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PMMA biosensor for nucleic acids with integrated mixer and electrochemical detection

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ABSTRACT

This paper discusses the design, microfabrication and use of an electrochemical biosensor based on a polymer substrate for cost effectiveness and disposability. As model analyte, amplified *hsp70* mRNA from *Cryptosporidium parvum* was chosen. Microfluidic channels were fabricated in poly(methyl methacrylate) (PMMA) using hot embossing with a copper master. The electrochemical transducer, an interdigitated ultramicroelectrode array (IDUA) was also realized directly on the PMMA surface. First, the unstructured PMMA surface was UV functionalized. An 8 min UV treatment resulted in a carboxylic acid density of approximately 8 nmol/cm^2 on the PMMA surface. The surface carboxylic acid groups were then conjugated to cystamine using water-soluble carbodiimide chemistry. Gold (200 nm) was then evaporated onto the thiol-functionalized surface. Using standard photolithography techniques, the IDUA containing 10 μm wide electrodes with 5 μm gaps was then formed followed by a gold etch. The PMMA surface containing the microchannel was subsequently bonded to the PMMA surface containing the IDUA using UV-assisted thermal bonding. The additional UV treatment also served to decrease the water contact angle of the surface from 62.5° ± 0.7° to 48.4° ± 0.2° thus, aiding with the capillary flow in the device. The *hsp70* mRNA was isolated from *C. parvum* oocysts and amplified using nucleic acid sequence-based amplification (NASBA). The amplicon was detected in a sandwich hybridization assay with capture probe-coated superparamagnetic beads and reporter probe-tagged liposomes. The liposomes entrapped potassium ferro/ferrihexacyanide to enable amperometric quantification of the amplicon on the IDUA. Amplified mRNA from only 1 oocyst was detectable with this PMMA biosensor. The final detection device measured approximately 10 mm × 40 mm × 3 mm and contained two detection channels for dual analyses.

1. Introduction

PMMA is a popular substrate due to its machinability, low cost and optical clarity (Castaño-Álvarez et al., 2005; Chen et al., 2003; Li et al., 2005a,b; Liu et al., 2004; Muck et al., 2004; Qi et al., 2002; Wei et al., 2005; Yu and Chien, 2005). Microchannels are commonly formed in PMMA using laser ablation (Klank et al., 2002; Yao et al., 2005) and hot embossing (Brown et al., 2006; Grass et al., 2001; Lee et al., 2001; Li et al., 2005a,b; Soper et al., 2005). Once the channels are formed, they are typically bonded to an unstructured piece of PMMA using a method such as thermal bonding, solvent bonding or a combination of the two. Thermal bonding is a straight-forward method where the two pieces of PMMA are clamped together at a temperature above the glass transition temperature (T_g) resulting in a fusion of the PMMA mating surfaces. Unfortunately, thermal bonding can result in distortion of the channels while the PMMA is above the glass transition temperature (Chen et al., 2004; Tsao et al., 2007). Solvent bonding uses a thin layer of solvent on one or both of the mating surfaces as a plasticizer (Wang et al., 2002). This method works very well providing only one of the surfaces is structured. UV-assisted thermal bonding is a method that lowers the T_g of the mating surfaces using UV irradiation while the bulk PMMA remains unchanged (Tsao et al., 2007). A bonding temperature higher than the T_g of the mating surface, but lower than that of the bulk PMMA can then be used to fuse the mating surfaces together.

The increased use of PMMA as substrate for microfluidic systems has also lead to surface modification of PMMA becoming an emerging area (Bi et al., 2006; Brown et al., 2006; Cheng et al., 2004; Dominick et al., 2003; Fixe et al., 2004a,b; Henry et al., 2000; Hideji Ichijima et al., 1991). The use of UV for the carboxylation of PMMA has shown to be a relatively simple and clean surface modification technique (Situma et al., 2007; Soper et al., 2005; Tsao et al., 2007). Once a surface is carboxylated it can be further modified with other functional groups such as amines and thiols using linkers such as ethylenediamine (Ligaj et al., 2006) and cystamine (Ngo, 1986), respectively.

