A parallel microfluidic channel fixture fabricated using laser ablated plastic laminates for electrochemical and chemiluminescent biodetection of DNA

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Herein is described the fabrication and use of a plastic multilayer 3-channel microfluidic fixture. Multilayer devices were produced by laser machining of plastic polymethylmethacrylate and polyethyleneterephthalate laminates by ablation. The fixture consisted of an array of nine individually addressable gold or gold/ITO working electrodes, and a resistive platinum heating element. Laser machining of both the fluidic pathways in the plastic laminates, and the stencil masks used for thermal evaporation to form electrode regions on the plastic laminates, enabled rapid and inexpensive implementation of design changes. Electrochemiluminescence reactions in the fixture were achieved and monitored through ITO electrodes. Electroaddressable aryl diazonium chemistry was employed to selectively pattern gold electrodes for electrochemical multianalyte DNA detection from double stranded DNA (dsDNA) samples. Electrochemical detection of dsDNA was achieved by melting of dsDNA molecules in solution with the integrated heater, allowing detection of DNA sequences specific to breast and colorectal cancers with a non-specific binding control. Following detection, the array surface could be renewed via high temperature (95 °C) stripping using the integrated heating element. This versatile and simple method for prototyping devices shows potential for further development of highly integrated, multi-functional bioanalytical devices.
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I. INTRODUCTION

Microfabricated high throughput diagnostic devices are valuable tools for clinical analyses, point of care diagnostics, genomics, proteomics, and biosensor applications.^{1–3} The development of micro total analysis systems (μ TAS) or “lab on chip” concepts can be limited by the cost and complexity of manufacturing these devices, which can greatly affect their accessibility. Not only do such devices need to rapidly quantify multiple analytes, they are often required to limit reagent consumption, but also have minimal power requirements, and simultaneously be designed for additional integrated laboratory techniques, which are normally performed by larger analytical instruments, such as mixing, sample preparation, and heating steps.^{4,5} The first μ TAS platforms introduced in the early 1990s were fabricated from Si or glass microchips with embedded microchannels that were assembled by relatively expensive and time consuming bonding techniques. These microchips can be constructed with hundreds of parallel channels and be combined with capillary electrophoretic separation capabilities for fluid handling. High density commercial microarrays are readily available for complex genetic analysis that can detect single nucleotide polymorphisms, genetic mutations, and have built-in polymerase chain reaction capabilities.^{6,7}

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Plastic materials, which can be processed by techniques including injection molding or embossing, can be quite inexpensive and have been investigated as alternative materials for Si and glass chips.^{8–11} Efforts have also been focused on forming channels in polymeric plastics by methods such as cast molding using a prefabricated master stamp. The sealing of these modified polymer microchannels is much simpler than with Si or glass, often being accomplished with low temperature thermal annealing onto a wide variety of substrate materials allowing for the construction of cheap disposable devices which can be more readily mass produced. Different polymeric materials including polycarbonate, polymethylmethacrylate, and polydimethylsiloxane have been employed. The electrophoretic preconcentration and separation of double-stranded DNA fragments,¹² the detection of environmental pollutants,¹³ and numerous clinical and biological markers have all been reported in connection with disposable plastic chips.¹⁴ A variety of detection schemes have been used with both glass and plastic chips including, mass spectrometry, optical, and electrochemical methods. Electrochemical detection is particularly attractive due to its potential for miniaturization both for detector and control instrumentation. Additionally, electrochemical detection is capable of remarkable sensitivity with minimal power requirements.^{15–17}

Both the development of CO₂ laser treatment of polymethylmethacrylate (PMMA) (Refs. 18 and 19) and the application of these modified plastics^{20,21} have been reported. A recent example of the use of laser ablation for microchannel development based on a 3 layer PMMA laminate utilized two different types of lasers.²² A 10.6 μm CO₂ laser was used to open vias in the top layer and pattern the microchannel in the middle layer. The stack was clamped together, and the second Ti:sapphire laser (800 nm, femto-second pulsed) was used to ablate the surface of the bottom layer located in the channel through the top layer to create surface patterns. A CO₂ laser was also used for ablation of plastic laminates for fabrication of microfluidic channels in a chip subsequently interfaced with a surface plasmon resonance biosensor.²³ This sensor was used for quantification of small molecules in saliva.

