

***In situ* oligonucleotide synthesis on poly(dimethylsiloxane): a flexible substrate for microarray fabrication**

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ABSTRACT

In this paper, we demonstrate *in situ* synthesis of oligonucleotide probes on poly(dimethylsiloxane) (PDMS) microchannels through use of conventional phosphoramidite chemistry. PDMS polymer was moulded into a series of microchannels using standard soft lithography (micro-moulding), with dimensions <100 µm. The surface of the PDMS was derivatized by exposure to ultraviolet/ozone followed by vapour phase deposition of glycidoxypropyltrimethoxysilane and reaction with poly(ethylene glycol) spacer, resulting in a reactive surface for oligonucleotide coupling. High, reproducible yields were achieved for both 6mer and 21mer probes as assessed by hybridization to fluorescent oligonucleotides. Oligonucleotide surface density was comparable with that obtained on glass substrates. These results suggest PDMS as a stable and flexible alternative to glass as a suitable substrate in the fabrication and synthesis of DNA microarrays.

INTRODUCTION

The uptake of DNA microarrays into the academic and commercial arena over the past 10 years has been rapid owing to their usefulness in high-throughput, parallel gene expression, single nucleotide polymorphism analysis and comparative genome hybridization (1–6). Further applications of this technology encompass topics such as optimization of antisense oligonucleotides (7), molecular hybridization studies (8,9), mutation identification via gene resequencing (10–12) and analysis of DNA–protein interactions (13). Microarrays are now widely available from a number of companies.

Microarrays are fabricated by spotting or ink-jetting cloned DNA or pre-synthesized oligonucleotides onto solid surfaces; or they are fabricated through a variety of *in situ* techniques including ink-jetting, photolithography and novel electrochemistry (14,15). In all cases, the oligonucleotide probes are synthesized using well-established phosphoramidite chemistry (16,17).

Poly(dimethylsiloxane) (PDMS) is a mouldable silicone rubber, which has attracted growing attention over the past decade as a material for device fabrication in the microfluidics and lab-on-a-chip markets (18). Acceptance by the scientific community has been largely due to the introduction of soft lithography (19) allowing the rapid production and prototyping of PDMS chips. In addition to its ease of manufacture, PDMS has several other advantages for application to microfluidic devices, such as low cost, high flexibility, low polymerization temperature, high optical transparency, ability to seal readily against many substrates and biological inertness (20).

The inert characteristics of PDMS stem from the unreactive and hydrophobic nature of its surface, formed from a $[-\text{OSi}(\text{CH}_3)_2-]_n$ backbone. Numerous reports describe modifications of the PDMS surface to enhance its chemical reactivity, the most common of which include oxygen plasma (21,22), corona discharges (23) and ultraviolet/ozone (UVO) (24), and have successfully led to the production of an oxidized, hydrophilic surface (25).

Until very recently, the use of PDMS in oligonucleotide synthesis was limited to micro-contact printing [i.e. using PDMS as a tool for stamping oligonucleotides onto glass surfaces (26,27)], but a recent work by Liu *et al.* (28) has briefly introduced the possibility of using PDMS as a substrate onto which pre-synthesized (*ex situ*) oligonucleotides are covalently attached. Given the prevalence of PDMS as a material for biological microfluidic devices, in this report we introduce a method for *in situ* oligonucleotide synthesis directly onto modified-PDMS surfaces using conventional phosphoramidite chemistry.

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MATERIALS AND METHODS

All reagents were purchased and used without further purification. Microfluidic channels were manufactured using the Dow Corning PDMS kit Sylgard 184 (Farnell, UK). Dow Corning primer OS 1200 (Farnell, UK) was used to improve the adhesion of PDMS to glass. Menzel-Gläser microscope slides were purchased from Fisher. Photolithographic reagents included SU8-25 (MicroChem Corp., Newton, MA), 1-methoxy-2-propanol acetate (Sigma-Aldrich) and S1818 photoresist (Shipley).

Surface treatment reagents, 3-glycidoxypropyltrimethoxysilane (GPTMS) and *N*-(3-propyltriethoxysilyl)-4-hydroxybutyramide (HBAPTES) were purchased from Fluorochem, poly(ethylene glycol) (PEG) (MW 200; Sigma-Aldrich), ethanol (95%; Fisher). Phosphoramidite and oligonucleotide synthesis reagents, 5'-dimethoxytrityl-*N*-benzoyl-2'-deoxyadenosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (dA), 5'-dimethoxytrityl-*N*-benzoyl-2'-deoxycytidine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (dC), 5'-dimethoxytrityl-*N*-isobutyryl-2'-deoxyguanosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (dG) and 5'-dimethoxytrityl-2'-deoxythymidine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (dT), acetonitrile and tetrazole activator (all Transgenomic), iodine (Sigma-Aldrich), acetic acid (Fisher), pyridine (anhydrous; Fisher) and trifluoroacetic acid (TFA) (Sigma-Aldrich).

For hybridization experiments, aminoethanol, polyvinylpyrrolidone, BSA pentax fraction V, Tris-HCl, EDTA and SDS were all obtained from Sigma-Aldrich. KCl, MgCl₂, ficoll (400) and STE buffer were purchased from Fluka. 5'-GCGTAGATGAGGGGTCGATGATGGCATTGCAGA-3' and 5'-GGCCACAAGTATCACTAAGC-3' custom oligonucleotide targets were synthesized at 1.0 μm scale, high-performance liquid chromatography (HPLC) purified and supplied 5'- and 3'-Cy5-labelled, respectively (Eurogentec, UK). All aqueous solutions were prepared with HPLC grade water (Sigma-Aldrich).

Mould preparation

Mask design. The layout for the microstructures was designed in a computer-aided design program and reproduced in chrome on glass by DeltaMask (The Netherlands). This chrome mask was used for the photolithographic definition of SU8 photoresist. The microfluidic design comprises 64 channels, each 80 μm wide, branching symmetrically from central inlet and outlet channels. Blocked channels, occurring every fourth channel (achieved by plugging the channel), act as a reference for gauging background fluorescence. As no reagents enter these channels, any resulting fluorescence must be either intrinsic to the material or due to non-specifically adsorbed target.