The integration of electrochemical detection approaches into microfluidics rather then incorporating optical detection has clear advantages including a lack of photobleaching of marker molecules

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and simple hardware requirements. We have previously demonstrated highly sensitive detection of nucleic acid molecules using interdigitated ultramicroelectrode arrays (IDUA) fabricated on Pyrex® 7740 as a substrate and overlayed polydimethylsiloxane (PDMS) channels (Min and Baeumner, 2004). Here, a titanium adhesion layer was used between the gold and substrate surface. In general, gold has poor adhesion properties to most surfaces including PMMA. Thus, an intermediate adhesion layer of another metal such as titanium (Goral et al., 2006; Sexton et al., 2008) or chromium (Sexton et al., 2008) is commonly used between the substrate and the gold layer. However, for an electrochemical detection system, a bimetallic system results in a galvanic cell with the less noble of the two metals being solublized. Since it cannot be guaranteed that the adhesion layer is not coming in contact with the solution, it can result in limited lifetime of the electrode. Alternatively, mercaptopropyltrimethoxysilane (MPTMS) has been used as an effective alternative to a metallic adhesion layer by providing the substrate surface with a thiol monolayer (Ling et al., 2003). The gold electrodes are then adhered using gold–thiol interactions. Although limitations were found for the usable applied potential, the electrodes were more stable and had a longer lifetime compared to metallic adhesion systems.

In this study, cystamine was conjugated to the UV-modified PMMA surface using water-soluble carbodiimide chemistries, resulting in a thiolated surface. A liposomal detection system was employed to aid in signal amplification (Min and Baeumner, 2004). The liposome was tagged with a DNA probe complimentary to the target RNA. Superparamagnetic beads tagged with a target complimentary capture probe were used to immobilize the target and liposome complex over the IDUA.

2. Materials and methods

2.1. Cryptosporidium parvum

Lysed *C. parvum* oocysts (Iowa isolate) were supplied by Wisconsin State Lab of Hygiene (Madison, WI). The oocysts were counted using flow cytometry and then lysed in a lysis binding buffer (100 mM Tris–HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) using five 1 min freeze-thaw cycles. Oligo$(dT)_{25}$ beads (Dynal, Oslo, Norway) were placed in the lysed sample and then allowed to hybridize for 5 min with gentle shaking. It was then aspirated on a magnetic stand. The sample was then rinsed twice with wash buffer A (10 mM Tris–HCl, pH 7.5, 0.15 mM LiCl, 1 mM EDTA, 0.1% LiDS) and twice with wash buffer B (10 mM Tris–HCl, pH 7.5, 0.15 mM LiCl, 1 mM EDTA). The washed beads were then resuspended in 5 μL of nuclease-free water and the mRNA was amplified using the NuliSens NASBA kit (bioMerieux, Durham, NC). The NASBA primers and protocol have previously been shown to yield high amplification efficiency (Esch et al., 2001). The NASBA amplicon was then stored at −80 °C until needed.

2.2. Device design

The device contained a hybridization chamber and a detection channel. A sawtooth mixer (Nugen et al., 2008; Nichols et al., 2006) was incorporated into the hybridization chamber to aid in mixing during loading. The detection channel contained no sawtooth structures but contained the IDUA. The electrode was designed with a 5 μm gap between the fingers and the finger width of 10 μm. The finger width was wider than previously used in order to increase the surface area of the electrode to maintain adhesion during use (Goral et al., 2006). A second inlet channel was designed to insert the detergent for induced lysis of liposomes to enable the amperometric detection of ferro/ferrihexacyanide released from the liposomes.

2.3. IDUA formation

The PMMA (Lucite International, Southampton, UK) was cut into 50 mm × 50 mm pieces and then sonicated in 50% 2-propanol for 10 min prior to use. The adhesion of gold electrodes on the PMMA surface was accomplished by thiolating the PMMA surface (Fig. 1). The surface thiolation required an initial UV treatment to induce carboxyl formation. Cystamine could then be conjugated to the carboxylated surface using water-soluble carbodiimide chemistry.

