A Multiple Drugs of Abuse Test for Saliva Specimen on a Single Lab-on-a-Film-Chip Using Electrochemical Immunoassay

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ABSTRACT

A lab-on-a- film-chip (LOFC) developed by Nano-Ditech Corp is a disposable microfluidic immunoassay device. Multiple drugs of abuse test for saliva specimen has been developed on a single lab-on-a-film-chip as an alternative to a nitrocellulose membrane based immunochromatography assay. The assay is a competitive test format since the target analytes have small molecular weights. The drug-conjugates immobilized on a carbon electrode compete with free drugs in saliva specimens for a binding site of specific drug antibody conjugated with alkaline phospatase. The binding signal for each analyte is generated by captured antibody conjugated with APase on a separate carbon electrode and is analyzed by electrochemical assay. Three analyte, amphetamine, cocaine and opiate, are simultaneously detected from drug spiked saliva samples with the cutoffs of 20 ng/ml, 40 ng/ml and 50 ng/ml, respectively.

Keywords: LOFC, multi drug abuse test kit, saliva

INTRODUCTION

Clinical specimens such as urine, hair, blood, sweat or oral fluid, are currently used for the drugs of abuse test and the urine specimens are used most commonly. However, using saliva as a sample has some advantages over using a urine sample; less chance for adulterating test samples and convenience for collecting samples on-sites. It is also possible to detect drugs from the time of ingestion when saliva is used, while at least several hours from ingestion are required to detecting the drug and/or its metabolites in urine. These may be the reasons that lately there is a trend that end users prefer using the saliva-based test than the urine-based test.

Most of commercially available saliva-based drug test kits employ nitrocellulose membrane-based lateral flow immuno assay using colloidal gold particles coated with a drug specific antibody and a drug conjugated protein. Since the drug molecules are small, a competitive assay is a preferred detection format. In a competitive assay, the test signal decreases as the concentration of an analyte in a specimen increases. When the concentration of a drug in a specimen is over the cutoff level, the test signal disappears. A positive or negative result is determined based on the presence or absence of the test line. However, this judgment is often subject to many factors including; operator's eye sight, intensity of test line particularly a faint signal, interference due to a residual gold color background and brightness of a testing area.

As an alternative to the nitrocellulose membrane based lateral flow immunoassay, we have developed a Lab-On-a-Film-Chip (LOFC) technology [1]. LOFC is a microfluidic electrochemical immunoassay device capable of generating electrochemical signal. In conjunction with a reader, electric signals are collected and processed to generate quantitative results or qualitative results using a cutoff concept. In LOFC technology, fabricated micro channels do both capillary action of nitrocellulose membrane and fluid pulling function of absorption pad. Depending on designs of the fabricated channels, the flow rate and the timing of addition or release of reagents can easily be controlled. Splitting the channel and adding multiple chambers (or electrodes) along the channel allows detection of multiple analytes from the same source of the specimen. Since the channel is narrow and the detector area is small, washing of unbound enzyme coupled antibody on the electrode can be achieved with the residual sample.

We have developed a multiple drugs of abuse test using a saliva specimen on a LOFC. Three common abuse drugs, amphetamine (AMP), cocaine (COC) and opiate (OPI), spiked in saliva were evaluated on this device. The channel communicating a reagent chamber for a mixture of 3 specific antibodies coupled with alkaline phosphatase as detectors, a substrate chamber for enzyme substrate and a detection chamber including 3 electrodes for each drugs had been fabricated as shown in the Fig. 1. Each drug conjugated to a carrier protein was deposited on the 3 separate electrodes. Three drugs were spiked