SU-8 Photolithography. A 100 mm <100> *n*-type silicon wafer with 900 nm of thermally grown oxide was dehydration baked for 15 min at 110°C on a hotplate (Model 1000-1, Electronic Micro Systems Limited). An aliquot of 10 ml of SU8-25 was spun onto the wafer using a commercial spinner (Model 4000, Electronic Micro Systems Limited) and a two-step spin process to a thickness of 50 μm. The wafer was baked at 65°C for 3 min and then at 95°C for 15 min. After cooling, the wafer was exposed through a chrome-on-glass mask to 10 mW/cm²

of broadband UV light for 30 s using a mask aligner (Quintel Model Q4000), then post-exposure baked at 65°C for 1 min followed by 95°C for 4 min.

The wafer was subsequently developed for 5 min using two baths of 1-methoxy-2-propanol acetate, each wafer submerged for 2.5 min per bath and agitated, followed by rinsing in propan-2-ol before blow drying with dry nitrogen. Post-baking was performed at 150°C for 10 min and then protected using a spun layer of S1818 photoresist, baked at 110°C for 1 min. The wafer was then diced using a MicroAce Series 3 wafer saw (Loadpoint, Swindon, UK) with a S1025 diamond saw blade. The moulds were manually separated and the photoresist protection layer removed by rinsing first in acetone and then in propan-2-ol before blow drying with dry nitrogen. After separation the moulds were inspected by eye for any major defects or gross damage.

PDMS channel preparation

The procedure for the fabrication of PDMS structures, using soft lithography, has been described previously (19). Briefly, PDMS was prepared by mixing base polymer and curing agent with a ratio of 10:1, followed by a degassing step under reduced pressure for 30 min. A small amount of this pre-polymer mixture was poured onto both an SU8 mould and a pre-treated microscope slide, acting as the support. Pre-treatment of the microscope slide was carried out using an adhesion promoter (Dow Corning OS1200).

Moulds were supported by a strip of 5-mm-thick polycarbonate in order to improve their durability in addition to ease of handling. The overall thickness of the cast, which was minimized in order to avoid swelling under the influence of solvents, was controlled by having the mould supported by strips of Kapton tape on either side of the mould (Figure 1). This resulted in an overall thickness of ~180 μm.

The microscope slide and mould were brought into contact, sandwiching a film of PDMS and held firmly in place while the PDMS was allowed to cure. Curing was done overnight at room temperature followed by a final cure at 70°C for 1 h. On completion, the mould was gently lifted off the cast and the strips of Kapton tape were removed prior to use.

PDMS surface derivatization

The PDMS microchannels were initially oxidized through exposure to UVO through use of a UVO-Cleaner Model 42

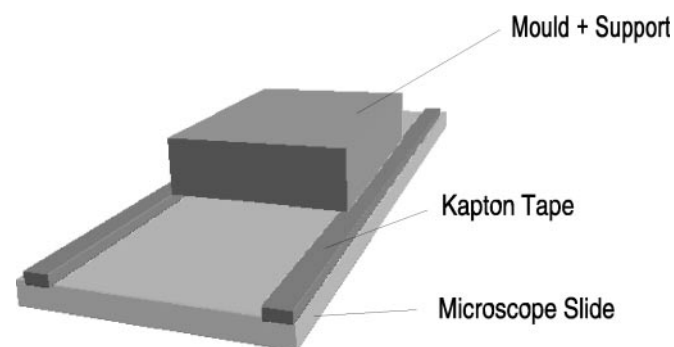


Figure 1. Schematic diagram showing casting procedure for PDMS microchannels using SU8 micro-moulding.