A UV/ozone stripper (SAMCO International, Inc., Sunnyvale, CA) was used for the oxidation. Initially, the effect of UV, ozone and UV/ozone were evaluated individually for a 10 min treatment. For cystamine conjugation, the PMMA was treated with UV (10 mW/cm^2 at 254 nm) for 8 min.

The PMMA pieces were then placed in agitated DI water for 30 min for a post exposure rinse before being dried with nitrogen. For the surface conjugation, 1 mL of a 0.05 M MES, pH 6.0 containing 300 mM EDC (G-Biosciences, St. Louis, MO) and 300 mM sulfo-NHS (G-Biosciences, St. Louis, MO) was placed in the middle of a 4-in. Petri dish. The PMMA was then placed onto the solution with the UV-exposed side in contact with the liquid. After 25 min, the PMMA was rinsed and then placed face down in another Petri dish with 1 mL of 0.05 M sodium carbonate buffer, pH 9 containing 300 mM cystamine (Alfa Aesar, Ward Hill, MA). Following a 3 h conjugation period, the PMMA was again rinsed with DI water and dried with nitrogen.

The gold electrodes were formed on the thiolated PMMA surface using standard photolithographic methods followed by a gold etch. The thiol-functionalized PMMA was coated with gold using a CHA Mark 50 evaporator (CHA Industries, Freemont, CA). Briefly, the gold was deposited at 0.25 nm/s for a total thickness of 200 nm. Following evaporation, the positive photoresist S1827 (Shipley Co., Marlborough, MA) was then spun onto the gold-coated PMMA at 3000 rpm for 30 s. The piece was baked in a 90 °C oven for 20 min to allow the solvent to evaporate. Following baking, the photoresist was UV exposed for 5 s through a mask containing the electrode pattern using a contact aligner (Hybrid Technology Group). The piece was then placed back in the 90 °C oven for an additional 5 min. Following the post-exposure bake, the exposed resist was removed in the developer, MIF-321 (Shipley Co., Marlborough, MA) for 2 min and then rinsed with DI water before being dried with N_2. The electrode was formed by submerging the piece in gold etchant (Transene, Co. Inc., Danvers, MA) for approximately 3 min. The remaining surface was then again washed with deionized water and then dried with nitrogen.

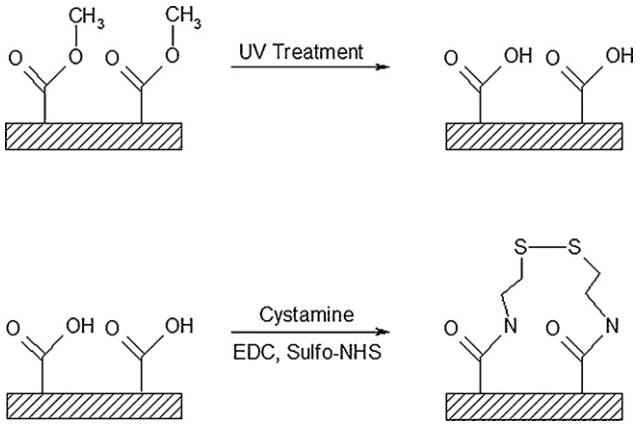

Fig. 1. Surface modification of PMMA. The surface of PMMA was initially carboxylated using 10 mW/cm^2 of UV at 254 nm for 8 min. The carboxylic acids were then conjugated to cystamine using EDC/sulfo-NHS. The thiolated surface could then provide adequate adhesion for the gold electrode.

The PMMA and remaining photoresist were then exposed to UV for 8 min. This step served to both modify the PMMA for bonding as well as expose the remaining photoresist to allow for its removal *in situ* following bonding.