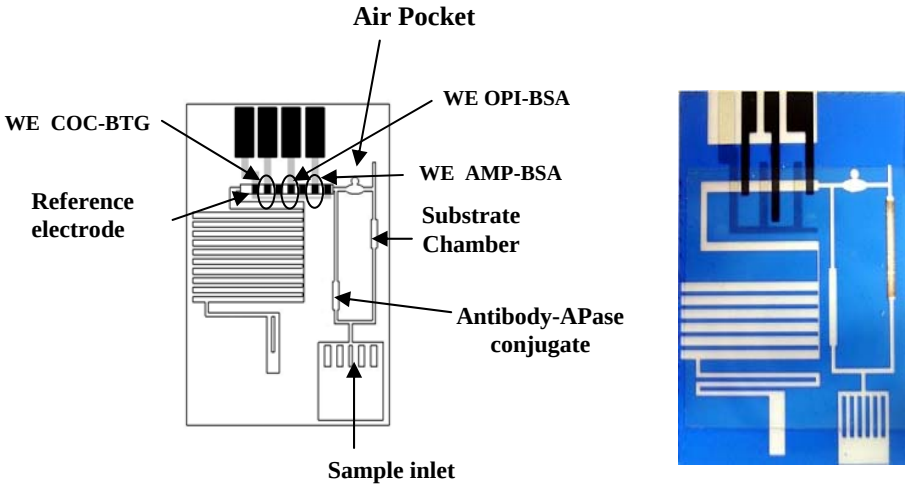

Figure 1. Multi-electrode LOFC structure used in this study, WE COC-BTG; working electrode coating with Cocaine-BTG conjugate, WE AMP-BSA; working electrode coating with Amphetamine BSA conjugate, WE OPI-BTG; working electrode coating with Opiate BSAconjugate.

around the cutoff concentrations in saliva and tested on the LOFC device. Using the recommended cutoff concentrations of SAMHSA [2] of 50 ng/ml, 20 ng/ml and 40 ng/ml for AMP, COC and OPI, respectively, the LOFC device clearly detected the presence of specific drugs in saliva samples.

2. MATERIALS AND METHODS

2. 1. Reagents

Antibodies against drug molecules were purchased from Biostream Inc. (NJ, USA). Alkaline phosphatase (APase) was purchased from Biozyme (Cal, USA). Chmeicals for antibody conjugation with APase, SATA (N-succinimidyl S-Acetylthioacetate) and sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) were purchased from Pierce (IL, USA) and Thermo Scientific (IL, USA), respectively. Drug conjugate with carrier protein for competition immuno assay, Amphetamine-BSA and Benzoylecognine-BTG were purchased from Immunetics (NJ, USA) and Opiate-BSA was purchased from Pyxis (Cal, USA). Standard drug molecules for positive saliva control specimens were purchased from Cerilliant (TX, USA). Alkaline phosphatase substrate, pAPP was purchased from Universal sensors (Ireland).

2.2. Instruments

PRO-LAM 320 (AKILES, Korea) was used to laminate dried photo resistant (DPR) films and cover tapes. UV flood system of Loctite Corporation (CT, USA) was used to irradiate and polymerize DPR film. Rota-Spray of VPC (MN, USA) was used to develop unmasked DPR layer

by spraying etching solution (0.1% sodium carbonate solution). All electrochemical test results in this study were acquired using a PG580 Potentiostat-Galvanostat (Uniscan instruments LTD, Derbyshire, UK) with UiEChem version 1.57 program.

2.3. Conjugation of Drug antibody with Alkaline Phospatase.

Drug antibodies were conjugated with APase immunoassay following the manufacturer's instruction (Pierce, IL.USA) and used as indicators in a competitive. To generate sulfhydryl groups on antibodies (IgG-SH), 1 ml of antibody solution (4 mg/ml in PBS) was mixed with 10 µl of the SATA (55 mM in DMSO) and incubated at room temperature for 30 minutes. Unbound SATA was removed from reaction product by dialysis against 50 mM potassium phosphate buffer. To generate sulfhydryl groups, SATA modified antibody was deacetylated by adding 100ul of deacylation buffer (0.5 M hydroxylamine, 25 mM EDTA in PBS) and incubated for 2 hours at room temperature. The final reaction mixture was passed through a desalting column equilibrated with conjugation buffer (10 mM EDTA in PBS).

To make maleimide activated APase, sulfo-SMCC (200 µg) was added to 4 mg of APase in 1 ml and the mixture was incubated for 60 minutes at room temperature and desalted by passing through a desalting column. To conjugate IgG-SH to maleimide activated APase, equal volume of two reaction products were mixed and incubated overnight at 4 C^{o}. The final reaction mixture was passed through a desalting column equilibrated with 20 mM phosphate buffer.