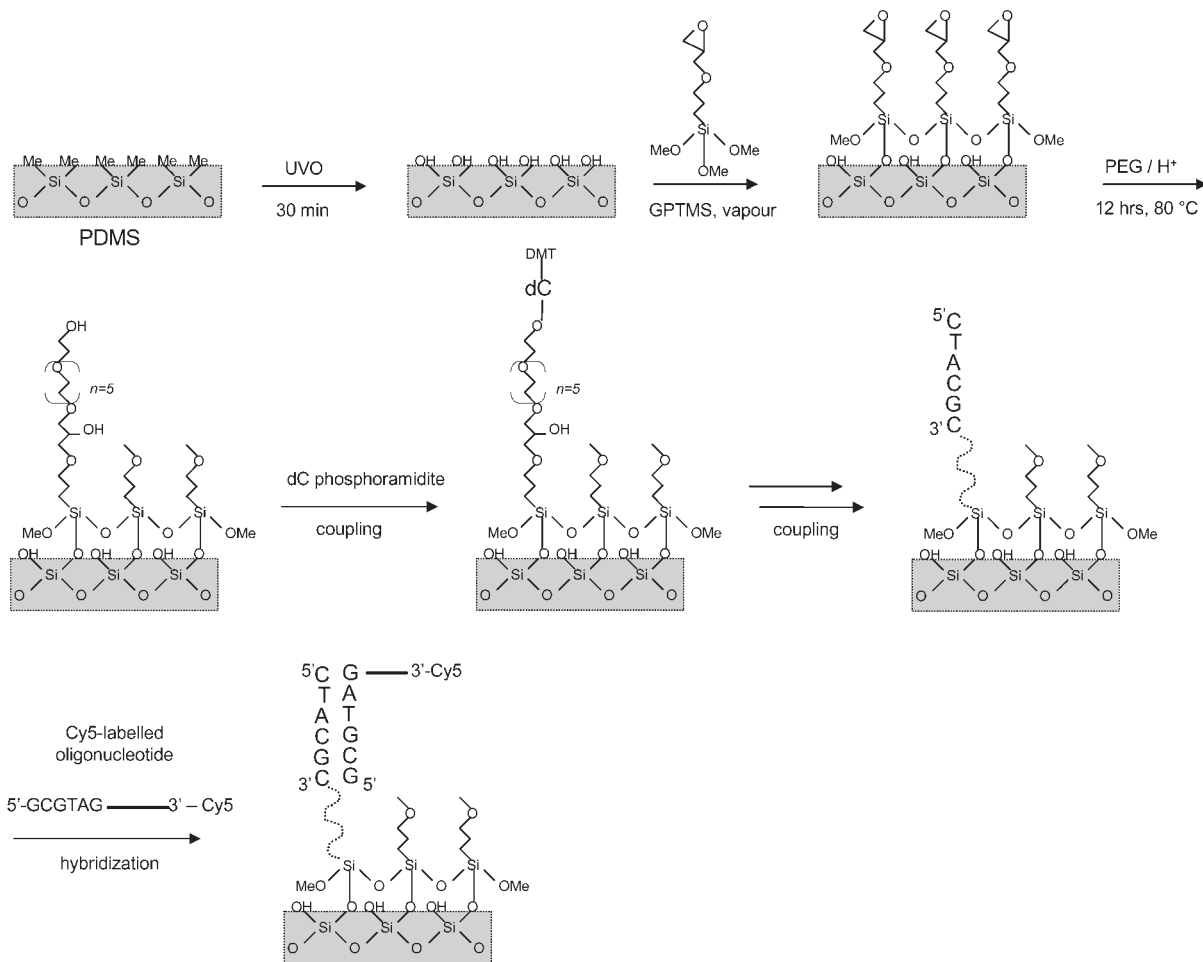


Figure 2. Overview of derivatization, silanization and DNA attachment chemistry on PDMS substrates.

(Jelight Company Inc., CA) for 30 min at a lamp–substrate separation of 5 mm. Data from X-ray photoelectron spectroscopy (XPS) and contact angle imaging (data not shown) verified a significant change in PDMS surface energy and atomic content in accordance with the reported literature (M. J. Moorcroft and A. Crossley, unpublished data) (23,24,29,30) and is consistent with a significant increase in silanol (SiOH) density.

Vapour-phase silanization was performed in a vacuum furnace (Instron SFL, UK) by introducing 5 ml of GPTMS into the ampoule alongside the oxidized-PDMS substrate. The furnace was pre-programmed with a 10 h cycle which evacuated the chamber and heated the oven to 10 mbar pressure and 175°C, respectively. On completion, the PDMS substrates were removed and the ampoule checked to ensure successful evaporation of the silane. In contrast, solution-phase silanization was performed through immersion of the oxidized-PDMS substrate into 100 ml of 1% (v/v) HBAPTES in ethanol and gently agitated for 15 min. After rinsing, the substrates were cured in an oven for 5 min at 100°C.

Subsequent derivatization of the silane with PEG spacer was performed as reported previously (31). Silanated-PDMS substrates were placed into a glass Petri dish containing ~20 ml of PEG with a few drops of concentrated H₂SO₄

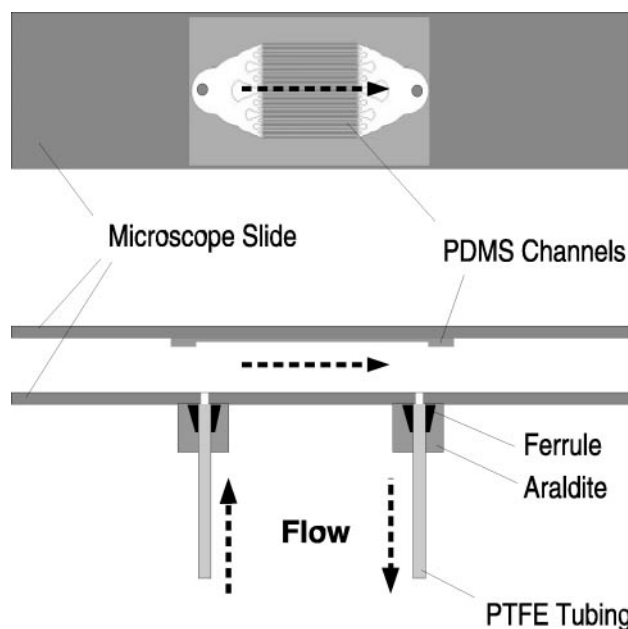


Figure 3. Schematic diagrams showing side and top views of fluidic manifold, designed to allow DNA reagents to flow through PDMS channels.

catalyst. The slides were heated overnight in an oven at 80°C, rinsed with methanol to remove excess PEG and stored in a desiccator before use. A simplified schematic diagram of the derivatization chemistry is presented in Figure 2.

Reagent/fluidic manifold

Fluidic connection between a DNA synthesizer (Applied Biosystems 394 DNA/RNA Synthesizer) and the PDMS channel structure was achieved through a second microscope slide with inlet holes and fluidic couplers attached (Figure 3). The PDMS channels were aligned with the inlet holes and the two sides

were clamped together. As PDMS seals reversibly onto glass, the fluidic header was reusable.

Oligonucleotide synthesis

Synthesis of oligonucleotides was achieved using a standard Applied Biosystems 392 DNA/RNA Synthesizer. A slight modification of the pre-programmed cycles was required to incorporate extra rinsing and purging to ensure complete clearance of microchannels between reagents.

