Gold-black Micropillar Electrodes for Microfluidic ELISA of Bone Metabolic Markers

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Summary

Modification with gold black considerably increased the detection current of protruding micropillar electrodes in microfluidic electrochemical enzyme-linked immunosorbent assay (ELISA).

There has been a growing interest in the miniaturization of analytical systems for biochemical sensing applications because miniaturized systems could offer many potential advantages over the conventional assay platforms. Owing to their simplicity

and portability, miniaturized electrochemical sensor chips facilitate bedside diagnosis and assays at home, such as self-monitoring of blood glucose in diabetic patients[1]. However, one of the major problems with detection using miniaturized systems is the low detection current. A typical miniaturized system consists of two-dimensional (2D) planar electrodes placed on the bottom of a microchannel. Although electrochemical sensing using a microchannel is generally considered to be highly efficient, the reactions are in fact limited to the vicinity of the planar electrodes and, despite the small size of the channel, a large proportion of the analyte molecules may pass over the electrodes without being used for the analysis[2]. In this study, we performed a preliminary evaluation of the efficiency of a 2D planar working electrode using a typical electrochemical microfluidic system. A planar working electrode (500 μm × 500 μm) was formed in a microchannel (width: 500 μm and height: 55 μm), and a solution of 1 mM L-ascorbic acid, as a model electroactive analyte, was passed over the electrode at a flow rate of 10 μl/min. A simple calculation made using the obtained results revealed that only ~10% of the analyte was consumed by the 2D electrode under this condition. If we assume that the flow mode is 2D Poiseuille flow, 10% of the analyte is equivalent to the number of molecules that flow within ~10 μm above the surface of the electrode. Fabrication of a narrow microchannel with a 10-μm height

and 500-μm width in a reproducible manner is not necessarily easy. Furthermore, reducing both height and width may increase the pressure drop and decrease the detection current of the electrode.

Micro/nano fabrication technologies such as nano electrode arrays[3], platinum-black electrode arrays[4], and electrodes modified with nanotubes[5] and nanoparticles[6] have been used to prepare lab-on-a-chip biosensors. Although the use of some of these technologies enlarges the surface area of the electrode and improves its detection current and sensitivity, these modifications have been applied to inherently 2D planar electrodes, and are not necessarily able to collect a considerable amount of analyte molecules passing through the region that is at a distance of more than 10 μm from the electrode surface in a microchannel. Meanwhile, electrodes with protruding three-dimensional (3D) micro-scale structures, which may be instrumental in collecting analytes that pass over the electrode in a microchannel, have scarcely been used in lab-on-a-chip biosensors. In a few previous studies[7,8], the potential application of 3D electrodes in ELISA had not been investigated; nevertheless, their results clearly indicate the feasibility of using protruding 3D electrodes in microfluidic biosensors. Needless to say, it is expected that the detection current of microfluidic biosensors can be increased by increasing the aspect ratio of the 3D microstructures. However, the

fabrication of high-aspect-ratio structures is not easy and there are practical limitations. In this study, to overcome this technical challenge, we deposited nanoporous structures on 3D micropillar electrodes by electroplating and assessed their performance. The electrochemical behavior of the nanoporous 3D electrodes in ELISA of bone metabolic marker proteins was studied by cyclic voltammetry and amperometry.

A PDMS substrate with a microchannel was placed on top of a glass substrate with electrodes (Fig. 1). A detailed description of its fabrication procedure is provided in the Electronic Supplementary Information. Briefly, a planar three-electrode system was patterned using the conventional photolithography techniques, including sputter-deposition of metals, photoresist patterning, and chemical etching. 3D micropillars with a height of 50 μm and diameter of 30 μm were fabricated using a thick film photoresist SU-8 on the active area of a gold working electrode. A new gold layer was sputter-deposited on the micropillars of the working electrode through a stainless steel mask with a square hole of 500 μm × 500 μm. The surface of the micropillars was modified with gold black at a current density of –60 μA/mm^2 for 5 min in a vigorously stirred solution containing 83 mM hydrogen tetrachloroaurate(III) tetrahydrate and 1.58 mM lead(II) acetate[9]. Scanning electron microscopy was used to reveal the formation of the nanoporous gold black layer on the gold surface of the 3D

micropillar electrode (Fig. 1b).

