Highly sensitive detection of human cancer antigens by an immunogold-silver assay chip coupled with a polythiophene-based optical sensor*

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***Abstract*— This work presents a novel method for protein or cancer antigen detection in clinical samples by an immunogold-silver assay microfluidic biochip coupled with a polythiophene-based organic photodetector. The method has showed a detection limit below 1ng/mL and the low cost and high sensitivity of both organic photodetector and immunogold-silver assay make this method amenable for realization in a portable handheld probe tip biosensor.**

I. Introduction

Highly sensitive detection of proteins is often an important step towards early diagnosis of many diseases, cancer in particular, because even a small number of molecules of proteins are often sufficient to affect the normal function of cells [1, 2]. Hence, the low abundance of protein biomarkers in body fluids or tissues demands for highly sensitive techniques, and the realization of these detection techniques in inexpensive point-of-care devices is still challenging.

Generally, high detection sensitivity is achieved in the clinical laboratories using laborious assays coupled with bulky optical instrumentation. Due to its easy protocol, cheap reagents, robustness, and low detection limit, the immunogold-silver assay holds a great promise for inexpensive but sensitive biosensors. The simplicity of the immunogold-silver assay makes it amenable for microfluidic devices handling human fluid samples [3]. Furthermore, for a point-of-care device, the complex optical instrumentation can be replaced with the inexpensive organic/polymer based optical detectors [4, 5]. The organic photodetectors (OPDs) can easily be fabricated onto low-cost glass or polymer substrates, on contrary to their silicon-based counterparts, and have been shown to retain high detection sensitivity and specificity in microfluidic biosensors testing clinical samples [6].

In this work, the immunogold-silver assay is conducted in a glass biochip with enhanced reduction of unspecific protein adsorption, and this biochip is integrated with a bulk heterojunction OPD of poly[4,8-bis-substituted-benzo[1,2-b:4,5-b'] dithiophene-2,6-diyl-alt-4-substituted-thieno[3,4-b]thiophene-2,6-diyl] (PBDTTT) and (6,6)-phenyl-C_{70}-butyric-acid methyl-ester ($PC_{70}BM$). This integrated approach is intended to be the active sensing part of a probe tip based sensor and method shown in Fig. 1. The PBDTTT:$PC_{70}BM$ OPD, although has been widely studied in solar cell applications, has never been reported in biosensors or diagnostic devices so far. Remarkably, this OPD can show external quantum efficiencies approaching 70% for 600-700 nm [7], which makes it interesting for measuring output of the immunogold-silver assay (~650 nm). For the detection tests, the human carcinoembryonic antigen (CEA), a glycoprotein marker extensively studied in clinical diagnosis of different cancers [1, 2], was selected as the model analyte.

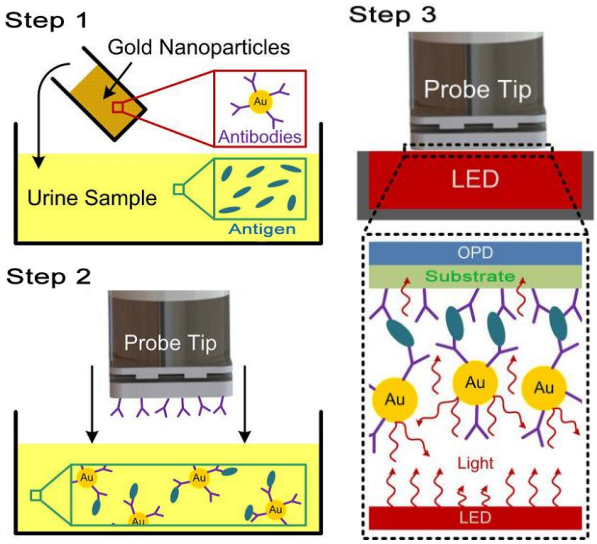

Figure 1. Schematic of the probe tip sensing method incorporating gold nanoparticles, light emitting diode (LED) and sensitive organic phodetector (OPD). The antigen-recognition probes are immobilized onto glass or polymer substrate surface of the integrated OPD.