It was observed in soaking tests that PDMS swelled in some of the solvents frequently employed in oligonucleotide

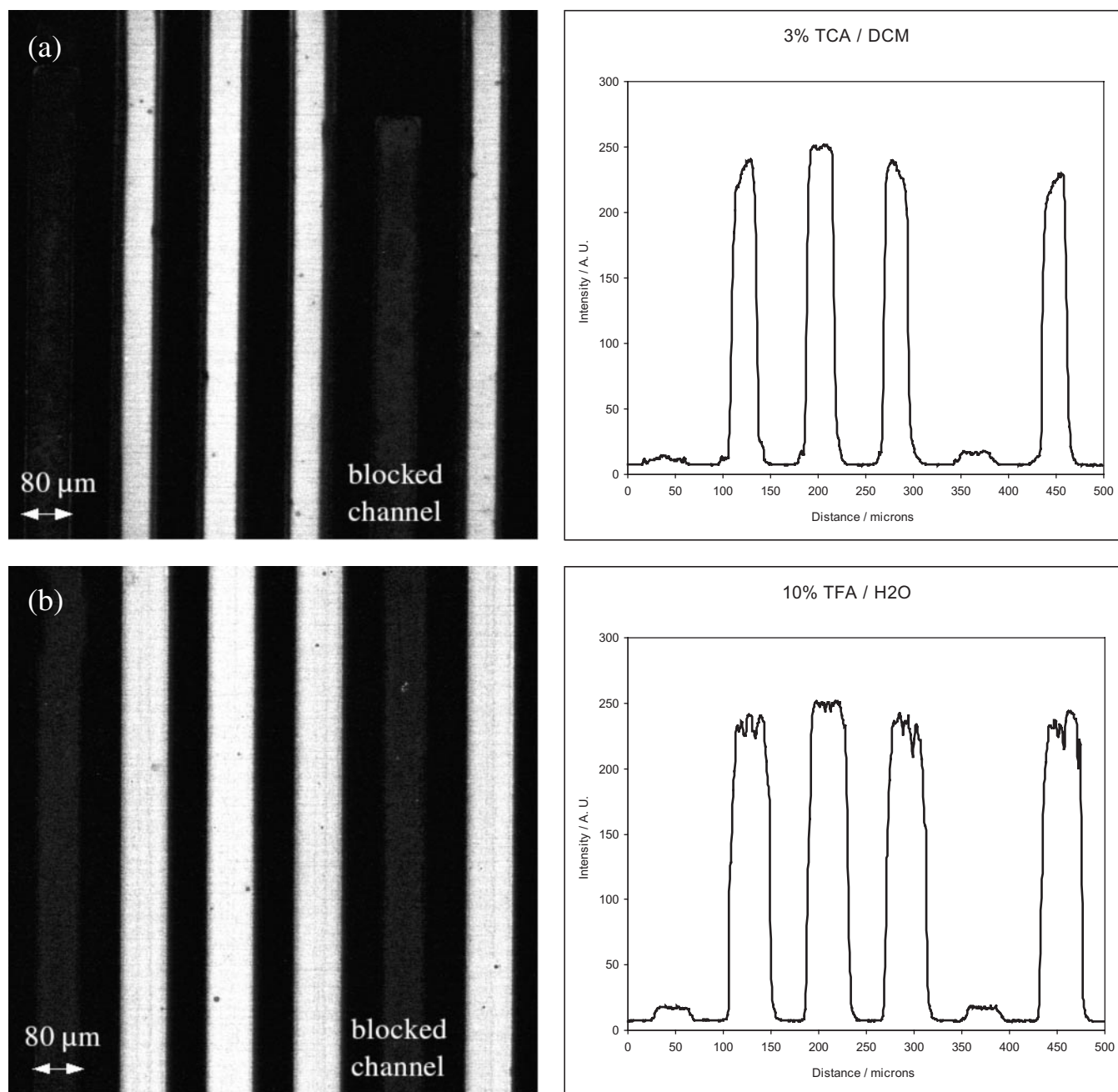


Figure 4. Confocal images with corresponding intensity plots showing synthesis/hybridization of 6mer oligonucleotides (5'-CTACGC) on PEG/GPTMS/PDMS microchannels using (a) 3% TCA/DCM and (b) 10% TFA/H₂O deblocking reagents. The blocked channels provide a control surface for the background fluorescence signal. The resulting stripes in (a) are thinner due to channel swelling occurring during synthesis.

synthesis. Swelling in tetrahydrofuran (oxidizer) and dichloromethane (DCM) (deblock) solvents, in agreement with the published data (32), was found to be too great to permit their use while acetonitrile and water were found to cause negligible swelling. Alternative solvents, therefore, were substituted for the oxidation and deblocking steps. For the oxidation reagent, a solution containing 0.1 M iodine dissolved in 9:1 pyridine/acetic acid (v/v) was prepared (33), while the deblocking reagent comprised an aqueous solution of 10% (w/w) TFA.

Hybridization and fluorescence analysis

Oligonucleotides were deprotected according to the method of Polushin *et al.* (34) by immersion of the entire PDMS substrate into a 50% ethanolamine, 50% ethanol solution for 25 min at 60°C immediately after synthesis. On removal, the substrate was rinsed thoroughly with ethanol and dried with nitrogen.

For 6mer experiments, 1 μM of the 5'-Cy5-GCGTAGATG-AGGGGGTCGATGATGGCATTGCAGA-3' target was prepared in hybridization buffer [0.1% (w/v) ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% BSA pentax fraction V, 20 mM Tris-HCl, 50 mM KCl, 10 mM MgCl₂ and 1 mM EDTA, pH 8.3]. This target-probe pair was used because of the stringent requirement of pairing all 6 nt of the probe, 5'-CTACGC, for successful hybridization (35). For 21mer experiments, 1 μM of the 5'-GGCCACAAGTATCACTAAGC-Cy5-3' target was prepared in STE-SDS buffer [100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA and 0.01% (w/w) SDS, pH 7.8] for hybridization against a 5'-GCTTAGT-GATACTTGTGGGCC-3' as probe. STE buffer was purchased from Sigma-Aldrich and the SDS added on receipt. Both oligonucleotide targets were prepared and stored at -20°C in the dark before use. In initial experiments, 6mer probes were synthesized on top of TAGT to act as a steric spacer; in later experiments the 6mer was synthesized on a PEG spacer. In all cases, hybridization reactions were carried out away from sunlight at 25°C for 30 min, by introducing 100 μl of the solution onto the substrate and ensuring removal of any trapped air bubbles in the channel area. After hybridization, thorough rinsing with hybridization buffer ensured removal of non-specifically bound oligonucleotide target.

