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Paper-based electrochemical immunoassay for rapid, inexpensive cancer biomarker protein detection

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Inexpensive, reusable electrochemical sensor chips were fabricated from gold CDs. All reagents were loaded onto a paper disk sequentially, then placed on the chip to detect cancer biomarker prostate specific antigen (PSA) in serum at pg mL^{-1} levels in ~15 mins.

Technically simple, inexpensive devices for cancer biomarker measurement have the potential to enable a future era of personalized medicine in which patient treatment is guided by molecular diagnostic tests. [1-4] Paper has been used as a cheap, versatile material for development of clinical diagnostic tools for analytes in urine and blood.[5-10] Microfluidic paper analytical devices (µPADs) offer rapid testing of biological samples and promise real time diagnostic tests to assist physicians in delivering personalized treatment and improving prognoses for patients. [4,11,12] In fact, paper has been used for decades in inexpensive over-the-counter pregnancy test strips.[13] These simple, inexpensive tests have saved thousands of clinic visits, allowing physicians to allocate their time to more urgent care. Paper devices have great potential for both resource-limited and resource endowed communities in situations where time and cost may be important constraints.

While many paper-based device use colorimetry,[14-16] electrochemical detection is usually more sensitive and easier to achieve quantitatively with simple electronics. For example, electrodes printed on paper electrochemical devices (µPEDs) were used to measure Pb^{II} at 1 ppb levels.[17] µPEDs were used with a commercial glucose sensor to detect glucose, lactate, cholesterol, and ethanol,[18] and a 3-D µPED was developed to measure multiple biomarker proteins.[19] We recently developed a µPED for genotoxicity screening of environmental samples using electrochemiluminescence.[20]

Specific proteins in serum become elevated at the onset of cancer and can be used as biomarkers for early detection and therapy monitoring.[1,4] However, immunoassays should be further miniaturized and simplified to be applicable to resource-limited situations. In this paper, we describe a novel, low cost, non-microfluidic paper immunoassay interfaced with a reusable electrochemical chip for rapid measurement of cancer biomarker proteins. Modified filter paper disks were interfaced previously with commercial screen printed electrodes to measure dopamine in

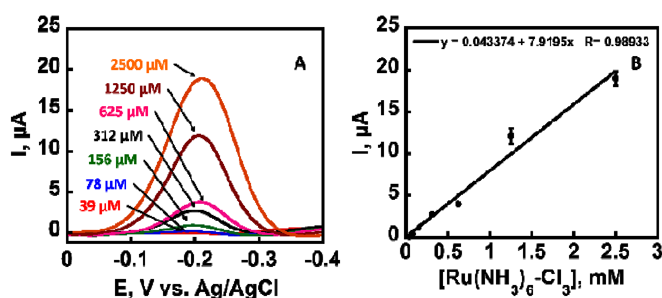

Fig. 1 Characterization of paper disk sensor for a reversible redox system: (a) DPV response for various concentrations of $Ru(NH_3)_6$-Cl_3 in 0.1 M KCl; (b) Calibration plot for various concentration $Ru(NH_3)_6$-Cl_3 of in 0.1 M KCl (n=3). DPV was done at 4 mV step, 25 mV pulse and 15 Hz frequency vs. Ag/AgCl from 0 V to -0.4 V.

human serum samples,[21] and glucose when integrated with a reagent-loaded silicon tube, glucose.[22] Here, we adopt an approach that pre-loads capture antibodies (Ab_1) for immunoassay onto a paper disk (Whatman Protran nitrocellulose) serving as a tiny 8 µL electrochemical cell. Unlike previous approaches that requires pipetting, a dip-and-wash protocol (Scheme 1) is used to enable sandwich immunoassays. The wet disk with bound analyte and detection labels is placed on top of the electrochemical chip for measurement. The electrode chips were fabricated using wet chemical etching of gold CDs in a laboratory environment not requiring any specialized equipment or cleanroom and costs US $0.35 each in materials including the CD as previously described.[23-25] These inexpensive non-lithographically fabricated chips feature Au working and counter electrodes and a hand screen printed Ag/AgCl reference electrode for on-chip detection. Using the dip-and-wash protocol, the device achieved detection limit of 6 pg mL^{-1} for PSA in serum in 15 min assays, with good sensitivity in suitable ranges for most cancer biomarker proteins.[1,4] To our knowledge, this is the first paper based sandwich-type immunoassay reporting a reusable sensing element, a tiny (8 µL) measuring volume, and no solution dispensing or flow. The approach offers a low cost per test while minimizing sensor to sensor variability.