II. Probe Tip Based Detection Technique

The probe tip sensor depicted in Fig. 1 aims to be a portable handheld device providing rapid detection of protein antigens when contacting the biological sample (i.e. urine, saliva or serum). Firstly, the method would require a sample

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pre-preparation step, where a solution of gold nanoparticles (AuNPs) coated by antibody is added into the biological sample. After incubation allowing interaction of the antibody-coated AuNPs with the specific antigen, the probe tip sensor is dipped into the biological sample. The probe tip surface containing the sensitive OPD is previously modified with antigen-specific antibodies, which capture the antigen bonded to the immunological AuNPs. Further, the probe tip is situated close to a light source emitting a specific wavelength detectable by the integrated OPD. Due to the presence of AuNPs immunologically attached to the probe, the emitting light is then blocked from the immobilized particles. Higher amount of attached AuNPs would thus mean lower photocurrent response of the OPD. The light blocking effects from the immobilized AuNPs can be enhanced by depositing the silver enhancer solution, thus completing the immunogold-silver assay [3]. The photocurrent signal could be transmitted via wireless to the user interface or immediately read in a liquid-crystal display.

III. Materials & Methods

To demonstrate the basic detection principle of the probe tip sensor, an experimental setup was constructed with three modules (see Fig. 2), a commercial red light-emitting diode (LED) used as the light source, a glass- polydimethylsiloxane (PDMS) chip in where the immunogold-silver assay is developed, and a fabricated OPD made of PBDTTT:$PC_{70}BM$ bulk heterojunction. The immunogold-silver assay chip, placed between the LED and OPD, is exposed to the red light and, after addition of the silver enhancer solution (containing silver acetate and hydroquinone) on the AuNP-immune complex, the transparent chip turns to opaque. The AuNPs catalyze the reduction of silver ions to metallic silver, and the resultant opaque silver-AuNPs film partially blocks the transmission of red light through the glass biochip.

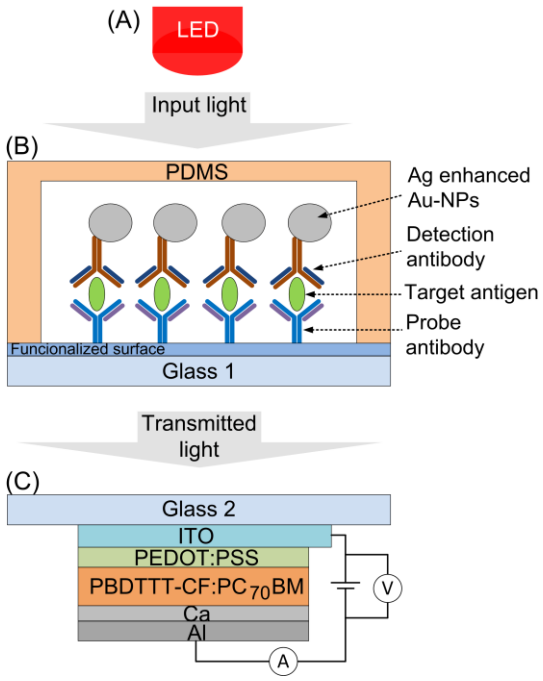

Figure 2. The biosensing scheme for the detection of human carcinoembryonic antigen using the immunogold-silver assay. The measurement setup comprises: (A) red LED as light source, (B) immunogold-silver assay chip and (C) PBDTTT:$PC_{70}BM$ OPD.

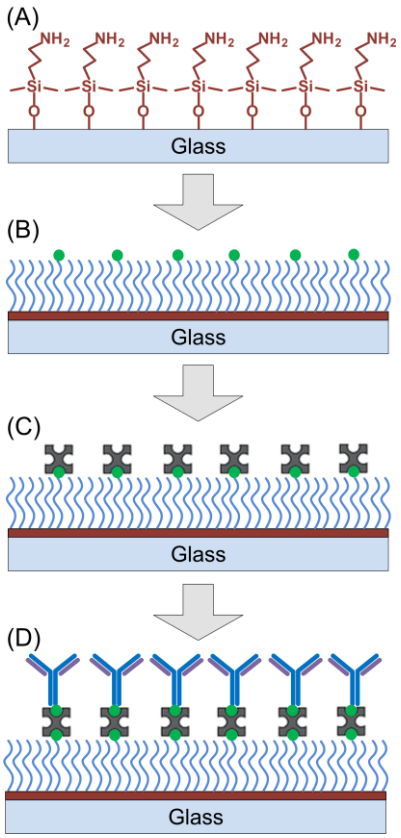

Figure 3. The procedure and technique of immobilizing the antigen-recognition probe onto the glass chip. (A) Silanization; (B) Surface passivation with biotin-polyethylene glycol; (C) NeutrAvidin interaction; (D) Biotinylated antibody binding.

