DNA capture-probe based separation of double-stranded polymerase chain reaction amplification products in poly(dimethylsiloxane) microfluidic channels

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Herein, we describe the development of a novel primer system that allows for the capture of double-stranded polymerase chain reaction (PCR) amplification products onto a microfluidic channel without any preliminary purification stages. We show that specially designed PCR primers consisting of the main primer sequence and an additional “tag sequence” linked through a poly(ethylene glycol) molecule can be used to generate ds-PCR amplification products tailed with ss-oligonucleotides of two forensically relevant genes (amelogenin and human *c-fms* (macrophage colony-stimulating factor) proto-oncogene for the CSF-1 receptor (CSF1PO)). Furthermore, with a view to enriching and eluting the ds-PCR products of amplification on a capillary electrophoretic-based microfluidic device we describe the capture of the target ds-PCR products onto poly(dimethylsiloxane) microchannels modified with ss-oligonucleotide capture probes. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4729131>]

NOMENCLATURE

AMEL = amelogenin

APTES = 3-aminopropyltriethoxysilane

CE = capillary electrophoresis

CSFPO = *c-fms* (macrophage colony-stimulating factor) proto-oncogene

dNTP = 2'-deoxynucleotide triphosphate

ds = double-stranded

FAM = 6-carboxyfluorescein

PCR = polymerase chain reaction

PDITC = *p*-phenylene diisothiocyanate

PDMS = poly(dimethyl siloxane)

PEG = poly(ethylene glycol)

r.f.u. = relative fluorescent units

ss = single-stranded

STR = short tandem repeats

I. INTRODUCTION

Modern microfluidic polymerase chain reaction (PCR)/capillary electrophoretic (CE) devices, which include nanoscale architectures for analyte differentiation, offer a solution to simpler

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and faster front-end DNA profiling devices. These devices combined with analysis of short tandem repeats (STRs) will improve both forensic human identification and medical diagnostics, in particular genetic alteration linked with mutation in STR regions^{1–3} in terms of speed, cost, and accuracy of the results. Modern STR analysis systems^{4,5} are based on genome DNA amplification with subsequent electrophoretic separation/detection of multiple STR loci using complex and expensive CE devices. For accurate and sensitive detection of multiplex PCR amplification products (up to 16) a four-channel fluorescent detection system is commonly required.^{6,7} Furthermore, precise adjustment of all PCR amplification products is used to avoid any overlap in the common fluorescent detection channel. The development of primer systems with non-overlapping PCR amplification products is therefore imperative for any further advancement in this area. One such approach that will overcome these limitations is to selectively capture PCR amplification products with subsequent time-resolved controllable dehybridization/release. This approach gives more flexibility in primer design and construction, and means a system can be designed which only uses a single fluorescent dye for CE allele visualization.

To date there has been little literature describing the development of microfluidic CE systems which encompass a preliminary PCR product concentration/purification strategy.^{8–12} Many strategies are based on a common technique which involves both the concentration of PCR products and separation from unreacted primers, nucleotide triphosphates, and dyes. In these cases, linear or crosslinked acrylamide hydrogel plugs located between the sample and waste channels in a classic double T-junction CE microfluidic system¹³ are used. Typically, the hydrogel plugs are covalently modified with either short oligonucleotide capture probes^{9,14} or with streptavidin.^{11,15} Oligonucleotide affinity preconcentration/purification has been demonstrated for both synthetic single-stranded (ss) oligonucleotides and ss-products of sequencing reactions,^{8,14} as well as double-stranded (ds) PCR products.^{9,11} In the former case,¹⁴ a proof of concept was achieved with a fluorescently labelled complementary target strand. The authors¹⁴ also showed separation of a mixture of ss-oligonucleotides after stacking two hydrogel plugs with different capture probes.

The purification of ds-products of amplification has also been achieved by the reaction of ds-products with immobilized ss-capture probes via a helix invasion reaction,^{9,11} which involved the formation of a triple-stranded DNA structure through Hoogsteen base pairing.¹⁶ Sample cleanup based on a biotin/streptavidin affinity technique has also been performed.^{11,15} Here, streptavidin-modified acrylamide plugs were used to cleanup and preconcentrate products of multiplex amplification of 9 forensically relevant STRs¹¹ and human respiratory viruses.¹⁵ In each case, the PCR products to be purified were labelled with a biotin tag which later became trapped during electrophoresis through the streptavidin modified hydrogel plugs.¹⁵ Subsequent thermal denaturation of the DNA duplexes resulted in the release of fluorescently labelled complementary single strands, followed by analysis using CE. In spite of the high level of product concentration and increased sensitivity of CE, it was noted that acrylamide capture plugs were quite mobile and tended to expand in the microfluidic channel at increased temperature causing broadening of peaks.