2.4. Surface modification validation

Toluidine blue O (TBO) has previously been used to quantify carboxylic acid on a polymer surface (Goddard et al., 2007; Kang et al., 1996). TBO is a dye which adsorbs to carboxylic acids in a low pH solution and then desorbs at a higher pH. This assay was performed on 0.6 cm diameter PMMA discs from the same stock sheet as the PMMA to be used for electrodes deposition. The dye assay was performed on the PMMA prior to being UV treated, following UV treatment, and following cystamine conjugation. The PMMA discs were agitated in a 0.5 mM TBO solution, pH 10, for 4 h in order to allow for dye adsorption to the carboxylic acids. Following the adsorption step, the discs were rinsed in pH 10 filtered water to remove free dye. The discs were then vortexed in 200 μL of 50% weight acetic acid for 10 s to desorb the dye. The concentration of dye in the desorbing solution was determined by absorbance at 633 nm and comparison to a standard curve of TBO in 50% weight acetic acid.

In addition to the dye assay, the water contact angle was measured on the PMMA. The unmodified PMMA was compared to the UV treated PMMA as well as a UV treated PMMA which was rinsed in deionized water for 1 h. The PMMA pieces were placed horizontally on a VCA Optima XE goniometer (AST Products Inc., Boston, MA). Two microliters of distilled water were then deposited on the surface and the droplet image was immediately captured. The contact angle was determined using the installed software.

Thiol surface density was determined using the Ellman's reagent assay. The cystamine was initially reduced by incubation in a reducing buffer (0.1 M sodium acetate, pH 4.5; 0.1 M NaCl; 0.1 M DTT)