A. Biological probe immobilization & reaction chip

For performing the immunogold-silver assay, the glass biochip was functionalized with probe antibody specific to CEA. The surface functionalization process is summarized in Fig. 3. Firstly, the glass surface is subjected to silanization, and this is achieved by covalently linking an amine-containing silane (i.e. 3-aminopropyltriethoxysilane, APTES) to the glass (Fig. 3a). Thus, the glass surface was treated with APTES at ~1% v/v in ethanol for 10 min at room temperature. This was followed by rising with toluene and ethanol to remove non-covalently absorbed silane. Subsequently, to substantially reduce binding of unspecific proteins to the glass surface, the chip surface was passivated, and a biotin-containing polyethylene glycol (PEG) was used as the passivation layer (Fig. 3b). Hence, the silanized glass was incubated overnight at room temperature with methoxy-PEG-succinimidyl valerate and biotin–PEG–succinimidyl valerate, prepared in solution with sodium bicarbonate buffer. Any unbounded PEG/PEG-biotin is then washed out with distilled water. Further, the glass substrate passivated with PEG/PEG-biotin was allowed to react with neutravidin for 10 min (Fig. 3c), with unbound neutravidin molecules removed by washing the substrate with phosphate-buffered saline (PBS). Finally, the neutravidin linkers allowed specific immobilization of biotinylated probe antibody onto the glass biochip (Fig. 3d).

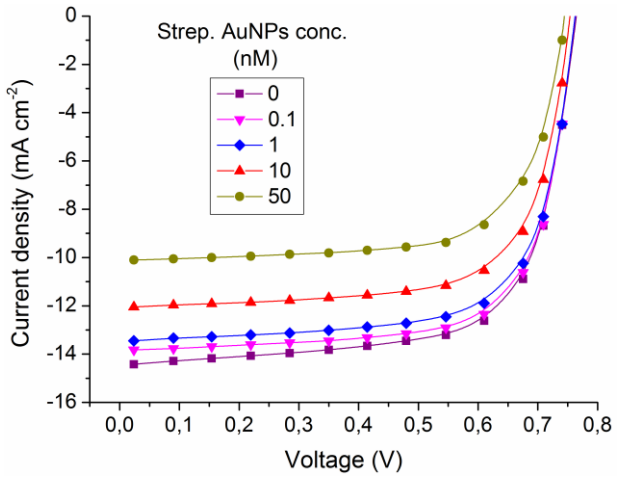

Figure 4. Change in current-voltage response of the PBDTTT:$PC_{70}BM$ OPD due to different streptavidin-gold conjugate concentrations, which were detected using after silver enhancement process.

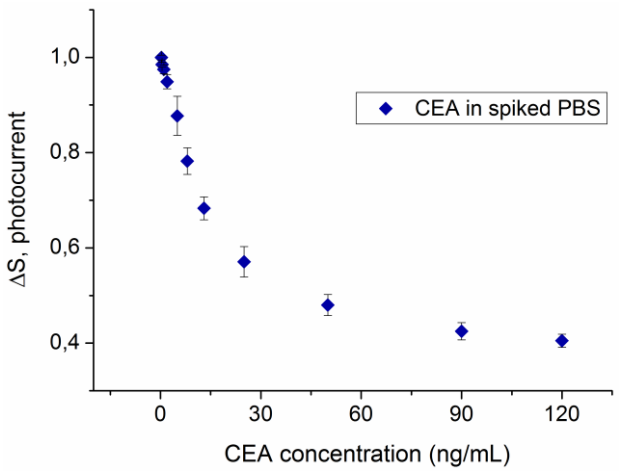

Figure 5. Detection of human carcinoembryonic antigen (CEA) in phosphate-buffered saline (PBS), using the immunogold-silver assay chip coupled with the PBDTTT:$PC_{70}BM$ OPD operated with no bias.