An interesting approach has been demonstrated by Hauser *et al.*¹⁷ The authors applied chimeric primers consisting of single-stranded L-DNA tag linked to the 5' termini of standard primers (D-DNA) to perform enzymatic amplification of nucleic acids followed by microarray capture of the obtained products. However, to avoid competition of binding of any unreacted primers during hybridization of the L-DNA tagged PCR product on the microarray, the authors required the use of an additional PCR purification step.

To overcome the issues outlined above the work presented here aims to directly modify a poly(dimethylsiloxane) (PDMS) microfluidic channel surface *in situ* with synthetic ss-oligonucleotide capture probes. Our approach was first to silanize a plasma oxidized PDMS surface with 3-aminopropyltriethoxysilane (APTES) followed by the activation of the terminal amine moieties with *p*-phenylene diisothiocyanate (PDITC) and subsequent attachment of amino-modified ss-oligonucleotides via thiouretane bond formation. These ss-oligonucleotide capture probes were specially designed to capture novel amplification products of forensically relevant amelogenin (AMEL) and human *c-fms* (macrophage colony-stimulating factor) proto-oncogene for the CSF-1 receptor (CSF1PO) genes. An entirely new primer system was developed for such a system which

allowed for the hybridization of unpurified ds-PCR products with the capture probes immobilized on a PDMS surface.

II. METHODS

A. Microfluidic channel fabrication

PDMS microfluidic channels were fabricated according to standard soft-lithography techniques for rapid prototyping described in Ref. 18. A master mold of a standard Y-junction microfluidic device was prepared on a Si wafer (non-porous, polished Si (100) wafers (p^{++} type, boron doped, 0.0008 – 0.0012 Ω cm), Siltronic, France) with SU-8 2025 photoresist (MicroChem Corp., USA). The microfluidic channels in the master mold were 200 μ m wide and 40 μ m deep. A PDMS replica was made using a Sylgard 184 silicone elastomer kit from Dow Corning, USA by pouring a mixture of PDMS base and curing agent in a ratio 10:1 (w/w) onto the master mold. The entire assembly was then baked in an oven for 30 min at 120 °C. After peeling off the DMS replica from the master mold this, and a flat sheet of PDMS, were oxygen plasma treated at 0.2 Torr of O_2 , 18 W power for 45 s (PDC-32G-2 Plasma Cleaner, Harrick Plasma, USA)). Irreversible bonding and sealing of the device were then achieved by pressing each surface against each other for 5 min at room temperature.

B. Microfluidic channel surface immobilization with ss-oligonucleotides capture probes

APTES, ethanol (96%), PDITC, pyridine, dimethylformamide (DMF), and ethanolamine were all purchased from Sigma-Aldrich, Australia and used as received. Amine-modified ss-oligonucleotides were purchased purified from IDT DNA Technology, USA. The sequences and melting temperatures of the primers capture probe oligonucleotides and their complementary sequences are presented in the Table I.

Immediately after plasma oxidation and bonding (see Fig. 1 for details), all microfluidic channels of the device were filled with a solution of APTES (2% (v:v)) in ethanol and left at room temperature for 20 min. Subsequently, the now APTES modified microchannels were

TABLE I. Sequences and melting temperatures of primers and capture probes.