In this report, we describe the fabrication of a plastic multilayer 3-channel microfluidic fixture via CO₂ laser machining of plastic laminates by ablation. The focus of this paper is the unique application of this technology and integration of functional materials toward parallel microfluidic channel and arrayed electrochemical sensors with controlled working electrode areas as well as the generation of electrochemiluminescence for biosensing applications. Acrylic adhesive coatings on the plastic sheets provided a route to multilayer structures via simple hydraulic pressing at room temperature. An array of individually addressable gold or ITO electrodes, and a resistive platinum heating element, was introduced in the fixture, along with a common platinum counter electrode and a common Au pseudo-reference electrode. Thermal evaporation directly onto plastic laminates through a stencil mask, fabricated using the same laser ablation equipment, allowed for formation of electrode regions on the laminates. This approach allowed for rapid and inexpensive development of robust devices, ideal for autonomous sensing, while providing multiple options for working electrode material to meet specific assay requirements.

II. EXPERIMENTAL METHODS

A. Plastic laminate sensor array

An electrochemical cell sensor array was integrated into a plastic package which included a microscale fluid delivery network, heater, and temperature sensor. The integrated system is referred to as a fixture with an overall size of $(0.2 \times 1.3 \times 2.3)$ in.³ and is shown schematically in Figure 1. The fixture was fabricated by laminating functional and passive laser-cut layers of polyethyleneterephthalate (PET) and PMMA. The basic fluidic channel was made by cutting the channel pattern using a CO₂ laser ablation system (Model PLS 6.75 with a 60 W cartridge, Universal Laser Systems, Scottsdale, AZ) into a custom sheet of PET coated with acrylic adhesive (0.002 in. thick for the PET and ~ 0.0005 in. thick for each adhesive; Fralock, Valencia, CA) on both sides forming the side walls and defining the channel's three dimensions. The top and bottom walls were formed from plain PET sheets (0.004 in. thick). Into one or both of

FIG. 1. Schematics of fixture structure. Black in the cross-section views indicates location of fluid. Cross-section cutout view labels indicate system electrodes.

these plain PET sheets fluidic vias were cut to allow for access to external tubing or channels located above or below. Alignment of the layers was accomplished with registration holes and pins. The layers were pressed together between two flat steel plates in a hydraulic press.

Complex systems were formed by joining simple channels located on multiple layers and by functionalizing some of the non-adhesive layers. A Ti/Au (200/2000 Å) working electrode array, or Ti/Au/ITO (200/2000/1000 Å) working electrode array, with a shared pseudo-reference Ti/Au electrode was patterned onto the bottom wall of the detection channel. The Ti/Pt (200/2000 Å) shared counter electrode was patterned onto the top wall of the detection channel, while a Ti/Pt (200/2000 Å) heater was patterned onto the opposite side of the same layer. Details of fabrication follow below.

Patterning of Au and ITO electrodes was accomplished with electron beam evaporation through a stencil mask. The stencil was made from a ground 0.02 in. thick quartz disk (*GM Assoc.*, Oakland, CA) from which the pattern was cut with a CO₂ laser. Alignment holes and pins ensured adequate registration between stencil and substrate during the deposition. As this method does not yield precise and well defined features, it was necessary to add a mask to the fixture over the electrodes that defined the area of the working electrode exposed to the solution in the channel. This layer also served to insulate the traces from other working electrodes that crossed the channel path. This method of electrode patterning did, however, allow for rapid and inexpensive modifications to the electrode configuration, being complementary to the rapid development nature of the fluidic portion of the fixture.

ITO is desirable as an electrode material, because it allows for a wider positive potential window in aqueous electrolyte solution over metallic electrodes, and is transparent, allowing for optical detection assays. In order to minimize resistive losses from ITO traces and avoid cracking of the oxide deposited onto the flexible laminate, the ITO array was prepared by depositing ITO working electrode pads in contact with Ti/Au electrode leads (see Figure 3(a)). This was done using RF sputtering through a laser-cut fused silica stencil with a target comprising 90% indium oxide and 10% tin oxide. The process parameters were 25 sccm argon, 4.8 mTorr, and 200 W RF power. The deposition rate was measured at 1.59 Å/s with a final thickness of 1000 Å. No subsequent annealing was performed. Ti/Au also served as the common pseudo-reference electrode material.

B. Fabrication

The fixture consisted of nine laminate layers (three PMMA, six PET) numbered from zero, for the bottom most layer, up to nine. In Figure 2, only layers 1–4 are shown (for

FIG. 2. Schematics of the sensor and resistive heater portions of the fixture laminate.