Hybridization of fluorescent targets was analysed by a Leica TCS NT confocal microscope (Leica Microsystems Ltd, UK) with 2 μm pinhole aperture, in photon counting mode, immediately after hybridization. For these experiments, only qualitative observations were sufficient to observe the results, so no effort was made to standardize the PMT signal. Both the 10 \times and 5 \times objectives were used and laser power was kept low to allow replicate measurements without excess photo-bleaching.

RESULTS AND DISCUSSION

Optimization of synthesis reagents

Oligonucleotide probes of 5'-CTACGC were synthesized using conventional phosphoramidite chemistry via an ABI 392 instrument on PEG/GPTMS-treated PDMS channels using both DCM- and aqueous-based deblocking reagents as outlined in Materials and Methods.

Hybridization to a fluorescently labelled complement (Figure 4) shows an extremely intense signal throughout the channels. The sharp edges of the bands indicate a good degree of sealing between the PDMS surface and the silicon support. Although both solvents appear to give near-quantitative detritylation, the use of DCM solvent was ruled out as it produced unacceptable channel narrowing (or swelling) (Figure 4a) which ultimately led to complete blockage. Aqueous TFA, on the other hand, resulted in little or no swelling and exposure was easily varied to achieve optimal detritylation with acceptable levels of depurination even up to 21mer synthesis (see Synthesis of 21mer probes subsection). In order to validate that there was some specificity of the Cy5-labelled target to the probe and as a control for non-specific binding, an oligo-A₆ was synthesized. After hybridization to the same 5'-CTACGC target, the resulting fluorescence is shown in Figure 5.

Effect of surface treatment

A wide variety of surface treatments have been reported that can be used to prepare substrates for oligonucleotide synthesis, including a variety of silanes, choice of spacers, etc. (36). Here, we present a brief study of silanization and spacer effects that demonstrates the flexibility and the versatility of PDMS substrates with alternative surface treatments.

Previously, the use of PEG as a spacer has been reported to increase hybridization duplex yields through relieving steric interaction between the incoming target and the substrate surface (9,37). To study this effect on PDMS, 6mer 5'-CTACGC probes were synthesized in microchannels with and without pre-treatment by using PEG spacer.

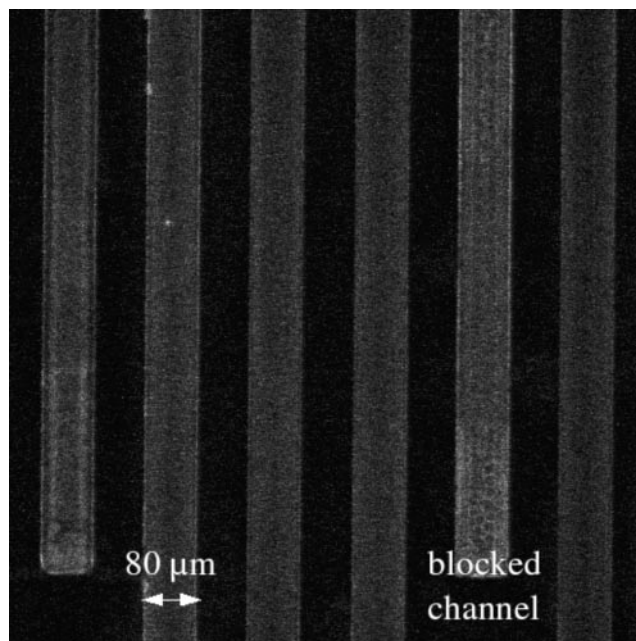


Figure 5. Demonstration of target/probe fidelity and lack of non-specific binding through synthesis of an oligo-A₆ (5'-AAAAAA) on PEG/GPTMS/PDMS followed by attempted hybridization to 5'-CTACGC target. Lack of differentiation between blocked and unblocked channels indicates non-hybridization.

It is shown in Figure 6 that the addition of a PEG spacer dramatically increases surface uniformity and fluorescence intensity, consistent with the conclusion that steric factors may play a significant role in limiting duplex yields.

A number of reports have shown differences in surface homogeneity resulting from solution- and vapour-phase silanization methods, which may be explained by the formation of multilayer and monolayer silane networks, respectively (38,39). To test this behaviour on PDMS, the surface was reacted with solution-phase HBAPTES and