	Sequence	Tm, °C ^a
Capture probes and complementary sequences		
Capture probe No. 1	5'-Amino-TGG TCC TTG TCT TAT GTC CAG ATT G-3'	65.4
Capture probe No. 2	5'-Amino-TCA CCC ACC TCC TCA TTG TAA-3'	64.2
Complementary sequence No. 1	5'-CY5-CAT TCT GGA CAT AAG ACA AGG ACC A-3'	65.4
Complementary sequence No. 2	5'-FAM-TTA CAA TGA GGA GGT GGG TGA-3'	64.2
PCR primers and PCR capture probes		
AMEL-F-looped	5'-CT ATT CTT TAC AGA -/PEG-linker/- <u>CCC TGG</u> <u>GCT CTG TAA AGA ATA GTG-3'</u>	65.3 (53.2 ^b)
AMEL-F-control	5'-CCC TGG GCT CTG TAA AGA ATA GTG-3'	65.3
AMEL-R	5'-Cy5-ATC AGA GCT TAA ACT GGG AAG CT-3'	65.1
AMEL-capture-probe	5'-GGC TCT GTA AAG AAT AGT-Amino-3'	54.8
CSFIPO-F-looped	5'-TTA AGA CAG GTT TAC CTC - /PEG-linker/ - <u>CCG</u> <u>GAG GTA AAG GTG TCT TAA AGT-3'</u>	65.1 (56 ^b)
CSFIPO-F-control	5'-CCG GAG GTA AAG GTG TCT TAA AGT-3'	65.1
CSFIPO-R	5'-FAM-ATT TCC TGT GTC AGA CCC TGT T-3'	66.2
CSFIPO-capture-probe	5'-GAG GTA AAC CTG TCT TAA-Amino-3'	54.7

^aMelting temperatures were calculated using Oligo Analyser 3.1 (IDT DNA Technology, USA) under the following conditions: an oligonucleotide concentration of 0.2 μ M, Na^+ concentration of 100 mM, Mg^{2+} concentration of 3 mM and deoxy-ribonucleotide triphosphates (dNTPs) concentration of 0.2 mM. The primer's main sequence is underlined.

^bMelting temperature of the intramolecular duplex, °C.

FIG. 1. Schematic of covalent immobilization of ss-oligonucleotide capture probes in a PDMS microchannel. Note the surface modification scheme is not to scale.

rinsed with ethanol, dried under a stream of nitrogen, and baked in an oven at 120 °C for 20 min. The next stage involved the modification of the APTES layer with a 10 mM solution of PDITC in pyridine/DMF 1:9 (v:v) for 1 h. The microchannels were then washed with ethanol and dried in an oven for 15 min at 120 °C. For modification of the surface with a ss-oligonucleotide capture probe, the PDITC-modified inlet microchannels were treated with an amine-modified ss-oligonucleotides (50 μ M) in sodium phosphate buffer (0.1 M) at pH 8. The rest of the PDITC-modified PDMS microchannel surface was deactivated by rinsing the surface with a solution of ethanolamine (50 mM) in sodium phosphate buffer (0.1 M) at pH 8.

Water contact angle (WCA) measurements were performed on the PDMS, APTES-modified PDMS, and PDITC-modified PDMS surface using the sessile drop method on a Surface Profile Analysis Tensiometer (PAT-1, SINTERFACE Technologies, Germany). The contact angles reported were the average of three measurements.

C. DNA amplification and hybridization

Human genome DNA was isolated from the authors' own blood sample with a QIAamp DNA blood mini kit (Qiagen, Germany) according to the recommendations of the manufacturer. All primers, including PEGylated (poly(ethylene glycol) modified) primers and fluorescently labelled Cy5 (red) and FAM 6-carboxyfluorescein (green) primers were purchased from IDT DNA Technology, USA and used at a concentration of 0.2 μ M (see Table I for PCR primers and PCR capture probe sequences). PCR was performed using a Qiagen multiplex PCR kit (Qiagen, Germany) with a final $MgCl_2$ concentration of 3 mM. A PCR amplification regime of 95 °C for 5 min, 30 cycles of 94 °C for 20 s, 63 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 3 min was used. DNA hybridization within the microchannel was performed in 2X SSC buffer at pH 7 with 0.05% (w/v) of sodium dodecylsulfate (SDS) (Sigma-Aldrich, Australia) at 40 °C. Release of the captured PCR products from the PDMS surface was achieved by putting the whole microfluidic device on a hotplate preheated to 95 °C while simultaneously pumping hot water through the channels with a micropipette.

Fluorescent images of the hybridization results were taken with an Olympus IX-81 fluorescent microscope (Olympus, Japan) using 548–580 nm and 450–490 nm band pass excitation and 610–660 nm and 510–550 nm emission filters for Cy5 and FAM dyes, respectively.

III. RESULTS AND DISCUSSION

A. PDMS surface activation and immobilization of ss-oligonucleotides

Surface modification of a PDMS microfluidic channel was carried out in three simple steps adapted from Ref. 19 [Fig. 1]. Convenient oxygen plasma treatment of both the PDMS

microchannel replica and flat PDMS sheet with subsequent intimate contact resulted in irreversible bonding but also activated the surface with silanol groups used for the silanization and chemical attachment of APTES.²⁰ It should be noted the time of bonding and subsequent filling of the microchannel with APTES is critical to achieve both strong and irreversible bonding.