FIG. 3. Photographs of (a) ITO working electrode array and common pseudo-reference Au electrode, (b) Au working electrode array and common pseudo-reference Au electrode with PET mask defining the electroactive region (layer 2), and the PET layer defining the microfluidic channels (layer 3), (c) assembled fixture with platinum counter electrode and resistive heating element (layer 4) relative to a mm scale, and (d) fixture mounted in card edge connector with RTD.

simplicity) and all were fabricated from PET material. Layers 0, 7, and 9 were PMMA (0.060 in. thick) supports. Layer 7 housed six O-rings (silicone, AS568A-002, *McMaster-Carr*, Santa Fe Springs, CA) for receiving 1/16 in. diameter rigid PEEK or PTFE tubing for introducing liquids at low pressures (tested to be leak free up to at least 90 psi) without the need for additional fittings. These O-rings created an inexpensive solution for interfacing disposable devices with an external microfluidic system. Layers 6 and 8 were double-sided adhesive-coated PET for adhering the other layers. These upper layers formed the fluidic interface with external tubing. Layer 0 was also used for interfacing electrically with a 34 conductor card-edge connector (*3M*, St. Paul, MN). Because the total thickness of the PMMA layer 0 and the metalized PET layer 1 exceeded the dimensional specifications of the card-edge connector, layer 0 was “thinned” using the CO₂ laser in raster mode just over the region where the electrical connection is made.

Layer 1 was single-side adhesive-coated PET (oriented down or AC; A-adhesive, C-carrier, the first letter is the bottom-most material). The working and reference electrode patterns were deposited onto the non-adhesive side as described above. The adhesive was used to join this layer to the support layer, layer 0. Layer 2 was also single-side adhesive-coated (oriented AC) PET. This layer defined the electrode areas exposed to the channel liquids, as described previously, and also compensates for slight misalignment of the metal pattern and fluidic channel layers. Layer 3 was double-sided adhesive-coated PET (ACA) and defined the fluidic channel geometry volume providing three parallel micro scale channels, each containing three working electrodes and a shared pseudo-reference electrode. A close-up photo of layers 0 through 3, for a Ti/Au array, is shown in Figure 3(b).

Layer 4 was PET (C only) with metal patterns on both sides. The bottom-side metal pattern was the platinum counter electrode which formed the top wall of the fluidic channel. The top-side metal pattern was a Pt and Au heater used for controlling the solution temperature over the working electrodes allowing melting of double stranded DNA (dsDNA) and release of bound analyte DNA from the immobilized capture probes. As contact with the card-edge connector was only possible on the top side, a set of electrical vias was used to connect the counter

electrode to a contact pad on the top side. This set, an array of 2×10 circles, each 0.08 in. in diameter, was cut into layer 4 prior to metal deposition. This contact resulted in negligible electrical resistance measured at 0.58 Ω .

Figure 3(c) shows the completely assembled fixture, including the two fluidic ports for each channel and the Pt counter and resistive heating element (layer 4), relative to a scale. The working electrodes are not visible as they are located under the counter and heater electrodes. Figure 3(d) is a photograph of the fixture interfaced with a card edge ribbon connector and the RTD element in place. A commercially available resistance thermal device (RTD, *Omega Engineering*, Stamford, CT) was fixed to the heater to provide feedback for closed-loop control of the solution temperature. An integrated platinum RTD was unsuccessfully implemented due to the unpredictable and unstable resistance. This was likely due to the flexible substrate and/or the inability to anneal the metal. The ribbon cable served to interface the fixture electrically to the heater and hand-held potentiostat circuitry.

The laminates were pressed after the addition of each layer at approximately 3000 psi at room temperature for 2 min in a manual hydraulic laboratory press (Model 3850, *Carver, Inc.*, Wabash, IN). A custom fixture, to ensure alignment of layers and uniform force distribution, was fabricated from two plates of 1/4 in. steel with surfaces machined and polished flat.

C. Closed-loop temperature control

The temperature of the three channels was controlled simultaneously above the dsDNA melting temperature by means of an integrated platinum resistive heater, RTD element, DC power circuit, data acquisition board (*National Instruments*, Austin, TX), and computer running a custom LabVIEW (*National Instruments*) program. The software-based control algorithm was a standard proportional-integral-derivative (PID) implementation. The input variable was the measured temperature from the RTD, and the controlled variable was a modulation of the pulse width train from the data acquisition board. The pulses were amplified and then passed to the resistive heater. The PID algorithm was tuned to reject overshoot but eliminate the integral error as quickly as possible. Cooling in the channel was passive and no control was implemented at the lower temperature.