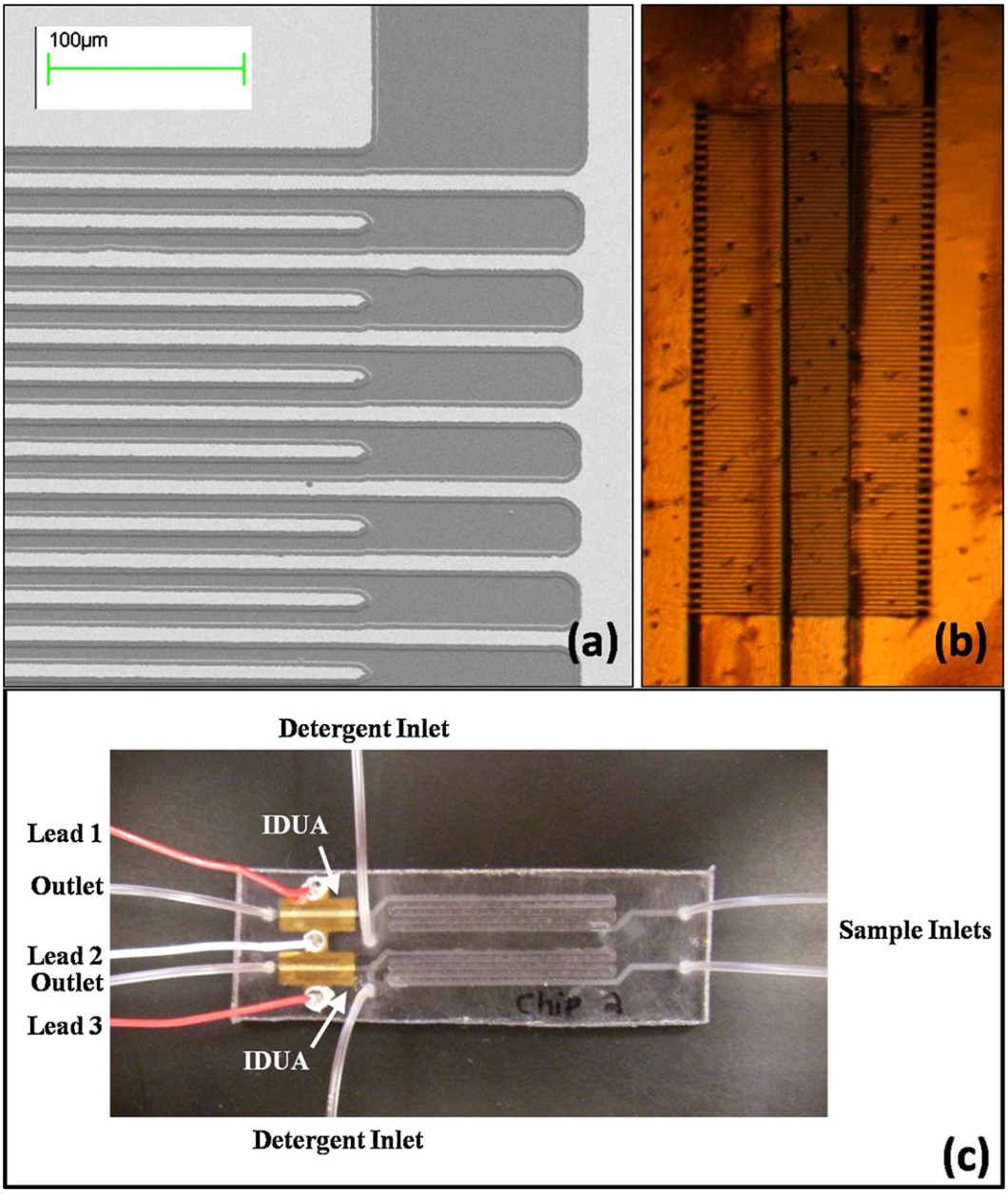

Fig. 2. (a) SEM of a gold IDUA formed on a PMMA substrate. (b) A PMMA sheet containing a hot embossed channel was then bonded to the PMMA containing the IDUA. The finished device contained a 500 μm channel positioned along the IDUA. (c) The finished chip containing two channels.

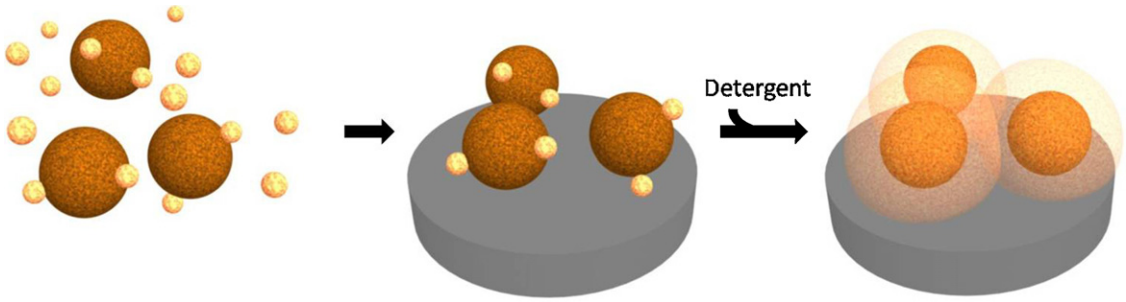

Fig. 3. Sandwich hybridizations bind the liposomes (yellow) to magnetic beads (brown). The bead/liposome complex is captured by a magnet placed over the channel. A detergent is then passed through the channel resulting in lysis of the liposomes. The liposome contents are then pumped over the IDUA for concentration determination (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

for 1 h at room temperature followed by rinsing with DI water and drying with N_2. *In situ* frames (Eppendorf, Hamburg, Germany) of known area (4.75 cm^2) were then placed on the PMMA surface in order to contain the reagent assay over a known surface area. A solution of 300 μL of 1.7 mM Ellman's reagent in 0.1 M sodium phosphate buffer, pH 8 was placed in the frame and allowed to incubate for 15 min at room temperature. Following incubation, the solution was withdrawn and placed in a 96-well plate and absorbance of the solution at 412 nm was compared to a standard curve constructed using L-cysteine.

2.5. Device fabrication

The PMMA channels were formed using hot embossing. The copper hot embossing master was fabricated as previously described (Nugen et al., 2008). The channel design consisted of five lengths joined in a serpentine, all of which incorporate a sawtooth micromixer. The sawtooth micromixer was previously shown to enhance mixing of tandem fluids (Nichols et al., 2006). The mixer was incorporated to aid in the hybridization of the *C. parvum* amplicon to the superparamagnetic capture beads and liposomes.