First, we used a 3D micropillar electrode modified with gold black and having no microchannel to perform ELISA of bone alkaline phosphatase (BAP) and then characterized the electrode by cyclic voltammetry. BAP is secreted by osteoblasts that are responsible for bone formation by the mineralization of the bone matrix. Assay using BAP and several other similar markers is more sensitive and effective in detecting osteogenesis as compared to other typical examinations such as bone densitometry[10, 11]. A drop of 20 μg/ml primary monoclonal antibody against human BAP was placed on four different working electrodes: two 3D electrodes with and without gold black, and two 2D electrodes with and without gold black. The antibody was then physically adsorbed for 10 min. After blocking with lactoprotein, a drop of 10 U/L BAP was placed on the electrodes and incubated for 10 min. After washing with PBS, 20 μg/ml secondary biotinylated antibody against human BAP and 0.5 U/ml β-galactosidase-streptavidin complex were subsequently placed on the electrodes and incubated for 10 and 15 min, respectively. Finally, a drop of 4.5 μM *p*-aminophenyl-β-D-galactopyranoside (PAPG) was placed on the electrodes. β-galactosidase converts PAPG to *p*-aminophenol (PAP). After 10-min incubation, cyclic voltammograms were recorded at a scanning rate of 20 mV/s from +0.8 to –0.4 V

vs. the on-chip Ag/AgCl reference electrode by using an Autolab PGSTAT12 potentiostat (Eco Chemie, Utrecht, Netherlands). Under all conditions, distinct peaks due to the oxidation of PAP were observed at approximately +0.1 V vs. the Ag/AgCl reference electrode (Fig. 2), which are consistent with the results of previous reports[12, 13]. The peak height for the 3D electrode was 6.3 times larger than that for the 2D electrode. The peak height increased further for the electrode modified with gold black; the peak height for the 3D electrode with gold black was 9.2 times larger than that for the 2D electrode without gold black. These results clearly show that the 3D micropillar electrode modified with gold black is beneficial for ELISA.

In the above experiments, we demonstrated the applicability of a 3D electrode for a single assay by directly performing the ELISA on the surface of the micropillars. Then, we used this 3D-electrode system to fabricate a more versatile ELISA device. Multiple samples can be measured using a single sensing electrode by separating the ELISA reaction site from the electrode and switching the analyte solution flowing into the electrode. Microbeads trapped in a microchannel have been typically used as a reaction site[14]. However, a possible challenge in this approach is to uniformly pack the microbeads to form a dam region in the microchannel for preventing biased flow and thus acquiring reproducible data. In this study, the reactions were performed on the

surface of PDMS micropillars uniformly fabricated in a serpentine channel (Fig. 1). The enzymatically converted analyte was detected downstream by amperometry. The SU-8 master for the PDMS mold was fabricated using a mask having the desired shape. The serpentine channel was 55 μm in height, 500 μm in width, and 50 mm in length. The PDMS micropillars were 55 μm in height and 50 μm in diameter. The inter-pillar distance (edge to edge) was 50 μm, and the number of PDMS micropillars was approximately 6000. As a preliminary experiment to test this microfluidic detection system, we directly immobilized β-galactosidase onto the PDMS micropillars via covalent binding with glutaraldehyde[15]. After activating the surface of the micropillars, 0.5 U/ml β-galactosidase solution was introduced into the microchannel at a flow rate of 1 μl/min for 15 min. Following this, PAPG with six different concentrations was introduced into the microchannel at 1 μl/min and enzymatically converted PAP was oxidized downstream at the planar working electrode. The potential of the working electrode was set to +0.3 V (vs. Ag/AgCl), and the steady oxidation current was recorded using the potentiostat. In this amperometric assay, a clear correlation was observed between the detection current and the concentration of PAPG (Fig. 3a). Although the enzyme may be denatured during the binding step, our results indicate that at least 4.5 μM PAPG is required for ELISA in this setup to obtain an enough detection

current.