2.4. Construction of LOFC

LOFC is a three-layered laminated film comprised of an electrode-printed polymer film, a dry photoresist film layer where various microfluidic channels are created using photolithography and an adhesive-coated polymer film as a

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cover layer. To make working and reference electrode on the LOFC [3], carbon and Ag/AgCl pastes (purchased from Gwent and Asai) were printed on the polyethylene terephthalate (PET) film using a DEK 248 screen-printing machine (DEK, Weymouth, UK). Microfluid channels were fabricated on the electrode printed PET film using dried photo resistant film (Ordyl MP100, Tokyo Ohka Kogyo Co, LTD) by a photolithography method. A DPR film was laminated on the electrode printed PET film. A photomask pattern of designed channel was placed on top of the laminated DPR film and subjected to UV irradiation to polymerize unmasked region of DPR film. The UV irradiated film was placed in the rotary spray etching machine to intaglio the non-irradiated regions with 0.1N sodium carbonate buffer, pH 10.5. After washing out the etching solution with deionized water, the channel-fabricated film was dried for 2 hours at 45 C^{o} before dispensing biochemical reagents.

The drug conjugates were immobilized on the carbon electrodes by physical adsorption [4]. Each drug conjugate (0.6 mg/ml) was prepared in 10 mM phosphate buffer, pH 7.2. A 0.5 µl of drug conjugate was dropped on the carbon electrode (4.0 mm^2) and dried for 1 hour at room temperature. A 1 ul of APase-conjugated drug antibodies (80 µg/ml), in enzyme stabilizer was deposited on the conjugate chamber. The APase substrate (200 mM pAPP) was deposited on the substrate chamber and then the channel was covered with an adhesive coated polymer film (ARcare 8190, Adhesive Research, PA, USA).

2.5. Detection of illicit drugs in saliva sample using electrochemical immuno assay

To make positive control saliva solution, saliva was collected from drug free volunteers using saliva collection swab. Collected saliva was centrifuged to remove debris and then spiked with standard drug molecules at various concentrations. A 20µl of saliva sample was applied to the sample well of the LOFC. When the saliva solution arrived at the flow sensor, a hole was punctured on the air pocket between detection and substrate chambers to allow the substrate flow into the electrode area. Sixty seconds after puncturing the air pocket, a potential of 150 mV was applied to the electrodes, and oxidative amperometric signal on the carbon electrodes versus the reference Ag/AgCl electrode was measured for 60 seconds.

3. RESULTS

3.1. Cyclic voltammograms (CV) of drug conjugates

To measure oxidative amperometric signal generated by electro active *p*-aminophenol, products of alkaline phospatase reaction on pAPP, Voltammetric cycles were carried out between -500 and + 500mV at a scan rate of 50mV/sec. From the CV test result, the oxidative current peak was appeared at 150 mV of applied potential. Drug conjugates immobilized on carbon electrodes for the competitive immuno assay should not generate any oxidative signal at the potential of 150 mV. As shown in Figure 2, there is no difference among CV between electrodes immobilized with 3 different drug conjugates and a bare electrode. This result indicates none of drug conjugate generated any electro active product at 150mv of applied potential voltage.

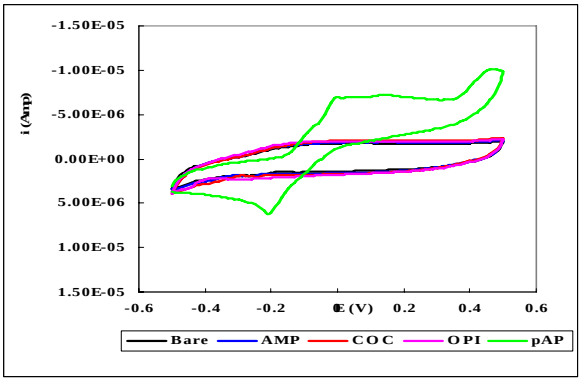

Fig. 2. Cyclic voltammograms of carbon electrodes; bare (Bare), immobilized with AMP-BSA (AMP), COC-BTG (COC) and OPI-BSA (OPI) conjugates at 50 mVs^{-1} in sodium carbonate buffer (pH 11.2, 100 mM KCl, 1mM $MgCl_2$).

3.2. Detection of single drug on LOFC

Three illicit drugs were evaluated separately on the LOFC device deposited with a single antibody conjugated with APase using a drug molecule spiked saliva samples. Sample (20 µl) of different concentrations of the drugs in saliva was applied on the sample port and the current was measured. Each sample was tested in replicate of 4 and the average current was plotted against the concentration. As shown in the figure 4, electric signals detected on the carbon electrode coated with a drug-protein conjugates were dose dependant. The signals generated with samples containing the drug below the cutoff and above the cutoff were clearly separated when cutoffs of 50 ng/ml, 20 ng/ml and 40 ng/ml were applied for AMP, COC and OPI, respectively (Fig. 3).