For delivery of assay reagents to the glass biochip, a microfluidic structure made of PDMS was in addition prepared. A mixture of a Sylgard 184 precursor and a curing agent at a ratio of 10:1 (w/w) was degassed in a vacuum chamber for 30 min and poured onto a SU-8 master template. After curing the structured PDMS layer was peeled off from the mold and added on top of the glass biochip by non-permanent bonding, as exploiting the strong adhesion of cured PDMS to glass. Openings for the inlet and outlet were arranged on the PDMS for reagent and sample flow.

B. Polythiophene-based optical sensor

The structure of the polythiophene-based OPD, as schematically shown in Fig. 2, was realized onto transparent indium tin oxide (ITO)/glass substrates. A thin layer of poly(3,4-ethylenedioxythiophene): polystyrene sulfonate (PEDOT:PSS), used as received, was first spin-coated onto the ITO/glass. This was followed by spin coating the PBDTTT-CF:$PC_{70}BM$ blend from the solvent mixture of 1, 8-diiodoctane (3% v/v) in o-dichlorobenzene. The weight ratio of PBDTTT-CF (used as received) to $PC_{70}BM$ (used as received), both dissolved in the solvent mixture, was 1:1.5. The resultant PBDTTT-CF:$PC_{70}BM$ film had a thickness of approximately 100 nm, as measured with a profilometer. Following deposition of the active layer, the Ca/Al electrode was added on top of the polymer structure by means of thermal evaporation under vacuum pressure. The fabricated devices were encapsulated using UV-curable epoxy.

C. Immunogold-silver assay

Diluted CEA standard solutions in PBS buffer and diluted anti-CEA detection antibodies conjugated with AuNPs were added into the glass-PDMS biochip, and allowed to incubate over the antibody-functionalized glass surface to form the immune-sandwich complexes. Bovine serum albumin (BSA) was previously added to the antigen and AuNPs-conjugate solutions to reduce probability of unspecific binding and aggregation. After washing out unbound antigens and AuNPs-conjugates from the biochip, the silver enhancer solution, containing equal volumes of silver acetate and hydroquinone, was interacted with the immunologically attached AuNPs. Immediately after 15 min the autocatalytic reaction between gold conjugates and silver enhancer was stopped by removing all the solutions from the biochip. Rapidly the biochip was then aligned between the red LED and PBDTTT:$PC_{70}BM$ OPD, and output photocurrent from the OPD was measured with a Keithley source measure unit, with the recorded data transferred to a PC via USB-GPIB.

IV. Results & Discussion

To demonstrate the effectiveness of immunogold-silver staining onto the PEG/PEG-biotin passivated surface, the glass-PDMS biochip was first challenged with streptravidin-gold nanoparticles conjugate. For the tests, this conjugate was diluted at different concentrations in solutions of PBS and BSA. The results shown in Fig. 4 demonstrated decreasing photocurrent response of the OPD with increasing concentration of streptavidin-AuNPs conjugate. This indicated that higher number of AuNPs, attached to the PEG/PEG-biotin surface due to strong streptavidin-biotin interactions, had led to higher opacity of the reduced silver film. These observations correlated well with apparent absorbance measurements conducted in parallel using a UV/Vis plate reader.

The subsequent analytical tests towards CEA detection were performed after the neutravidin linker and biotinylated detection antibody were immobilized onto the PEG/PEG-biotin surface of the biochip (steps 3 and 4 in Fig. 3). The normalized photocurrent (ΔS), determined as the ratio of the photocurrent measured at different CEA concentrations to the current measured at 0 ng/mL CEA, is plotted in Fig. 5. Triplicate assays were conducted for detecting each CEA concentration, and no bias voltage was applied for the OPD current measurements. Based on the normalized photocurrent variations the detection range for CEA was 0.3-120 ng/mL, with 0.3 ng/mL as the lowest detected CEA concentration. This limit of detection was lower than 1 ng/mL obtained with another immunogold-silver assay glass chip, which has employed no glass surface passivation and was coupled with an amorphous silicon photodetector instead of using an OPD [8]. The high short-circuit photocurrent response reached by