Next, we used our designed microdevice for ELISA of another bone metabolic marker, tartrate-resistant acid phosphatase-5b (TRACP-5b). BAP and TRACP-5b are used as markers for bone formation and resorption, respectively. The PDMS substrate with the micropillars in the serpentine channel was placed on a bare glass slide. After activating the surface of the PDMS micropillars as described above, 20 μg/ml primary antibody against human TRACP-5b was introduced into the microchannel at a flow rate of 1 μl/min for 10 min. The entire surface of the microchannel was then blocked with lactoprotein, and TRACP-5b with five different concentrations was introduced into the microchannel at 1 μl/min and allowed to react with the immobilized TRACP for 10 min. After washing with PBS, 20 μg/ml secondary biotinylated antibody against human TRACP-5b was introduced into the microchannel for 10 min. Then, 0.5 U/ml β-galactosidase-streptavidin complex was introduced at a flow rate of 1 μl/min and allowed to react with the secondary antibody labeled with biotin for 15 min. The PDMS substrate was subsequently peeled off from the glass slide and placed on the substrate with the 3D working electrode. Finally, 4.5 μM PAPG was introduced into the microchannel at 1 μl/min and the detection current was measured. The enzymatically converted PAP was detected using the 3D micropillar electrode modified

with gold black and the 2D planar electrode without gold black. In both the cases, a linear relationship was observed (Fig. 3b). Because the average TRACP-5b activity in normal women is 2.83 ± 1.1 U/L and it increases with age, it is important to detect any abnormal increase in the activity at an early stage[16]. The detection current of the micropillar electrode modified with gold black increased considerably by ~10 times as compared to that of the 2D electrode. On the other hand, the higher surface electrodes increase noise in general. If the signal-to-noise (s/n) ratio is defined such that the obtained current represents the signal and the corresponding standard deviation represents the noise, then, the s/n ratios, calculated using the data plotted in Fig. 3(b), are 12.5 ± 4.7 for the 2D electrode and 12.1 ± 5.2 for the 3D electrode. As such, there is no significant difference between the s/n ratios of the 2D and 3D electrodes. The 3D electrode with a larger output current should be beneficial because it enables the use of a portable and cheap electrochemical instrument, without requiring specially designed noise-reduction circuits.

In conclusion, a microfluidic device with 3D micropillar electrodes was developed for ELISA. Gold black was electrochemically deposited onto the surface of the micropillars. This modification considerably increased the detection current of the electrodes. The efficacy of the 3D micropillar electrodes was demonstrated by

performing electrochemical ELISA of bone metabolic marker proteins. In this study, we focused on the applicability of the 3D electrodes and performed ELISA off chip. In the future, we will use the electrodes in conjunction with lab-on-a-chip components such as valves for manipulating multiple samples and solutions for ELISA on a chip. Furthermore, because there are several bone metabolic marker proteins, and it is desirable to simultaneously detect them on an electrode, our future work aims to achieve the simultaneous detection of multiple analytes in serum using the designed electrodes[17, 18]. Our versatile and efficient 3D micropillar electrodes could potentially be a fundamental tool for the development of more sophisticated on-site diagnosis systems.

Acknowledgments

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Figure captions

Figure 1

Microdevice. (a) Schematic of device containing 3D micropillar electrode and PDMS

micropillars in serpentine microchannel. W.E., working electrode; R.E., reference electrode; A.E., auxiliary electrode. (b) Scanning electron microscopy images of 3D micropillar electrode (50 μm in height and 30 μm in diameter), gold black deposited on micropillars, and PDMS micropillars (55 μm in height and 50 μm in diameter). The SU-8 micropillars modified with gold black were used as the working electrode to detect an analyte, whereas the bare PDMS micropillars were used at the reaction sites for performing ELISA.

Figure 2

Detection of BAP by cyclic voltammetry. Cyclic voltammograms were recorded using four different electrodes at a scanning rate of 20 mV/s vs. the Ag/AgCl reference electrode. The arrow indicates the electrode potential for PAP.