D. Electrochemical instrumentation

All electrochemical measurements were performed on a PalmSens hand-held potentiostat (Palm Instruments BV, The Netherlands) and were measured versus the on-chip Au pseudo-reference and Pt counter electrodes.

E. Biological materials

Aqueous solutions were prepared with 18 M Ω water using a Barnstead Nanopure water purifier (Boston, MA). Synthetic oligonucleotides were produced by Integrated DNA Technologies, Inc. (Coralville, IA). Potassium ferricyanide, ExtrAvidin® horseradish peroxidase (HRP), ethanolamine, and 30% H₂O₂ were purchased from Sigma (St. Louis, MO). 2-[4-(2-Hydroxyethyl)-1-piperazine]ethanesulfonic acid (HEPES), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), 4-aminobenzoic acid, N-hydroxysuccinimide (NHS), and anhydrous acetonitrile (ACN) were purchased from Acros Organics (Beel, Belgium). 4-Nitrophenyl diazonium tetrafluoroborate and tetrabutylammonium tetrafluoroborate (Bu₄NBF₄) were obtained from Aldrich. Sulfuric acid, ethyl alcohol (95% denatured), phosphate buffer saline (PBS) solution, pH 7.4 (11.9 mM phosphates, 137 mM NaCl, and 2.7 mM KCl at 1 $\times$ concentration), saline sodium citrate (SSC) solution, pH 7.0 (300 mM NaCl and 30 mM sodium citrate at 2 $\times$ concentration), and potassium chloride were purchased from Fischer Scientific (Pittsburgh, PA). 3,3',5,5'-Tetramethylbenzidine (TMB) conductivity solution (containing TMB mediator and HRP substrate) and BSA peroxidase stabilizer solution were obtained from BioFX Laboratories, Inc. (Owings Mills, MD). Carboxylphenyl diazonium was synthesized as previously described.²⁴ All reagents were used as received.

F. Electrochemiluminescence measurements

Electrogenerated chemiluminescence (ECL) reactions were performed in an ITO working electrode fixture with a $1\times$ PBS, pH 7.4, solution containing 5 mM tris(2,2'-bipyridyl) ruthenium and 50 mM tripropylamine. The fixture was placed in a custom black box during ECL experiments and imaged with a Nikon D90 digital camera and 60 mm lens (AF-S Micro NIKKOR 60 mm f/2.8 G ED). Images were acquired for 1 s each.

G. Selective modification of electrodes

Each of the three individually addressable Ti/Au working electrodes in a single microfluidic channel was selectively patterned using electroaddressable diazonium chemistry, as shown in Figure 4. Details of electrochemical characterization of diazonium salt deposition in the fixture are included in supplementary material.²⁵

First, channels were briefly flushed with ACN. The adhesive is slightly reactive to ACN, so contact was limited to prevent delamination. A nitrophenyl film was then assembled onto one electrode in each channel via chronoamperometry by application of -1.0 V vs. Au pseudo-reference for 60 s in 1 mM nitrophenyl diazonium and 0.1 M Bu_4NBF_4 in ACN, as shown in Figure 4(a). (See supplementary material Figure S1 (Ref. 25) for representative current response profiles during diazonium deposition.) This was followed by thorough rinsing of the channels with water and modification of a second electrode in each channel with a carboxylphenyl film (Figure 4(b)) by applying a chronoamperometric step to -1.0 V for 60 s in 1 mM carboxylphenyl diazonium and 0.1 M Bu_4NBF_4 in ACN. The array channels were again rinsed with water and then treated with $10\text{ }\mu\text{M}$ of amine terminated DNA probe sequence 1, which is complementary to a sequence (2) known to be relevant to breast cancer, with 10 mM EDC, and 1 mM NHS, in 10 mM HEPES buffer, pH 7.5, for 2 h at room temperature (Figure 4(c)). See Table I for DNA strand IDs and sequences. The array was then rinsed thoroughly in water and treated with 0.1 M ethanolamine, 10 mM EDC, and 1 mM NHS, in 10 mM HEPES buffer, pH 7.5, for 5 min to conjugate any remaining free carboxyl groups. After rinsing with water, the third electrode in each channel was modified with a carboxylphenyl film (Figure 4(d)) by chronoamperometry at -1.0 V for 60 s in 1 mM carboxylphenyl diazonium and 0.1 M Bu_4NBF_4 in ACN. The array channels were rinsed with water and finally treated with $10\text{ }\mu\text{M}$ of amine terminated DNA probe sequence 4 (Figure 4(e)), which is complementary to a sequence (5) known to be relevant to colorectal cancer (CC), with 10 mM EDC, and 1 mM NHS, in 10 mM HEPES buffer, pH 7.5, for 2 h at room temperature, followed by rinsing with water. Channels of the array were then filled with $2\times$ SSC, and the array was stored overnight at 4°C . The array was used for electrochemical DNA detection the following day.