Filling should be carried out within a suitable time such that minimal hydrophobic recovery^{21,22} occurs and that the maximum amount of reactive silanol groups remain for APTES reaction. In this case, the best result was obtained by filling the microfluidic channel within 4–5 min after contact bonding. PDITC activation of the APTES-modified PDMS microfluidic channel was then carried out [Fig. 1], and it was found that the pyridine/DMF mixture employed did not swell the PDMS, as previously observed with most other organic solvents.²³

Successful modification of the PDMS with both APTES and PDITC was observed through changes in the WCA from $101.1^\circ \pm 2.2^\circ$ to $83.9^\circ \pm 3.3^\circ$ to $93.5^\circ \pm 4.0^\circ$, respectively. Here, the amine functionalities from the APTES have rendered the PDMS more hydrophilic. However, after modification with PDITC, the phenyl groups render the surface more hydrophobic again but due to the hydrophilic thiocyanate functionalities the WCA is not as hydrophobic as the original PDMS. In order to achieve microchannel patterning both inlet channels of the Y-junction microchannel device were simultaneously filled with 0.5 μl of amine-modified ss-oligonucleotide capture probes using a micropipette (Fig. 2(a)). Both the amino-modified ss-oligonucleotide immobilization and the final deactivation of the unreacted isothiocyanate groups with 2-ethanolamine were carried out in 0.1 M sodium phosphate buffer at pH 8. The deactivation step dramatically decreased the background fluorescent signal after hybridization with both the fluorescently labelled complementary strand and the PCR products (data not shown).

B. Evaluation of immobilization and hybridization efficiency and specificity

In order to evaluate the efficiency and specificity of covalent immobilization of the capture probes and the following hybridization procedures a simple hybridization experiment with fluorescently labelled ss-oligonucleotides complementary to the immobilized ss-capture probes was conducted. As depicted in Fig. 2(a), two different ss-oligonucleotides (capture probe #1 (CP #1) and capture probe #2 (CP #2)) (see Table I) were immobilized on a PDITC-modified PDMS surface of both the upper and lower inlet channels of a Y-junction channel device. In addition, the Y-junction contained an “empty” zone which contained ethanolamine deactivated PDITC-modified PDMS [Fig. 2(a)]. Hybridization with a mixture of complementary strands CS #1 and CS #2 (1.25 μM (each)) labelled with red Cy5 and green FAM dyes, respectively (for sequences see Table I) was performed by filling the whole channel system with the mixture for 10 min at 40 $^\circ\text{C}$. After hybridization, the channel system was rinsed with 0.2 $\times$ hybridization buffer and water and then dried with air.

Figures 2(b) and 2(c) show the fluorescent images through Cy5 and FAM excitation/emission filters of the upper and lower inlet microchannels with immobilized CP 1# and CP #2 after hybridization with their fluorescently labelled complementary strands. The fluorescence emission intensity plots [Figs. 2(b) and 2(c), right] clearly reflect intensity distributions of the fluorescent images across the respective microchannels. The data confirm specificity of the hybridization of CP #1 with CS #1 in the upper inlet microchannel at approximately 1250 r.f.u. The low intensity of the FAM green fluorescent signal at approximately 250 r.f.u. in the upper inlet microchannel is comparable with the background fluorescence outside the microchannel [Fig. 2(b), right]. This indicates that the upper microchannel shows specificity for only the Cy5 labelled target probe. Likewise, for the lower inlet microchannel with immobilized CP #2 after hybridization with CS #2 the data confirm specificity of the hybridization of CP #2 with CS #2 at approximately 500 r.f.u. [Fig. 2(c), right]. In addition, the low intensity of the Cy5 red fluorescent signal at approximately 180 r.f.u., in the lower inlet microchannel, is comparable with the background fluorescence outside the channel [Fig. 2(c), right] again indicating that this microchannel is only specific for the FAM labelled target probe. The “empty” zone, which contains only ethanolamine deactivated PDITC-modified PDMS (Fig 2(d)), is characterized by a low intensity of signal in both of the Cy5 (161 r.f.u.) and FAM (335 r.f.u.) fluorescent