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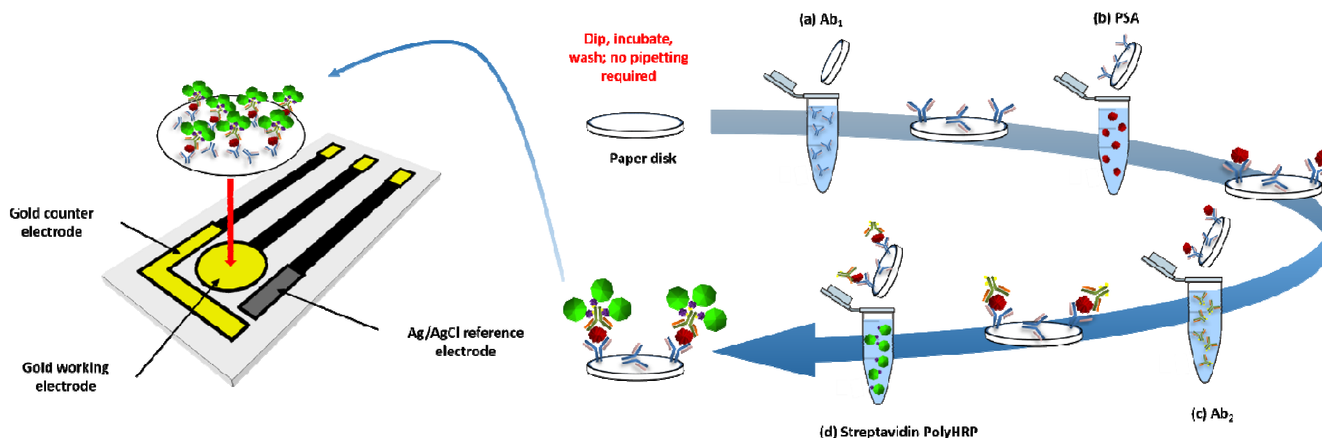

Scheme 1 Preparation of paper disk electrochemical immunoassay. (a) Adsorption of capture antibodies on paper disks; (b) incubation with sample after blocking with BSA; (c) incubation with biotinylated secondary antibody and (d) subsequent incubation with streptavidin PolyHRP detection label.

Chips fabrication starts with gold CDs with the polymer coating removed. A computer-printed ink template is heat transferred onto the gold before etching.[23] Chips are then washed with ethanol to expose contacts pads and sensor electrode, then washed with water and dried under nitrogen. The reference electrode was manually screen printed with Ag/AgCl ink (DuPont 5269) onto the designated area (Scheme S1, ESI). Surface gold oxide was removed from the sensor surface by cycling 3 times between -0.1 V to +1.2 V at 100 mV s^{-1} in 0.18 M sulfuric acid before each use.

Electrochemical response of the paper disk sensor was first characterized by assessing reproducibility of electrochemically addressable area in the absence of immunosensing. After dipping the paper disk in 5 mM ruthenium hexamine chloride $[Ru(NH_3)_6]Cl_3$ (RuHex) and 0.1 M KCl vs. Ag/AgCl, the paper disks, which require ~8 µL of solution to be completely wetted, were placed on the sensor to create a complete electrochemical cell. Differential pulse voltammetry (DPV) was done for a series of concentrations of RuHex (Fig. 1). A linear relation between concentration and peak current was obtained with detection limit 20 µM, demonstrating good analytical performance. Reproducibility of sensor area using paper disks varied <4% from sensor to sensor (Fig. S3, ESI). Well-defined quasi-reversible peaks of the ruthenium redox couple were found by cyclic voltammetry at 100 mV s^{-1} with peak separation of ~80 mV (Fig. S2, ESI), larger than the theoretical value of 59 mV for a reversible CV reaction at 25°C.