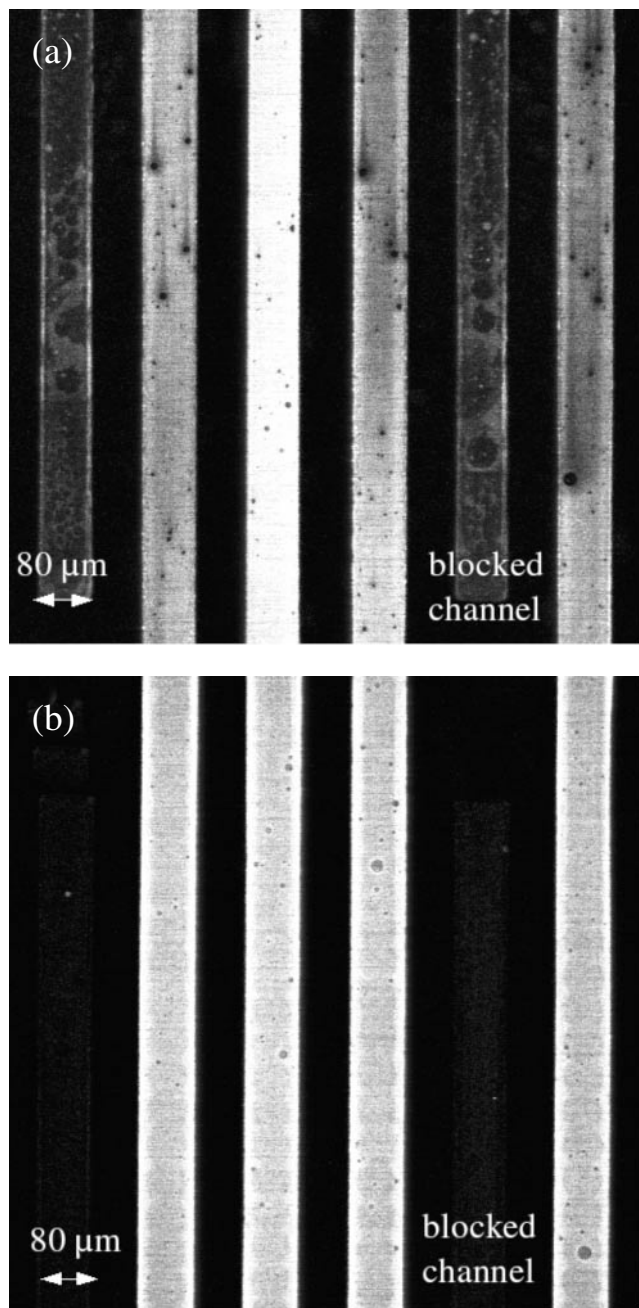


Figure 6. Confocal images showing the effect of (a) GPTMS only and (b) GPTMS with surface spacer, PEG, on the synthesis of 6mer oligonucleotides (5'-CTACGC) followed by hybridization. In (a), the epoxide groups on GPTMS were opened by washing with aqueous HCl before synthesis (31).

subsequently subjected to oligonucleotide synthesis as described above.

Figure 7 suggests the formation of a highly textured surface consistent with the suggestion of uncontrolled, multilayer silane polymerization in contrast to the homogenous, monolayer coverage achieved with vapour-phase methods, presented here (Figure 6) and elsewhere (38,39).

A number of studies show that oxidized-PDMS surfaces slowly undergo recovery from hydrophilic ($-OH$) to hydrophobic ($-CH_3$) properties via surface reorientation and migration of low molecular weight polymer from bulk (21,23,40–42). The speed of the recovery is related to the aggressiveness of oxidizing procedure, e.g. UVO versus plasma, but in general a measurable difference is usually noted within seven days. The effect of hydrophobic surface recovery of PDMS was monitored by observing its effect on oligonucleotide yield. Oligonucleotide probes of 5'-CTACGC were synthesized in PDMS microchannels that had been stored (i.e. allowed to 'recover') for varying lengths of time following treatment with UVO, GPTMS and PEG prior to synthesis. Fluorescence intensities following hybridization of aged samples show reasonable synthesis yields even after 60 days storage (Figure 8), suggesting that while there are still issues relating to longer-term storage, the hydrophobic recovery effect does not preclude oligonucleotide synthesis over shorter timescales. It seems likely that the recovery mechanisms common to derivatized-PDMS might be partially prevented through the anchorage of large, bulky silane/spacer moieties to the oxidized-PDMS surface.

Effect of substrate

Oligonucleotide synthesis on PDMS substrates was compared with synthesis on glass. By minor modification of the flow cell,

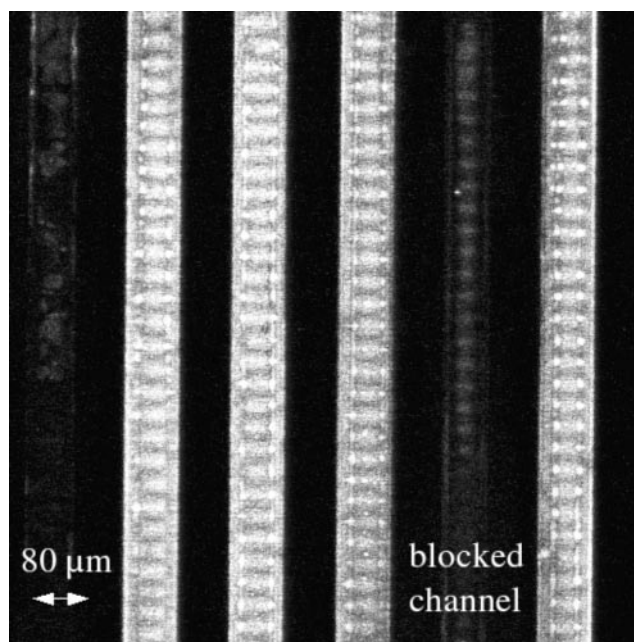


Figure 7. Confocal image following hybridization indicating non-uniform synthesis of 6mer oligonucleotides (5'-CTACGC) on HBAPTES deposited via solution-phase silanization.