FIG. 2. (a) Schematic of ss-capture probes immobilized on a PDMS surface of a Y-junction microfluidic device, (b) combined fluorescent image and fluorescent signal intensity distribution (distance from the Y-junction to the cross-section is around $500\ \mu\text{m}$), respectively, after the hybridization of CP #1 with CS #1 and CS #2 within the upper inlet channel, (c) combined fluorescent image and fluorescent signal intensity distribution, respectively, after the hybridization of CP #2 with CS #1 and CS #2 within the lower inlet channel, and (d) combined fluorescent image and fluorescent signal intensity distribution of the “empty” zone (the distance from the Y-junction to the cross-section is around $2000\ \mu\text{m}$) after the hybridization. Arrows indicate the direction at which the fluorescent signal intensity distributions were measured across.

channels (Fig 2(d), right). This in turn indicates that the deactivated PDITC-modified surface itself has a low tendency for non-specific absorption of DNA.

C. Design of primer system to capture forensically relevant ds-PCR products

Current literature describes only two methods for the surface capture of ds-PCR products directly after amplification.^{9,11,15} One approach is the biotin-streptavidin affinity system which

facilitates the binding of a capture probe and a target probe with very high affinity and strong bonding.²⁴ However, a drawback of this approach is that it is nearly impossible to release the target probe in a time resolved regime and there is always interference from the unreacted biotin-labelled primers with the streptavidin-modified capture support. A second approach employs the formation of a Hoogsteen triple helix (helix invasion) between homopurine sequences.¹⁶ However, in this case, the approach results in very weak binding and low specificity of the hybridization. Furthermore, it is often difficult to find suitable homopurine sequences for the DNA sequence of interest.

Here, we take an entirely new approach and have developed, and tested, a primer system allowing easily capture of ds-PCR products of amplification by hybridization with surface immobilized ss-oligonucleotides via classic Watson-Crick base pairing. Specifically, we have designed a primer system to amplify fragments of forensically relevant AMEL and CSF1PO genes. The sequences of the primers are based on primers used in PowerPlex 16 System (Promega Corporation, Madison WI, USA).²⁵ The forward primers of both pairs consists of the primer's main sequence (for amplification of the target DNA), an additional shorter sequence (for hybridization to the capture probe) and a PEG linker which links the 5'-termini of the primer's main sequence and the 3'-termini of the shorter sequence. The sequence required for hybridization to the capture probe is complementary to the primer's main sequence (see Table I), however, it is shorter in length. At room temperature such a primer forms a hairpin-like structure by base pairing between the primer's main sequence and the sequence required for hybridization to the capture probe. Partial complementarity of this sequence to the primer's main sequence provides a lower melting temperature of the hybridized (closed) intramolecular duplex in comparison to the melting temperature of the main PCR primer's sequence (annealing temperature of PCR). This intramolecular duplex must have a lower melting temperature than that of the primer's main sequence being hybridized to the target DNA. This is of critical importance in order to provide proper annealing of the primer's main sequence to the target DNA during the amplification reaction [Fig. 3]. For the control experiments, unmodified forward primers were used, as well as standard reverse primers which were labelled with the fluorescent dyes Cy5 and FAM. Primers and their melting temperatures are provided in Table I.

At the completion of the PCR all the products of amplification contained short tails of PEGylated ss-oligonucleotide sequences (attached to the amplified ds-PCR product) which could be hybridized (captured) to the surface immobilized complementary ss-oligonucleotide capture probes [Fig. 3]. Importantly, all unreacted PEGylated hairpin primers (no ds-PCR product attached) form self-complementary intramolecular structures that prevent hybridization of this primer sequence with the surface immobilized ss-oligonucleotide capture probes [Fig. 3].

Figure 4 shows a gel electropherogram of the singleplex (lanes 1-4) and multiplex amplification products (lanes 5 and 6) of AMEL and CSF1PO genes with PEGylated hairpin forward primers and fluorescently labelled (CY5 and FAM) reverse primers. The lengths of the obtained amplification products are in full accordance with the product length produced using traditional unmodified primers (data not shown). Furthermore, no unspecific products were observed for both the singleplex and multiplex reaction mixtures with modified hairpin primers.