3.3. Detection of multiple drugs on a single LOFC

The antibody-APase conjugate chamber was deposited with a mixture of 3 specific anti-drug antibodies conjugated with APase and saliva samples containing no drug or drug above the cutoff concentrations were tested. Signals on 3 carbon electrodes designated for the different drug molecules were measured. Samples were tested in replicate of 4 and the average signal was plotted along with the deviation. Electric signal from the specific electrode was dropped when the drug concentration was above the cutoff level (Fig. 4).

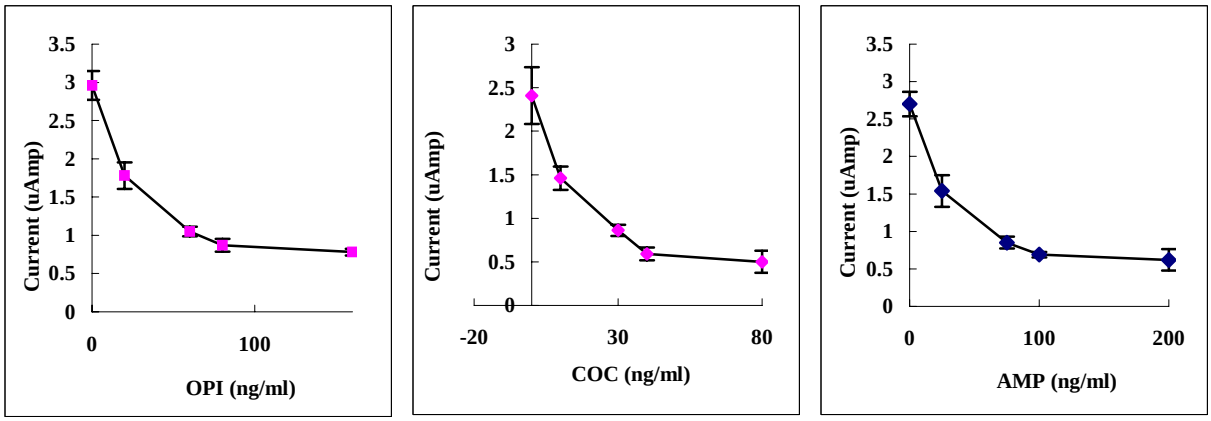

Fig. 3. Competitive immunoassay results of saliva spiked with different drug concentrations.

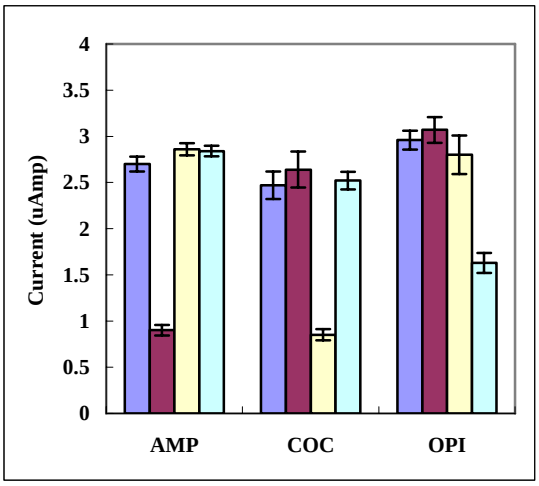

Fig. 4. Competitive immunoassay results for saliva containing a drug on a single chip capable of detecting multiple drugs. Each 20 µl of drug free (blue), 50 ng/ml AMP spiked (red), 20 ng/ml COC spiked (yellow) and 40 ng/ml OPI spiked (sky blue) saliva samples were used.

CONCLUSION

In this study , we have demonstrated that a multiple drugs of abuse test on a single lab-on-a-film-chip for saliva samples was successfuly developed using the cutoffs recommended by SAMHSA. It requires less than 30 µl of samples and can detect 20% of cut off concentration of drug analyte in saliva specimen. The standard deviations of the binding signal were fully acceptable to separate the signals between 25% over and 25% below cut off concentrations.

It has been shown that Lab-on-a-film-chip can be used for a versatile immunoassay format from excess to limited reagent immuno assay

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