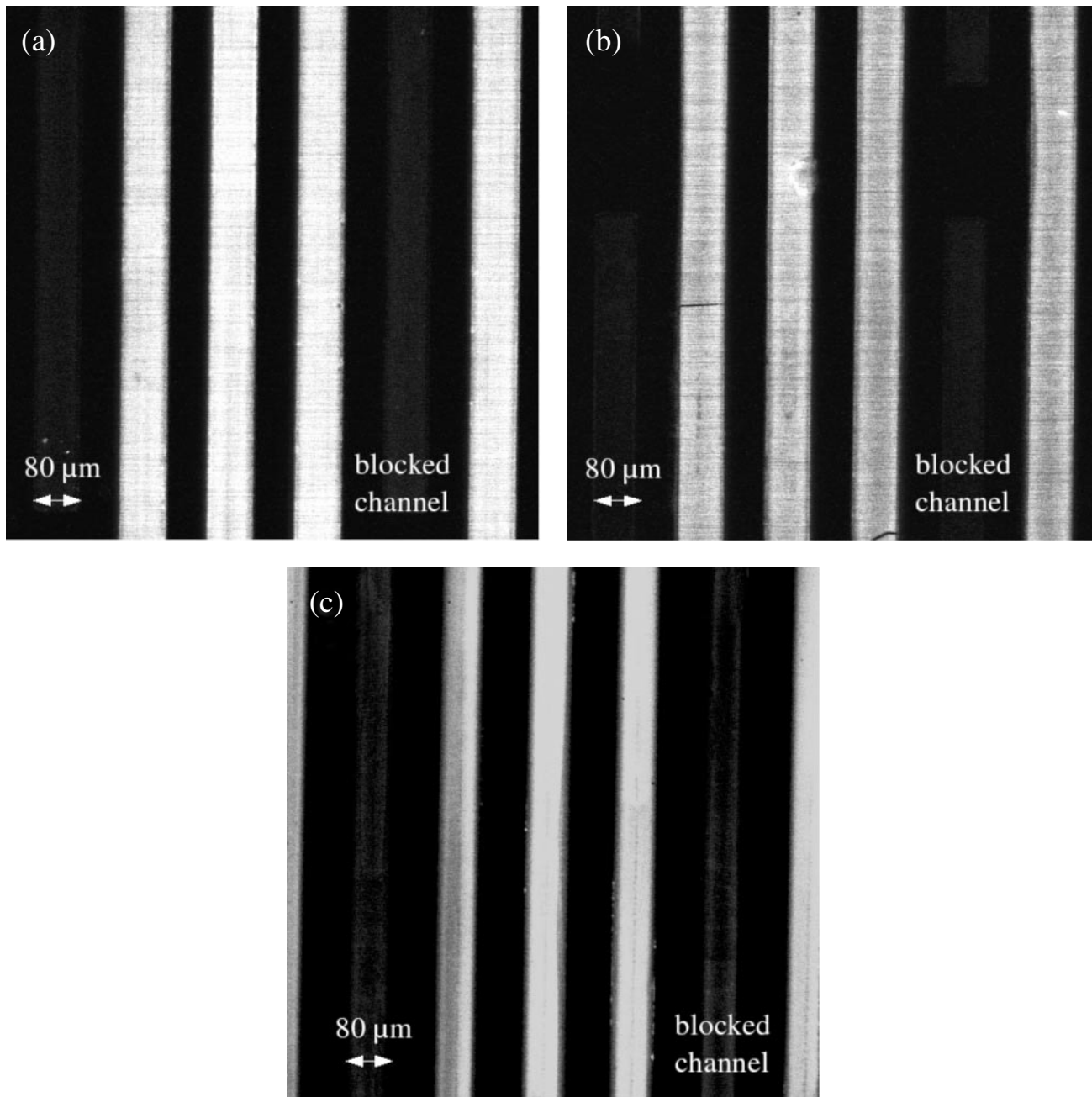


Figure 8. Influence of 'hydrophobic recovery' of derivatized-PDMS (PEG/GPTMS on UVO-treated PDMS) towards oligonucleotide synthesis of 6mer probes (5'-CTACGC) following (a) 7 days, (b) 14 days and (c) 60 days storage in a desiccator.

it was possible to use glass microscope slides as the synthesis substrate, as outlined in Materials and Methods. 5'-CTACGC probes were synthesized onto the glass surface, previously cleaned and treated with PEG/GPTMS; in this case PDMS was used only to provide flow channels for the oligonucleotide reagents.

From the fluorescence intensities (Figure 9), we conclude that the overall synthesis yields of PDMS and glass are similar within the limits of our measurement system. This is in accordance with the surface analysis of the derivatized-PDMS by XPS and contact angle data, which show chemical

composition and surface energies almost identical to those of glass (M. J. Moorcroft and A. Crossley, unpublished data) (23,24,29,30).

Synthesis of 21mer probes

Probe length was increased to incorporate the synthesis of 21mers in an attempt to estimate step-wise yields, validate alternate hybridization conditions and investigate longer-term sealing of PDMS during the course of extended reagent flow. 21mer 5'-GCTTAGTGATACTTGTGGGCC probes