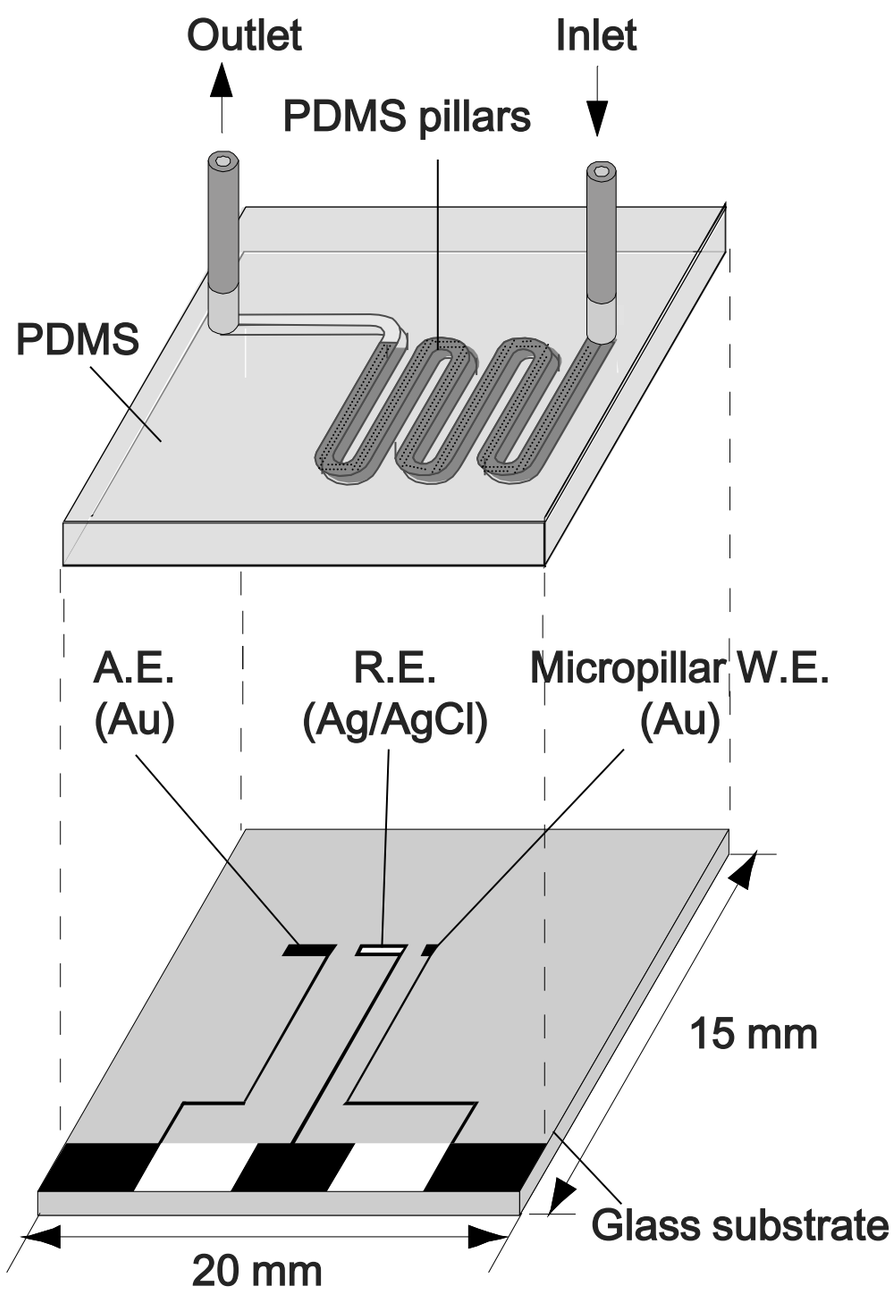
Figure 3

Microfluidic assay performed using device having PDMS micropillars in serpentine microchannel. (a) Dependence of detection current on concentration of PAPG. β-galactosidase was immobilized on the PDMS micropillars, and PAP converted from PAPG was detected downstream at the 2D electrode. (b) Dependence of detection

current on concentration of TRACP-5b. ELISA was performed on the PDMS micropillars, and PAP converted from PAPG was detected downstream at the 3D micropillar electrode with gold black and the 2D electrode.