The ability of the PEGylated ss-oligonucleotides (with attached ds-PCR product) to hybridize with the surface immobilized ss-oligonucleotides capture probes was evaluated inside a PDMS based microfluidic system. For this two different inlet channels of a standard Y-junction channel system were surface modified with two different ss-oligonucleotide capture probes; AMEL-Capture-Probe (upper channel, AMEL-CP) and CSF1PO-Capture-Probe (lower channel, CSF1PO-CP). These strands were complementary to the hybridization sequences of the corresponding ss-tailed PEGylated ds-PCR products, as described previously. A mixture of multiplex PCR products, without any post amplification clean up procedures, was diluted twice with 4× SSC buffer (pH 7.0) containing 0.1% (w/v) of SDS. The whole channel system was then filled with the hybridization mixture and kept for 4 h at 40 °C. Then the channels were rinsed with 0.2×x SSC buffer (pH 7), water, and dried under a stream of air. Figure 5(a) shows the fluorescent image at the junction point of the Y-junction system and fluorescent intensity distribution along the direction shown by the arrows. The red fluorescence intensity at approximately 500

FIG. 3. Schematic of the production of ds-PCR products tailed with a PEGylated ss-oligonucleotide. At the PCR denaturation stage, both the target DNA and PEGylated hairpin primers are melted. Then at the annealing temperature (approximately 65 °C) hybridization of both reverse and forward hairpin primer's main sequence with the target DNA occurs and elongation starts. The PEG-linker of the hairpin primer prevents the formation of the complementary strands to the "hybridization" sequence of the primer resulting in a ds-PCR product tailed with a PEGylated ss-oligonucleotide. During the following hybridization the ds-PCR products tailed with a PEGylated ss-oligonucleotide hybridize with immobilized ss-oligonucleotide capture probes. All unreacted hairpin-like primers form "closed" secondary structures at room temperature and are unable to hybridize with the ss-capture probe.

FIG. 4. A gel electropherogram of PCR amplification products with PEGylated hairpin modified forward primers. Lane 1—product of amplification of AMEL gene, lane 3—product of amplification of CSF1PO gene, lane 5—product of multiplex amplification of both AMEL and CSF1PO genes, lanes 2,4,6—negative controls, lane 7—DNA ruler.

FIG. 5. (a) fluorescent image (left) and fluorescent intensity distribution (right, the distance from the Y-junction to the cross-section is around 500 μm), respectively, of the hybridization of AMEL-CP and CSF1PO-CP immobilized onto the upper and lower inlet microchannels, with ss-tailed AMEL and CSF1PO amplification products labelled with Cy5 and FAM dyes, respectively. (b) fluorescent image (left) and fluorescent intensity distribution (right), respectively, of the hybridization of AMEL-CP and CSF1PO-CP immobilized onto the upper and lower inlet microchannels with AMEL and CSF1PO amplification products labelled with FAM and Cy5 dyes, respectively. Arrows indicate the direction at which the fluorescent signal intensity distribution was measured across.

r.f.u. (upper channel, top arrow) confirms specificity of the hybridization of the surface immobilized AMEL-CP with the AMEL ds-PCR products tailed with a PEGylated ss-oligonucleotide and labelled with Cy5 fluorescent dye [Fig. 5(a), right]. The low intensity of the red fluorescent signal in the lower channel modified with capture probes complementary to CSF1PO amplification products and the “empty” zone (ethanolamine inactivated PDITC-modified PDMS, middle outlet channel) reflects both specificity of hybridization and the absence of unspecific adsorption of the PCR products, as well as the fluorescently labelled unreacted primers [Fig. 5(a), right]. The signals from the “empty” zones in both cases are comparable to the background fluorescent outside of the microchannels. Figure 5(b) lower inlet microchannel does not show any green fluorescent signal expected as a result of the hybridization between the immobilized CSF1PO-CP with the complementary CSF1PO amplification product labelled with FAM fluorescent dye. The reason of this lack of green fluorescence may arise from the enhanced fluorescent performance of the Cy5 dye in comparison to the FAM dye in addition to a lower content of captured PCR products. Again, when the fluorescent dyes on the reverse primers were swapped so that the AMEL amplification product was now labelled with green FAM dye and the CSF1PO amplification product was now labelled with red Cy5 no green fluorescence was observed [Fig. 5(b) upper inlet microchannel]. However, an intensive red Cy5 fluorescent signal of approximately 500 r.f.u. was observed [Fig. 5(b) lower inlet microchannel], indicating successful hybridization of the CSF1PO-CP with the CSF1PO amplification product labelled with Cy5 dye.