The PMMA channel was hot embossed using a Fortin Hot Press (Fortin CRC Prepreg). The PMMA was sandwiched between the copper master and a blank copper plate and then pressed at 130 °C with a force of 1350 N for 5 min. For de-embossing, the newly structured PMMA and copper master were allowed to cool for approximately 1 min at room temperature. The PMMA was then manually removed from the copper master. Following de-embossing, the inlet and outlet holes were drilled using 0.8 mm steel bits. The PMMA was then again rinsed and dried with deionized water and N_2, respectively.

The channel structured PMMA and electrode modified PMMA pieces were bonded using UV-assisted thermal bonding (Tsao et al., 2007). This process uses photochemically induced main chain scission to lower the glass transition temperature of the PMMA surface. The extent and depth of the scission can be controlled with UV duration. A bonding temperature higher than that of the surface, but lower than that of the bulk PMMA can then be selected. This process allows for a solvent-free bonding with no thermal distortion of the PMMA channel. The PMMA adhering the electrode was not affected due to the masking by the electrode itself.

The two PMMA pieces, having been UV functionalized for 8 min, were aligned and placed in a pneumatic press at 80 °C with a force of 1350 N for 3 min. The bonded pieces were then removed and the inlet and outlet tubing was glued in place using a cyanoacrylate based adhesive (Henkel Consumer Adhesives, Inc., Avon, OH) (Fig. 2). The remaining resist in the channel and contact pads was removed with a 5 min treatment in 100 mM NaOH. The leads for the IDUA were coated with silver epoxy (MG Chemicals, Burlington, Ont.) and adhered to the contact pads.

2.6. Assay

To characterize the IDUA, various concentrations of potassium ferri/ferrohexacyanide were injected into the channel at a flow rate of 5 μL/min. A 400 mV potential was applied across the IDUA and the resulting current was measured on an Epsilon potentiostat (Bioanalytical Systems, Inc., West Lafayette, IN).

Potassium ferri/ferrohexacyanide encapsulating liposomes were prepared using reverse phase evaporation as described by Goral et al. (2006). The reporter probe (5′–3′) GTG CAA CTT TAG CTC CAG TT-CHOLESTEROL (Operon, Huntsville, AL) was incorporated into the lipid bilayer using a cholesterol tag. Streptavidin coated 1 μm diameter superparamagnetic beads (Dynal, Oslo, Norway) were conjugated with a biotin-tagged capture probe (5′–3′) BIOTIN-AGA TTC GAA GAA CTC TGC GC also as describe in Goral et al. (2006).

For the *C. parvum* assay, 1 μL of NASBA amplicon was combined with 1 μL of capture beads, 1 μL of liposomes, and 1 μL of a hybridization mixture (50% formamide, 10× SSC, 0.5% ficoll, 0.3 M sucrose, 8% dextran sulfate). The solution was pumped into the channel and allowed to hybridize for 15 min. During the hybridization, the solution was pumped in a reciprocating motion (0.5 μL; 1 μL/min) in order to aid in mixing. Following the hybridization step, a rare earth magnet was placed over the channel in order to hold the beads in place while the sample solution was pumped out and replaced with a washing solution (20% formamide, 4× SSC, 0.2% ficoll, 0.125 M sucrose, 5% dextran sulfate) at 1.5 μL/min. The magnet was about 1 mm removed from the middle of the channel. The first magnet was then removed and a second rare earth magnet was placed directly over the IDUA. The beads were then washed toward the IDUA again at 1.5 μL/min for 8 min. The placement of the second magnet captured the majority of beads directly upstream of the IDUA (Fig. 3). The washing step was needed to remove any unhybridized liposomes from the sample area. A detergent, n-octyl-β-D-glucopyranoside (OG) was then injected at 60 mM in water into the channel at 1 μL/min in order to lyse the liposomes and release the encapsulated potassium ferri/ferrocyanide solution which was in turn detected by the IDUA. The resulting current from the redox reaction was coulometrically measured and recorded. The resulting area under the current/time curve (nA s) was determined to be the assay signal.