H. DNA detection assay

Array channels were treated with $10\text{ }\mu\text{M}$ of ssDNA or dsDNA sequences (2, 5, and/or 7) in $2\times$ SSC for 2 h at room temperature. If melting of dsDNA was required the on-chip heater was used to raise the temperature of DNA in $2\times$ SSC solution to 85°C for 5 min prior to the 2 h room temperature incubation. For detection, a standard electrochemical-label sandwich assay was used (see supplementary material Figure S6). Following the 2 h treatment with analyte solution, the channel was thoroughly rinsed with $2\times$ SSC and treated with $10\text{ }\mu\text{M}$ of biotinylated DNA detection sequence 3 and 6 together (detection probes) in $2\times$ SSC solution for 2 h at room temperature. Solutions used for array modification and assay always covered all electrodes in a given channel. After treatment, the array was rinsed twice with $2\times$ SSC solution. The array channels were then incubated with a 1:1000 dilution of ExtrAvidin-HRP (label) in peroxidase stabilizer solution for 45 min. Afterwards, the channels were rinsed with $1\times$ PBS, pH 7.4. For detection, a given channel was exposed to TMB conductivity solution and the chronoamperometric current at each working electrode was measured at -300 mV vs. the common Au pseudo-reference. The current measured at each electrode 0.5 s after the potential step was collected and is reported as the current response. dsDNA was obtained by adding $15\text{ }\mu\text{M}$

FIG. 4. Selective functionalization of Au electrodes in a microfluidic channel for multianalyte DNA detection occurred via the (a) electroaddressable deposition of nitrophenyl diazonium onto Au electrode 1, (b) electrodeposition of carboxylphenyl diazonium onto Au electrode 2, (c) covalent coupling of an amine terminated ssDNA probe against a sequence specific towards breast cancer to Au electrode 2 by carbodiimide chemistry, (d) electrodeposition of carboxylphenyl diazonium onto Au electrode 3, and (e) covalent coupling of an amine terminated ssDNA probe against a sequence specific towards colorectal cancer to Au electrode 3, again by carbodiimide chemistry.

complimentary ssDNA to 10 μ M ssDNA in 2 $\times$ SSC and treating the solution at 95 $^{\circ}$ C for 5 min, followed by 60 $^{\circ}$ C for 2 min in a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA). The solution was then allowed to return to room temperature.

III. RESULTS AND DISCUSSION

Laser machining of plastic laminates by ablation, as described above, allowed for fabrication of research level sensor packages within 1 h (assembly only) to 8 h (all processes). The rate limiting step was deposition of the patterned metal as it required lamination of masking tape, laser cutting of patterns, selective removal of tape, metal deposition, and complete removal of the

TABLE I. Breast cancer and colorectal cancer detection assay DNA sequences.

Strand ID	Sequence
Breast cancer capture probe (1)	5'-NH ₂ -C6-GAC CTA GTC CTT CCA ACA GCT ATA AAC AGT-3'
Breast cancer target (2)	5'-AAA GTG TTT TTC ATA AAC CCA TTA TCC AGG ACT GTT TAT AGC TGT TGG AAG GAC TAG GTC-3'
Breast cancer detection probe (3)	5'-CCT GGA TAA TGG GTT TAT GAA AAA CAC TTT-Biotin-3'
Colorectal cancer capture probe (4)	5'-NH ₂ -C6-GGG CAC TCT TGC CTA CGC CAT C-3'
Colorectal cancer target (5)	5'-AAA ACT TGT GGT AGT TGG AGC TGA TGG CGT AGG CAA GAG TGC CC-3'
Colorectal cancer detection probe (6)	5'-AGC TCC AAC TAC CAG AAG TTT T-Biotin-3'
Control DNA (7)	5'-TTC AGA AGC GAA TTC CAT ATA GCA CAA GTA ATC GGA CAC GTA CTA ATT ACA-3'

remaining mask. Design changes could be made and implemented in minutes to hours. This extremely rapid device prototyping capability is not available using standard Si/glass processing, plastic casting, injection molding or embossing techniques. Further, use of the PMMA and PET laminates for device construction affords a low profile and robust package, permitting use in discrete autonomous sensing applications. Material cost for the plastic laminate fixture is similar to that reported for other plastic microfluidic devices, being estimated in the single US dollar range per device.

