6**, 71–74 (2009).

⁴S. R. Bates and S. R. Quake, *Appl. Phys. Lett.* **95**, 073705 (2009).

⁵I. Braslavsky, B. Hebert, E. Kartalov, and S. R. Quake, *Proc. Natl. Acad. Sci. U. S. A.* **100**, 3960–3964 (2003).

⁶J. F. Zhong, Y. Chen, J. S. Marcus, A. Scherer, S. R. Quake, C. R. Taylor, and L. P. Weiner, *Lab Chip* **8**, 68–74 (2008).

⁷L. Chung-Cheng, G. Sui, E. Arkadij, J. S. Chengyi *et al.*, *Science* **310**, 1793–1796 (2005).

⁸Y. A. Ben-Ari, Y. Glick, S. Kipper, N. Schwartz, D. Avrahami, E. Barbiro-Michaely, and D. Gerber, *Lab Chip* **13**, 2202–2209 (2013).

⁹N. Drayman, Y. Glick, O. Ben-nun-Shaul, H. Zer, A. Zlotnick, D. Gerber, O. Schueler-Furman, and A. Oppenheim, *Cell Host Microbe* **14**, 63–73 (2013).

¹⁰D. C. Duffy, J. C. McDonald, O. J. A. Schueller, and G. M. Whitesides, *Anal. Chem.* **70**, 4974–4984 (1998).

¹¹J. R. Anderson, D. T. Chiu, R. J. Jackman, O. Cherniavskaya, J. C. McDonald, H. Wu, S. H. Whitesides, and G. M. Whitesides, *Anal. Chem.* **72**, 3158–3164 (2000).

¹²G. M. Whitesides, *Nature* **442**, 368–373 (2006).

¹³J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. A. Schueller, and G. M. Whitesides, *Electrophoresis* **21**, 27–40 (2000).

¹⁴S. Neethirajan, I. Kobayashi, M. Nakajima, D. Wu, S. Nandagopal, and F. Lin, *Lab Chip* **11**, 1574–1586 (2011).

¹⁵M. Meier, R. Sit, W. Pan, and S. R. Quake, *Anal. Chem.* **84**, 9572–9578 (2012).

- ¹⁶J. C. McDonald and G. M. Whitesides, *Acc. Chem. Res.* **35**, 491–499 (2002).
- ¹⁷Y. W. Lee, J. Reed-Mundell, J. E. Zull, and C. N. Sukenik, *Langmuir* **9**, 3009–3014 (1993).
- ¹⁸F. Rusmini, Z. Zhong, and J. Feijen, *Biomacromolecules* **8**, 1775–1789 (2007).
- ¹⁹D. Burshtain and D. Mandler, *Phys. Chem. Chem. Phys.* **8**, 158–164 (2006).
- ²⁰C. D. Bain and G. M. Whitesides, *Ang. Chem. Int. Ed. Eng.* **28**, 506–512 (1989).
- ²¹M. Schaeferling, S. Schiller, H. Paul, M. Kruschina, P. Pavlickova, M. Meerkamp, C. Giammasi, and D. Kambhampati, *Electrophoresis* **23**, 3097–3105 (2002).
- ²²J. H. Fendler, *Chem. Mater.* **8**, 1616–1624 (1996).
- ²³J. J. Gooding and S. Ciampi, *Chem. Soc. Rev.* **40**, 2704–2718 (2011).
- ²⁴E. Katz and I. Willner, *Electroanalysis* **15**, 913–947 (2003).
- ²⁵M. Ronen, D. Avrahami, and D. Gerber, *Lab Chip* **14**, 2354–2362 (2014).
- ²⁶D. Kim and A. E. Herr, *Biomicrofluidics* **7**, 041501-1–041501-47 (2013).
- ²⁷M. Phaner-Goutorbe, V. Dugas, Y. Chevolot, and E. Souteyrand, *Mater. Sci. Eng., C* **31**, 384–390 (2011).
- ²⁸L. Netzer and J. Sagiv, *J. Am. Chem. Soc.* **105**, 674–676 (1983).
- ²⁹A. Ulman, *Chem. Rev.* **96**, 1533–1554 (1996).
- ³⁰S. K. Sia and G. M. Whitesides, *Electrophoresis* **24**, 3563–3576 (2003).
- ³¹S. R. Wasserman, H. Biebuyck, and G. M. Whitesides, *J. Mater. Res.* **4**, 886–892 (1989).
- ³²V. Artel, R. Cohen, I. Aped, M. Ronen, D. Gerber, and C. N. Sukenik, *Langmuir* **29**, 191–198 (2013).
- ³³J. Escorihuela, M.-J. Bañuls, S. Grijalvo, R. Eritja, R. Puchades, and Á. Maquieira, *Bioconjugate Chem.* **25**, 618–627 (2014).
- ³⁴N. Balachander and C. N. Sukenik, *Langmuir* **6**, 1621–1627 (1990).
- ³⁵A. Singh, D. H. Dahanayaka, A. Biswas, L. A. Bumm, and R. L. Halterman, *Langmuir* **26**, 13221–13226 (2010).
- ³⁶O. Gershevit, A. Osnis, and C. N. Sukenik, *Israel J. Chem.* **45**, 321–336 (2005).
- ³⁷I. Aped, Y. Mazuz, and C. N. Sukenik, *Beilstein J. Nanotechnol.* **3**, 213–220 (2012).
- ³⁸R. Banga, J. Yarwood, and A. M. Morgan, *Langmuir* **11**, 618–622 (1995).
- ³⁹M. E. McGovern, K. M. R. Kallury, and M. Thompson, *Langmuir* **10**, 3607–3614 (1994).
- ⁴⁰L. Cai, Y. Yao, J. Yang, D. W. Price, and J. M. Tour, *Chem. Mater.* **14**, 2905–2909 (2002).
- ⁴¹W. W. Cleland, *Biochemistry* **3**, 480–482 (1964).
- ⁴²Y. Glick, D. Avrahami, E. Michaely, and D. Gerber, *J. Vis. Exp.* **66**, e3849 (2012).
- ⁴³R. Singh and G. M. Whitesides, in *The Chemistry of Sulphur-Containing Functional Groups*, edited by S. Patai and Z. Rappoport (John Wiley & Sons Ltd, 1993), Chap. 13.
- ⁴⁴G. Kräuter, Y. Bluhm, Ch. Batz, and U. Gösele, *Adv. Mater.* **11**, 1035–1038 (1999).
- ⁴⁵See supplementary material at <http://dx.doi.org/10.1063/1.4930982> for additional information on device architecture and the effects of moisture on the silanization process.