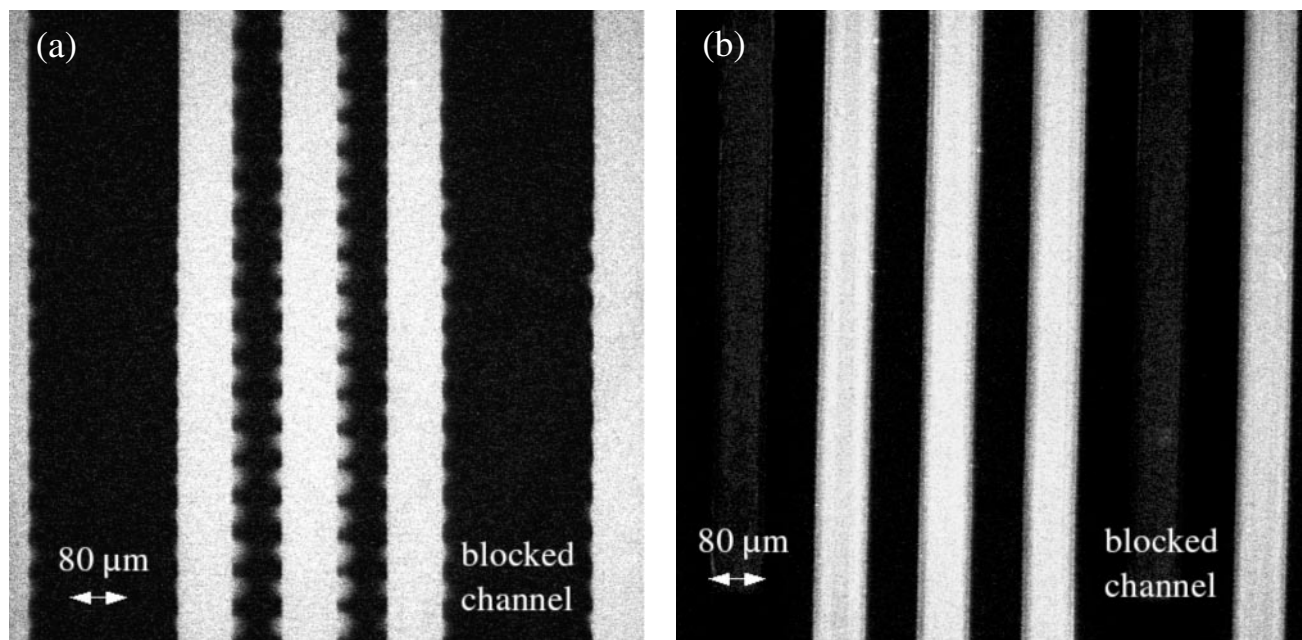


Figure 9. Qualitative comparison of confocal images of (a) glass versus (b) PDMS (both PEG/GPTMS) as a substrate for oligonucleotide synthesis of 5'-CTACGC following hybridization with complement target. The rippled effect in Figure 3.3.1 (a) is attributed to slight loss of sealing between the PDMS microchannels and glass microscope slides during synthesis.

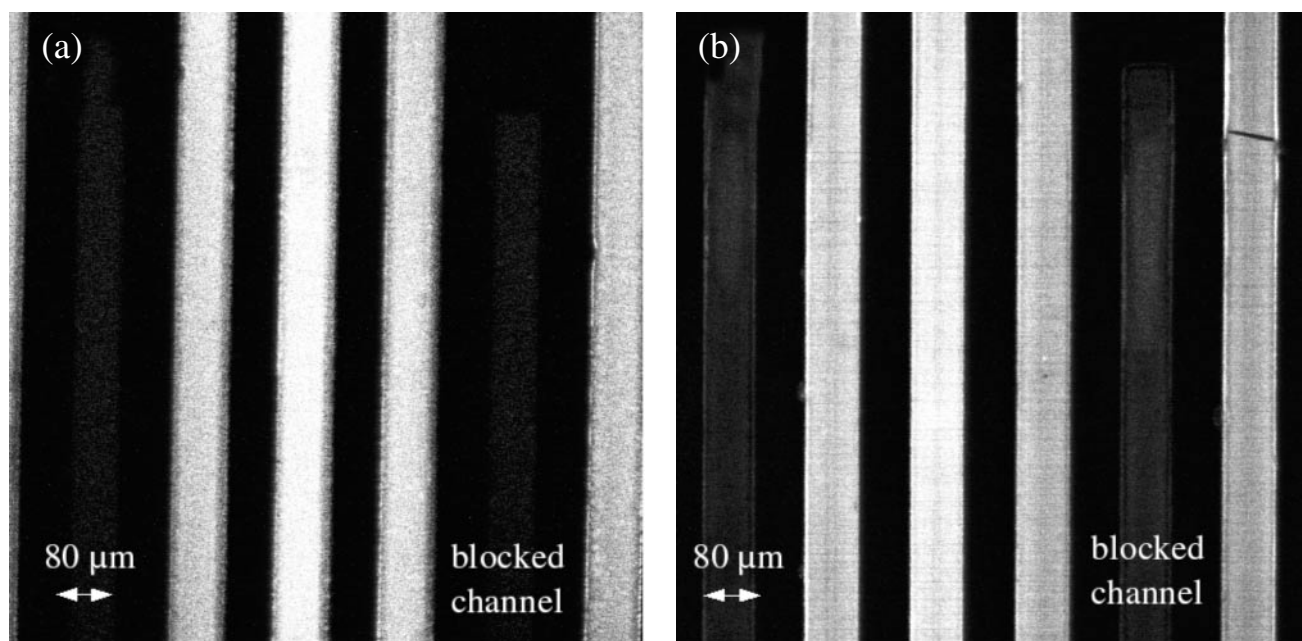


Figure 10. Synthesis and hybridization of (a) 21mer (5'-GCTTAGTGATACTGTGGGCC) and compared with (b) regular 6mer (5'-CTACGC) probes on PEG/GPTMS/PDMS microchannels after imaging under a confocal microscope.

were synthesized and hybridized against a different Cy5-target/buffer mixture as outlined in Materials and Methods. Fluorescence intensities were recorded and are presented in Figure 10.

Figure 10a shows evidence of successful 21mer probe synthesis in an alternative target/buffer system. These results suggest high step-wise coupling efficiencies in combination with excellent sealing/containment of fluids between the

PDMS and the silicon support over the course of 6mer and 21mer syntheses.

CONCLUSION

This paper outlines the design and fabrication of a simple, robust system to deliver oligonucleotide reagents down a

series of PDMS microchannels such that it can be readily coupled to a commercial DNA synthesizer. Following appropriate derivatization, it has been shown that successful *in situ* synthesis of up to 21mer probes on PDMS is possible, with yields and performance directly comparable with that obtained from conventional glass surfaces.

The effects of synthesis solvents, silanization, spacers, hydrophobic recovery and probe length on oligonucleotide yields were briefly investigated. Homogenous, high-yield probe densities were best obtained using PEG/GPTMS silane/linker on a UVO-oxidized PDMS surface. The main drawback of using PDMS is manifested in the need to chemically derivatize the inert polymer surface to allow oligonucleotide attachment, the main caveat of which is the concomitant change in its physical properties such as elasticity and, hence, sealing ability. While reported to be problematic in other applications, the effects of hydrophobic recovery on oligonucleotide synthesis yields are surprisingly not found to be prohibitive when studied over a 60 day period.

The work presented in this paper introduces the use of PDMS as a low-cost, stable and flexible alternative to solid glass substrates used in DNA microarray synthesis and provides a good example of the integration of a microfluidic device into a real-world analytical system. While this study only considers the use of one chemical system there is a scope for PDMS to be extended to encompass other solid-phase reactions such as peptide synthesis and other organic reactions.

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