Due to material choices of primarily PMMA and acrylic-based pressure sensitive adhesive, the device was limited to certain chemical exposure constraints. The fixture could be treated with freshly prepared hot piranha solution (70% H₂SO₄/30% H₂O₂) for less than 1 min. Brief exposure to acetonitrile (less than 3 min) was acceptable. In excess of these times, both reacted with the adhesive causing mechanical failure of the device from delamination. The system was compatible with water-based salt solutions at temperatures up to 100 °C for several days.

A. Electrochemical characterization of Au and ITO arrays

The electrochemical response of the thermally evaporated Ti/Au electrode array on PET was investigated using a 1 mM aqueous solution of the redox probe, ferricyanide (Fe(CN)₆^{3-/4-}). Cyclic voltammograms of the three Au working electrodes in each of the three microfluidic channels are shown in supplementary material Figure S2. Similar peak potentials and peak currents were obtained across all electrodes with the average formal potential (E^0) for Fe(CN)₆^{3-/4-} measured at -138 ± 2 mV vs. the common Au pseudo-reference, and the average peak separation, $\Delta E_p = 144 \pm 3$ mV. The maximum estimated resistance of the on-chip leads from the working electrodes was 5.1 Ω , and the overall measured resistance of each electrical trace from the working electrode to the potentiostat connector was approximately 7.8 Ω . These low resistance values indicate that the electrochemical cell is well designed such that deviations from thermodynamically determined potentials for initiating a redox event within the fixture, due to electrochemical cell efficiency (i.e., resistance overpotentials), are insignificant. The average relative variation of response between Au electrodes within a channel was 3.9%; the standard deviation of Au electrode response across the entire array was 6.2%. This low degree of variability in electrochemical response is attributed to the use of the PET mask (layer 2, Figure 2), which precisely defined the electrochemically accessible area of the working electrodes. The designed accessible area was 0.20 mm² (0.51 mm diameter). The optically measured area was 0.17 ± 0.02 mm², which was due to flow of adhesive during hydraulic pressing entering the hole periphery.

Similar responses were also obtained from fixtures with ITO (see supplementary material Figure S3) working electrodes. The average E^0 for Fe(CN)₆^{3-/4-} on ITO was -176 ± 2 mV

vs. the common Au pseudo-reference, and the average $\Delta E_p = 93 \pm 3$ mV. The average relative variation of response between ITO electrodes within a channel was 1.4%; the standard deviation of ITO electrode response across the entire array was 4.8%.

B. Electrochemiluminescence generated on ITO array

Electrochemiluminescence (ECL) involves the electro generation of chemical species that undergo subsequent electron transfer reactions forming excited states which emit light.²⁶ Incorporation of ECL labels on biomolecules and biomolecule detection systems for sensitive immunoassay and DNA detection are widely reported, and impact areas as diverse as microbiology, immunology, virology, and drug development.²⁷

ECL reactions were initiated at and imaged through ITO working electrode array fixtures. Ruthenium bipyridine ($\text{Ru}(\text{bpy})_3^{2+}$), with tri-*n*-propylamine (TPA) serving as co-reactant, was used to generate the ECL. In this system, both $\text{Ru}(\text{bpy})_3^{2+}$ and TPA oxidize at the same potential, followed by deprotonation of the $[\text{TPA}^\bullet]^+$ radical (Eqs. (1) and (2)). This results in a strongly reducing intermediate, $\text{TPA}^\bullet$, which reacts with $\text{Ru}(\text{bpy})_3^{3+}$ to yield $\text{Ru}(\text{bpy})_3^{\bullet 2+}$ (Eq. (3)). Relaxation to the ground state occurs by emission of a photon at ~ 620 nm (Eq. (4)).