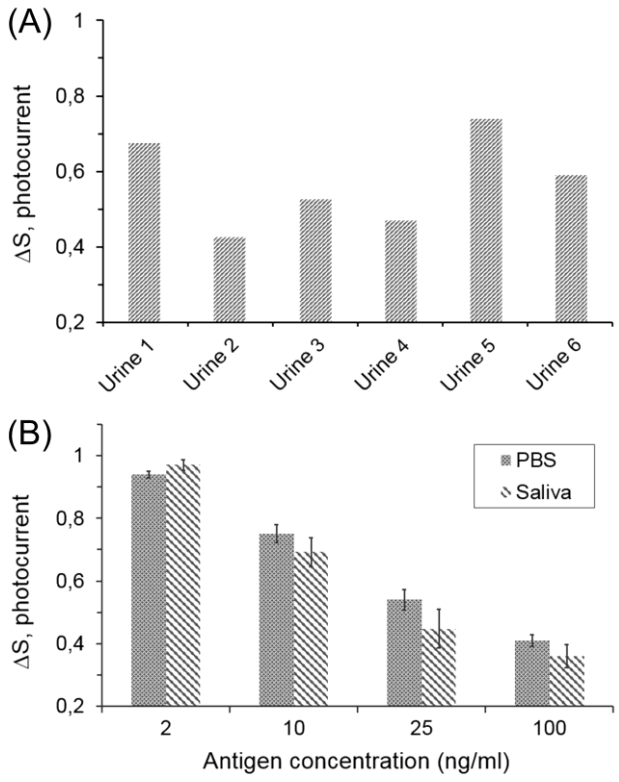

Figure 6. Analytical results of biological samples. (A) Normalized photocurrent response of the OPD due to detection of CEA in clinical urine samples. (B) Cancer antigen detection in artificial saliva.

the PBDTTT:$PC_{70}BM$ and the low protein adsorption conferred by the PEG/PEG-biotin chip surface may have decisively contributed to the enhancement of the detection sensitivity. Measurements of external quantum efficiency (EQE), following a determination method previously described in [6], resulted in a high value of 68% exhibited by the PBDTTT:$PC_{70}BM$ under an incident wavelength of 650 nm, the same emitted by the red LED used in the detection of CEA. An enhanced EQE meant an enhanced photocurrent response, as EQE is determined from the ratio of the current density collected at OPD electrodes to the flux density of incident photons. Furthermore, besides the high detection sensitivity, the small error bars (<5%) for each CEA concentration shown in Fig. 5 indicated high detection reproducibility of the biosensing scheme used in this study.

To investigate the amenability of the developed OPD-coupled glass-PDMS biochip for clinical diagnostics, six urine samples of adult subjects were tested by the immunogold-silver assay used here. The CEA in the urine samples were within the range of 13 ± 1.7 ng/mL to 69 ± 9.2 ng/mL as confirmed by a commercially available ELISA method. The results shown in Fig. 6a demonstrated that the developed method was able to distinguish the six different CEA concentrations among the tested urine samples. Moreover, to test another biological sample matrix, artificial saliva samples spiked with four different CEA concentrations were prepared and detected using the immunogold-silver assay. The preparation of artificial saliva followed the method described in [9]. Briefly, disodium phosphate, anhydrous calcium chloride, potassium chloride, sodium chloride, mucin and urea were all dissolved in distilled water. The results for ΔS (see Fig. 6b) showed that the photocurrent response of the OPD, testing the spiked saliva samples, showed good correlation with those obtained using spiked PBS samples used as the control.

V. Conclusion

A novel OPD-coupled immunogold-silver assay microfluidic biochip was demonstrated in this study. The biosensing method exhibited a detection limit of 0.3 ng/mL exploiting the use of the highly sensitive PBDTTT:$PC_{70}BM$ bulk heterojunction OPD for first time and performing the immunogold-silver assay onto a passivated glass chip with low unspecific protein adsorption. Besides retaining high sensitivity, the low cost of both OPD and immunogold-silver assay makes the biosensing method feasible to realize in a portable handheld probe tip-like biosensor. The results have shown the capability of the method to detect cancer antigens in real biological samples.

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Statement

The experimental procedures involving human urine samples were done in accordance with principles of the Helsinki Declaration (7th revision, 2013)

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