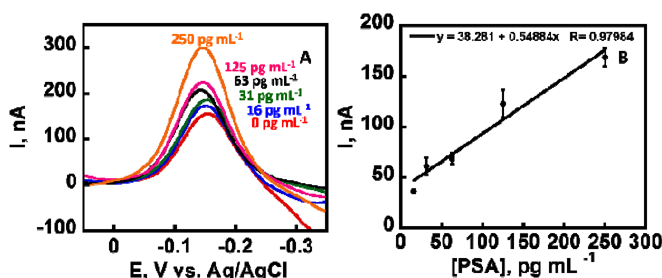

Fig. 2 DPV peaks for detection of PSA in undiluted serum. (a) peaks developed after dipping the completed assay on paper disk in 1 mM hydroquinone and 100 µM H_2O_2; (b) Calibration plot for PSA in undiluted serum (n=3). DPV was done at 4 mV step, 25 mV pulse and 15 Hz frequency vs. Ag/AgCl from 0.05 V to -0.35 V.

Peak current (i_p) versus square root of scan rate ($\upsilon^{1/2}$) was linear demonstrating diffusion control of the voltammetric peak. These data were used with Randles-Sêvcîk equation and the geometric area of the working electrode to estimate a diffusion coefficient (D) of 0.29 x 10^{-5} cm^2 s^{-1}, compared to 0.52 x 10^{-5} cm^2 s^{-1} in aqueous solution, which suggests the percolation of redox species within the paper.[26]

Sandwich type immunoassay for measuring PSA were developed by absorbing the corresponding capture antibody (Ab_1) onto the paper disk, which is then sequentially dipped into sample or standards containing the antigen, wash and biotinylated detection antibody (Ab_2) solutions. We chose a rapid signal amplification strategy featuring streptavidin PolyHRP that binds strongly to the biotinylated Ab_2 and provides a high molar ratio of HRP label

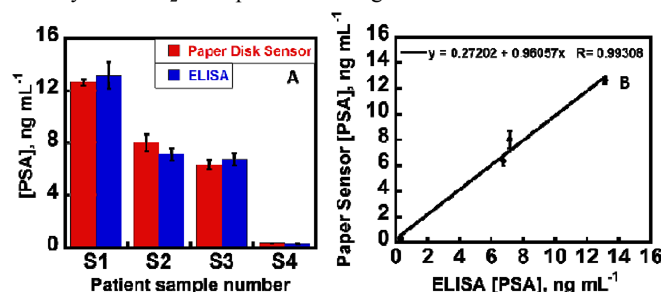

Fig. 3 Comparison of paper disk electrochemical immunoassay results to standard ELISA for PSA in patient serum samples. (a) Serum samples S1-S3 represent cancer patients and S4 a cancer-free patient. Error bars show standard deviation (n=3). (b) Correlation plot between paper disk array and standard ELISA with a slope of 0.96 ± 0.08.

enzymes (~400) per antigen-antibody binding event to improve sensitivity and limit of detection.[27] The fully developed assay on paper disks were dipped into a mixture of 1 mM hydroquinone mediator and 100 µM H_2O_2, then placed onto the sensor. DPV produced low noise peaks due to the oxidation of the iron heme of HRP labels by H_2O_2 to $Fe^{IV}=O$, whose reduction was mediated by hydroquinone mediator followed by the reduction of the oxidized mediator at the electrode surface (Scheme S2, ESI). Total assay time from PSA antigen capture to DPV measurement was ~15 mins. A complete calibration with 3 replicate measurements of 6 concentrations requires less than 10 mL of reagents including PBS and can be done in less than 2 hours. Multiple paper disks containing various concentrations during calibrations while a single senor chip is used Control DPV peaks from the full immunoassay procedure without PSA reflect the sum of residual non-specific binding and direct reduction of hydrogen peroxide. Representative

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DPV responses in Fig. 2 show peaks for PSA dissolved in undiluted calf serum in the pg mL^{-1} range. The detection limit (DL) for PSA determined as 3 times the average SD above the control was 6 pg mL^{-1}, well below the level in serum of cancer-free patients.