Using this ECL system, a visible bright orange-red emission was observed through the ITO working electrodes for applied potentials greater than approximately 1.4 V, as shown in Figure 5. This emission was restricted to the region in which the ITO pad is exposed to the reaction

FIG. 5. Images of electrochemiluminescent reactions initiated by application of +2 V vs. Au pseudo-reference. (a) Black-box image of ECL reactions at each electrode with layer 2 and 3 design overlays. Images of individually addressed ECL reactions with white-light image overlays: (b) one electrode in each channel; (c) all electrodes in a signal channel. Images were captured from the underside of the laminate array, through the ITO electrodes.

solution through mask layer 2 (Figure 5(a)). Interestingly, emission was not uniform across the entire area for all electrodes. This was not likely due to bubbles in the reaction solution as the regions have non-circular geometries. Further, it was not likely that this observation was a result of discontinuities in the ITO surface as microscopic examination revealed a uniform film. A non-uniform electric field due to geometric configurations may contribute to this observation. It was also possible to initiate ECL reactions in an addressable manner with one reaction occurring in each channel (Figure 5(b)), or all reactions occurring in a given channel (Figure 5(c)). Initiating and imaging this ECL reaction was not possible on the Au working electrode array due to the limited potential window of Au and the opaque working and counter electrodes. This demonstrates the ability of this fixture to utilize ECL as a detection tool for bioanalysis.

C. On-chip melting and detection of dsDNA on Au array

One critical requirement for a DNA sensor to analyze real-world samples is the capability to melt dsDNA to produce ssDNA. This process of heating the target sample must allow for melting of large DNA fragments with high melting temperatures, while ensuring that the target can hybridize to capture probes before reannealing. This process must be carefully controlled such that the solution does not vaporize and introduce bubbles that can disrupt fluidic flow and lead to non-uniform coverage of probe electrode surfaces.

An integrated platinum resistive heater under DC power with a RTD element and a PID closed-loop algorithm (described in Sec. II) was used to simultaneously control the temperature in the three microfluidic channels and melt dsDNA. Figure 6(a) shows a typical thermal profile and corresponding heater output during the 5 min treatment at 85 °C for on-chip melting of dsDNA. Initially, the power output is near 100%, allowing the temperature in the microfluidic channels to reach 95% of the set point within 25 s. Upon reaching the set point, the power output reduced to approximately 40% and the set point was closely maintained over the 5 min period with a standard deviation of 0.43 °C, and high and low temperatures of 86.9 and 83.7 °C, respectively, for the presented profile. This temperature was sufficiently high to melt the dsDNA ($2T_m = 66.4$ °C; $5T_m = 69.6$ °C) without vaporizing the analyte solution or damaging the immobilized DNA probe-functionalized electrode. Following the 5 min 85 °C treatment, power output to the resistive heater was set to zero, and the channels were allowed to return to room temperature, reaching 30 °C in ~ 3.5 min. This slow cooling allowed the analyte ssDNA to hybridize with surface immobilized probe DNA.

Electrochemical detection of ssDNA and melted dsDNA in the microfluidic fixture is presented in Figure 6(b). Control experiments showed that the electrochemical assay is specific for the target DNA sequence 2 (blue) when compared against the response from treatment with a random ssDNA sequence 7 (green). The current response for a given concentration of ssDNA was greater than that measured for the same concentration of on-chip melted dsDNA (red). This is likely due to competition for hybridization between surface immobilized probe DNA, and the in-solution free complementary strand to the melted target DNA sequence. Without employing the on-chip heater minimal current responses were observed for the dsDNA target (white), verifying that the melting of dsDNA is required for detection, and that the on-chip heater is effective at melting the dsDNA and allowing subsequent hybridization with the probe surface.

D. Multianalyte dsDNA detection

Selective functionalization of individual electrodes in the microfluidic fixture occurred by bias-assisted grafting of aryl diazonium salts,^{28,29} as described in Sec. II and supplementary material.²⁵ Following functionalization, the array was treated with a solution containing: dsDNA sequence 2 in channel A, dsDNA sequence 5 in channel B, and an equal molar mixture of dsDNA sequences 2 and 5 in channel C. The average response taken from 3 independent arrays following on-chip melting and electrochemical detection assay is shown in Figure 7. Significant current responses were obtained for each surface that was functionalized with the respective complementary DNA probe, averaging 260 ± 4.6 nA. In contrast, on-chip negative control surfaces (channels A-C, electrode 3) showed substantially lower currents that were similar from

FIG. 6. (a) Microfluidic electrode array thermal profile and heater output during on-chip dsDNA melting. Set point = 85.0 °C, 5 min treatment. Actual temperature = 85.1 ± 0.43 °C. (b) Electrochemical current response obtained from 10 μ M: ssDNA target sequence 2, dsDNA target sequence 2, and ssDNA control sequence 7 with on-chip melting (blue, red, and green, respectively), and dsDNA target sequence 2 without on-chip melting (white). Error bars are the standard deviation of measurements obtained from 3 electrodes.