3. Results and discussion

3.1. Surface modification

Initially the effect of UV, ozone, and the combination of UV and ozone was evaluated in order to determine the optimal method for functionalization. A 10 min treatment of each method resulted

in much higher surface functionalization for the UV treatment over the ozone and UV/ozone treatments. The water contact angle of unmodified PMMA was found to be 62.5° ± 0.7°. Immediately following the UV treatment, the water contact angle dropped to 22.9° ± 0.4°. The effect of UV on PMMA has been found to involve both side chain modification as well as main chain scission (Truckenmuller et al., 2004). The scission of the main results in the creation of smaller chains with some of them no longer bound to the surface. Following the rinsing step, which was used to remove the small soluble polymer chains, the contact angle was found to be 48.4° ± 0.2°.

The TBO dye assay determined that the initial UV treatment provided a carboxylation density of 8 nmol/cm^2 of carboxylic acids. The thiol density on the conjugated surface was found to be 0.77 nmol/cm^2 using the Ellman's reagent assay. The lower thiol density compared to that of the initial carboxylic acid density is most likely due to steric hindrance during cystamine conjugation.

3.2. IDUA fabrication and characterization

The IDUAs were examined using scanning electron microscopy in order to determine effective etch times (Fig. 2a). The UV-treatment prior to bonding resulted in observable etching between the IDUA fingers and is likely due to PMMA main chain scission (Ponter et al., 1994; Truckenmuller et al., 2004).

In fact, the initial bonding procedure, where all photoresist was removed prior to bonding, resulted in significant damage to many of the IDUAs. The yield of working bonded IDUAs was increased by leaving the photoresist on the IDUA during bonding to help protect the gold fingers. The photoresist was removed *in situ* by pumping 100 mM NaOH through the bonded channel. The NaOH was able to remove the UV-treated photoresist within 5 min.

3.3. IDUAs

The individual IDUAs were characterized using a dose–response to varying potassium ferro/ferrihexacyanide solutions (Fig. 4). As determined earlier, the smaller the gap size the lower the limit of detection (Min and Baeumner, 2004). However, the gap size used here on the PMMA substrate was sufficient to provide a low limit of detection of a single oocyst amplicon. The NASBA amplicons from

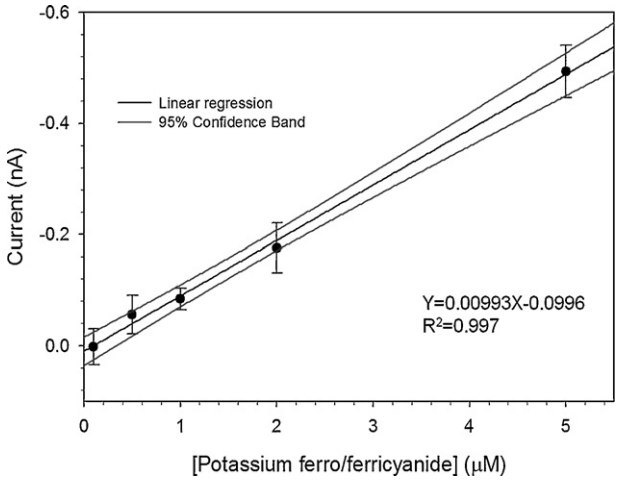

Fig. 4. Dose–response of an IDUA to increasing solutions of potassium ferro/ferrihexacyanide flowing through the channel. The potassium ferro/ferrihexacyanide was dissolved in a pH 7.5 phosphate buffer and pumped over the channel at 5 μL/min. Background values using only phosphate buffer were subtracted from the readings. Error bars represent the standard deviation of a minimum of three replicates.