For use in resource-limited situations, fluctuations in temperature might affect assay performance. Also, at elevated temperatures, biomolecules tend to collide more frequently to increase the rate of binding, so that assay time can be decreased. Nearly identical sensitivities and detection limits were achieved for 16 min assay at 25°C and 10 min assay at 37 °C (Fig. S4).

The accuracy of the paper based immunoassay was validated by determining PSA using human serum samples and was compared with a standard Enzyme-linked Immunosorbent assay (ELISA). Samples 1-3 represent pooled serum from prostate cancer patients, and 4 was serum from cancer-free patients. The samples were diluted 50-fold with PBS to fall within the dynamic range of the calibration. Assay results showed very similar levels of PSA by our device and ELISA (Fig. S3A, ESI). Linear correlation plot of the two methods gave slope of 0.96 ± 0.08 and intercept of 0.27 ± 0.66 confirming the strong correlation (Fig. S3B, ESI). Selectivity is also demonstrated by these experiments since PSA is measured accurately in the presence of the many hundreds of other proteins in serum. Results above show that combining paper-based immunochemistry with sensors wet etched from gold CD offers a simple, inexpensive route to rapid, sensitive, reproducible detection of individual protein biomarkers. A single sensor chip as fabricated herein costs less than US \$0.35 in materials with ~\$1.50 for the immuno-reagents, and requires no specialized equipment other than a potentiostat. The reusability of the sensor elements further reduces the cost per test. The ease of customization and prototyping allows development for a wide variety of applications. Paper-based electrochemical immunoassays have advantages in simplicity compared to conventional methods. The time per assay of ~15 mins is particularly relevant and suitable for scenarios that benefit from rapid results. The short incubation time is a result of high surface to volume ratio of the paper and tiny volume (8 μL) that confines immuno-reagents in the paper leading to shorted incubation times for binding.

Classical ELISAs have served as the gold standard for protein detection.[28] Magnetic bead based ELISA-type methods with electrochemiluminescence (ECL) detection offer easy sample preparation and multiplexed detection. While these commercialized systems can achieve pg mL^{-1} level detection limits,[29,30] they are too expensive and requires high level of expertise and maintenance inconsistent with usage in resource-limited settings. We previously developed a microfluidic magnetic bead based assay that combines massively labeled detection particles with a nanostructured electrochemical array for simultaneous multiplexed protein detection in ~50 mins at low fg mL^{-1} levels. By sacrificing the ultralow detection limits for speed, assay time can be decreased to 8 mins while achieving clinically significant dynamic ranges.[31] In comparison, the current paper disk approach reported here requires no moving parts or microfluidic chambers. The only hardware is the sensor chip and a measuring potentiostat (can be handheld) to detect clinically relevant levels in 15 mins. Reagents for the paper based assay could be readily deployed in resource-limited clinics in environments where temperature control is not available. However, the paper immunoassay can currently measure only a single protein at a time. Both magnetic bead based and paper immunoassays suffer from an approach requiring trained personnel. The paper immunoassay system is technically simpler, affordable and portable.

In summary, paper disk immunoassays using an inexpensive gold CD sensor has excellent potential as a cheap diagnostic tool for single protein monitoring in serum. The sensors are readily adaptable to detection of other cancer biomarker proteins by just changing the antibodies. The use of a simple amplification strategy enables immuno-detection of PSA at low pg mL^{-1} in ~15 mins. Further studies of temperature effects and the storage life of the antibody-loaded disks are being pursed. Reusability and portability of this paper immunoassay system and the possibility to use existing hand-held potentiostats presents a cost effective single biomarker cancer diagnostic tool for resource-limited settings.

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