channel to channel across the array, averaging 34 ± 5.5 nA. Signals from non-complementary ssDNA probe modified electrodes (channel A, electrode 2; channel B, electrode 1) were slightly higher than the response from the nitrophenyl surface (averaging 55 ± 13 nA) but were clearly distinguishable from the complementary probe surfaces, and consistent with non-specific binding currents measured previously (see Figure 6(b)). The three channel configuration can be connected in series, allowing for on-chip replicates performed in each channel, or potentially for detection of up to 8 targets with a single negative control surface. Alternatively, the 3 channels in this device can be used over different time periods allowing the device to be used 3 times before surface renewal or device replacement is required.

E. DNA probe surface regeneration

Following detection, the probe surface could be regenerated by stripping (water flushing at 85 °C for 5 min) to remove captured target and detection probe DNA. Chronoamperometric measurements in TMB conductivity solution were performed following stripping to ensure that no nonspecifically bound enzyme labels remained following hybridization assays. The TMB solution currents measured following stripping after detection assay (3 runs total) are shown in Figure 8 (green bars). These currents are similar to those obtained from arrays without any

FIG. 7. Multianalyte detection of dsDNA sequences specific towards breast cancer (BC) and colorectal cancer (CC) on a single chip. Each channel contains three selectively modified electrodes: Electrode (1) DNA sequence 1 (BC probe), electrode (2) DNA sequence 4 (CC probe), and electrode (3) nitrophenyl surface (non-specific binding negative control). Arrays were treated with: channel (a) 10 μ M dsDNA sequence 2 (BC target), channel (b) 10 μ M dsDNA sequence 5 (CC target), and channel (c) 10 μ M dsDNA sequence 2 with 10 μ M dsDNA target sequence 5. Error bars are the standard deviation of measurements obtained from 3 independent microfluidic electrode arrays.

prior exposure to HRP enzyme label, demonstrating nearly complete regeneration of the probe DNA electrode surface following stripping. Signals obtained from the dsDNA target (blue bars) decrease slightly with subsequent runs, decreasing by 3% from run 1 and 2, and another 12% from run 2 to 3. (Run 2 was performed 1 day after run 1. Run 3 was performed 4 days after run 1.) The signal from the random ssDNA sequence control (red bars) also rose slightly from

FIG. 8. Reuse of microfluidic electrode array for dsDNA detection. Arrays were treated with 10 μ M dsDNA sequence 2 in one channel (blue), and with 10 μ M ssDNA control sequence 7 in another channel (red). Error bars are the standard deviation of measurements obtained from the 3 electrodes in the channel, except for * which is the average value and standard deviation across all 6 electrodes used in the given run after stripping. Stripping occurred via water flushing at 85 $^{\circ}$ C (heated via the integrated resistive heater) for 5 min. Between runs the arrays were stored with 2 $\times$ SSC filled channels at 4 $^{\circ}$ C. Channels used for target or control DNA sequence treatments were alternated from run to run.

37 ± 11 nA to 43 ± 14 nA between runs 1 and 3. (Channels used for target or control DNA sequence treatments were alternated from run to run.) The number of effective reuses was limited as the sensor degrades/ages. Long-term aging and reuse studies of the sensor surface and the cartridge package, under investigation, are expected to elucidate quantifiable details.

IV. CONCLUSIONS

This report detailed the fabrication and use of a plastic multilayer three-channel microfluidic fixture with nine individually addressable sensing elements and an integrated resistive heater. Laser ablation of PMMA and PET sheets bound with acrylic adhesive facilitated rapid and inexpensive device prototyping. Multiple options for working electrode array material were available, providing versatility by way of electrode functionalization chemistry and meeting specific electrochemical detection assay requirements. Electrochemiluminescent reactions were addressability initiated and imaged through ITO electrodes. Electrochemical detection of dsDNA on Au electrodes occurred via melting of dsDNA sequences with the integrated Pt resistive heater. Following detection, the array surface could be renewed via high temperature stripping utilizing the on-chip heater. Selective patterning of the three electrodes within a given channel provided a non-specific binding negative control surface and two surfaces that could distinguish between dsDNA sequences specific to breast and colorectal cancers. The results presented herein demonstrate the utility of laser ablation of plastic laminates and quartz stencil masks for rapid fabrication of multilayer microfluidic devices. This technique shows potential for further development of highly integrated, multi-functional biosensors and DNA/protein microarrays.

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