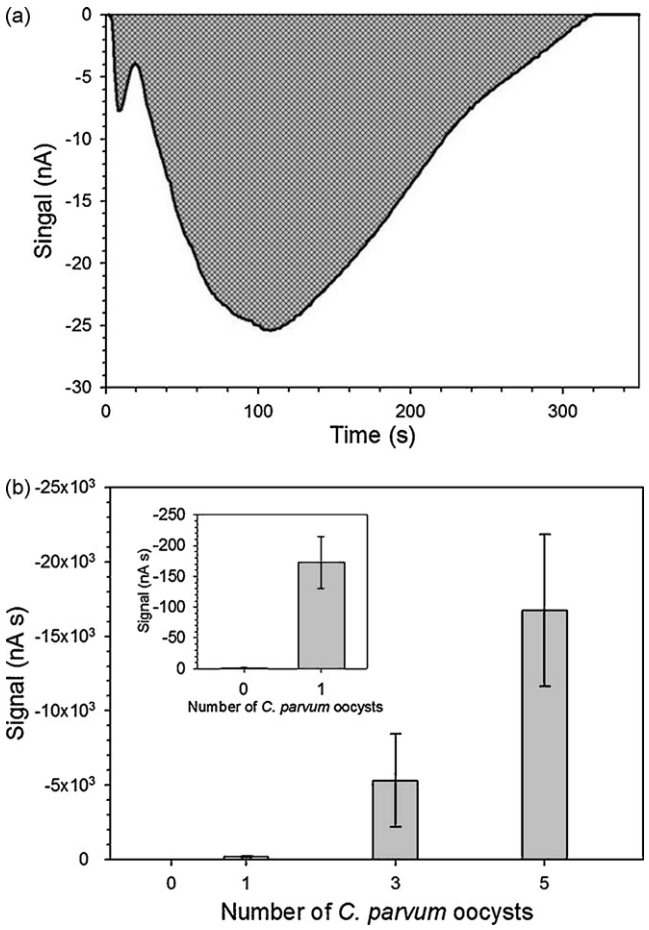

Fig. 5. (a) Electrochemical response following the injection of OG and lysing of immobilized liposomes. The area of the shaded region was determined and used as the assay result. (b) The response from assays using NASBA amplicon from 0, 1, 3 and 5 *C. parvum* oocysts. Error bars represent the standard deviation of a minimum of three replicates.

0, 1, 3 and 5 *C. parvum* oocysts were analyzed. Amplicons from 1, 3 and 5 oocysts were confirmed positive using a lateral flow assay as previously described (Connelly et al., 2008). The results showed the ability of the IDUA detecting the amplicon from a single *C. parvum* oocyst (Fig. 5). All oocysts concentrations were statistically distinguishable with a *P*-value below 0.05 when analyzed with a student *t*-test. Although peak heights of the individual signals varied, area calculations (Fig. 5a) gave reproducible results. The assays displayed very low backgrounds indicating a low level of non-specific binding of liposomes. This is likely due to negatively charged liposomes used in a modified PMMA channel with a negative surface charge due to surface carboxylic acids.

4. Conclusion

We have developed a PMMA based microfluidic biosensor with electrochemical detection ability. Although the polymer biosensor was designed to be disposable, the surface modification of the PMMA allowed the IDUA to adhere to the PMMA during repeated use. The use of a liposomal detection system encapsulating potassium ferro/ferrihexacyanide allowed for the detection of amplicon from a single *C. parvum* oocyst. Future experiments will investigate to narrow the gap and the finger width of an IDUA in order to generate even more sensitive electrochemical transducers for use in biosensing systems.

The UV-assisted thermal bonding proved gentle enough to allow most of the IDUA fingers to remain intact. When solvent bonding was used, the IDUAs were destroyed during bonding. Therefore, UV-assisted thermal bonding was shown to be a preferred technique for bonding PMMA with sensitive surfaces.

We have previously demonstrated successful isolation of the *hsp70* gene in similar microfluidic channels. We have also successfully amplified the *hsp70* mRNA from oocysts in environmental water samples (Connelly et al., 2008). Currently, we are developing an *in situ* amplification method which would complete a micro total analysis system from lysed cells to detection signal. Such systems will provide cheaper and more rapid testing as compared to current EPA methods.

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