In order to show reversible hybridization of the surface immobilized capture probes with the ss-tailed amplification products AMEL and CSF1PO capture probes were immobilized onto upper and lower inlet channels, respectively, of a device. The channels were then simultaneously filled via a micropipette with a 1:1 mixture of Cy5 dye labelled ss-tailed AMEL and CSF1PO

FIG. 6. (a1)–(a3) and (b1)–(b3) refer to the upper and lower microchannels, respectively, within a single microfluidic devices were (a1)–(a3) show the fluorescent images (left) and fluorescent intensity distributions (the distance from the Y-junction to the cross-section is around 500 μm) across the channels (right) of AMEL-CP immobilized onto the upper inlet micro-channel, after hybridization (a1) with complementary ss-tailed AMEL amplification products labelled with Cy5 dye, after denaturation (a2) and after rehybridization with the same amplification product. (a3). (b1)–(b3) show the fluorescent images and fluorescent intensity distributions across the channels (right) of CSF1PO-CP immobilized onto the lower inlet micro-channel, after hybridization with complementary ss-tailed CSF1PO amplification products labelled with Cy5 dye (b1), after denaturation (b2) and hybridization with non-complementary ss-tailed AMEL amplification products labelled with Cy5 dye (b3). Arrows indicate the direction at which the fluorescent signal intensity distribution was measured across.

amplification products in $2\times$ SSC buffer with 0.05% SDS. After 4 h of hybridization the device was washed with $1\times$ and 0.2 SSC buffer and fluorescent images were taken (Figs. 6(a1) AMEL and 6(b1) CSF1PO). The intensity of fluorescent signals inside the channels was approximately 400 r.f.u and 426 r.f.u. for AMEL and CSF1PO, respectively. A temperature assisted release of the captured PCR products was then carried out. To achieve this, the entire device was placed on a preheated hot plate at 95 $^{\circ}\text{C}$ and the channels were flushed via micropipette with hot water for 5 min and then dried with air. Subsequent fluorescent imaging of the channels resulted in a decrease in signal of more than 80% (223 r.f.u.) (Fig. 6(a2)) and 90% (204 r.f.u.) (Fig. 6(b2)) for the AMEL and CSF1PO channels, respectively. The same AMEL-CP and SCF1PO-CP modified channels were then rehybridized with only Cy5 labelled ss-tailed AMEL amplification products which gave rise to a subsequent increase in fluorescent signal only in the AMEL-CP channel (increase from 223 r.f.u. to 350 r.f.u.). This rehybridization signal is similar to the first hybridization event (370 r.f.u for the first hybridization (Fig. 6(a3)) compared to 350 r.f.u. (Fig. 6(b3)) for the second hybridization) indicating there still remains a degree of specificity within the microchannels.

Hybridization with PCR products obtained using unmodified forward primers and fluorescently labelled reverse primers did not give any positive fluorescent results. Again, this indicates the high selectivity and specificity of the hybridization interaction between the ds-PCR products with a PEGylated ss-oligonucleotide tail and the surface immobilized complementary ss-oligonucleotide capture probes.

IV. CONCLUDING REMARKS

We have developed a new, simple and straightforward approach which allows for the capture of ds-products of PCR amplification via hybridization with surface immobilized ss-oligonucleotides. Surface immobilization of ss-oligonucleotides capture probes was performed on a PDMS microfluidic channel surface after oxygen plasma treatment, silanization with APTES then reaction with PDITC and finally covalent anchoring of the ss-oligonucleotides. Integral to the performance of the device was the design and fabrication of novel PCR primers with a PEGylated ss-oligonucleotide tail. These primers were then used to generate ds-PCR amplification products of two forensically relevant genes (AMEL and CSF1PO) tailed with ss-oligonucleotides. The resulting PCR amplification product then required no additional purification steps and could be directly introduced into the microfluidic device modified with the

surface immobilized ss-oligonucleotides. Selective capture of the surface for forensically relevant DNA was shown through fluorescence imaging indicating high specificity and selectivity. In addition, the protocol for the chemical modification of the PDMS microfluidic channels showed a low tendency for nonspecific binding of both the fluorescent labelled PCR products and unreacted primers.

This work shows great promise in the advancement of the capture, purification, and detection of forensically relevant DNA for future portable in-field microfluidic devices, envisaged to be based on capillary electrophoretic techniques. Thus, further work is ongoing in the controlled time-resolved release of the target DNA from the surface and subsequent electrophoretic separation.

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