Fig. 1 Sonthaya Nui et al.

(a)



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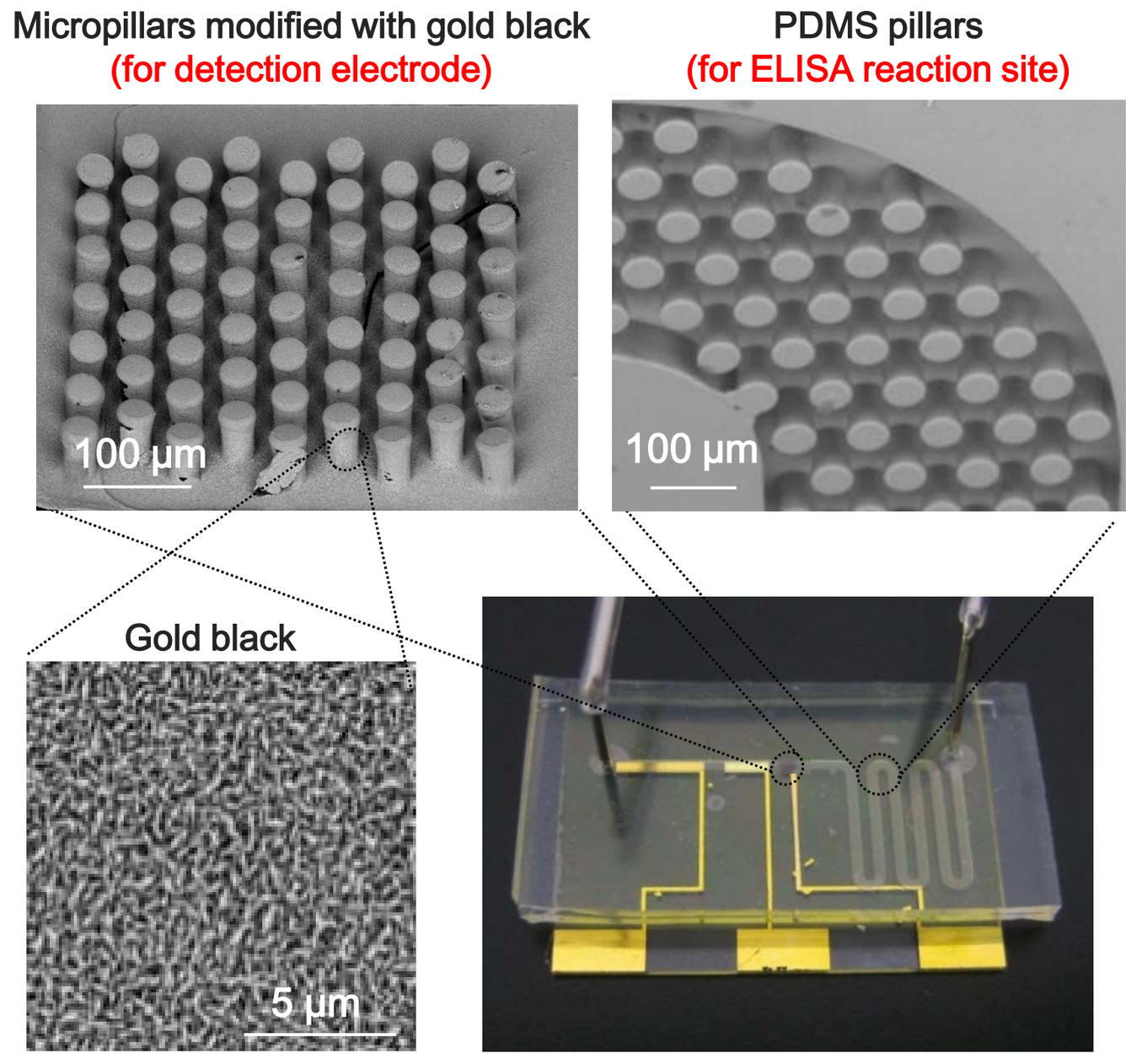


Fig. 2 Sonthaya Nui et al.

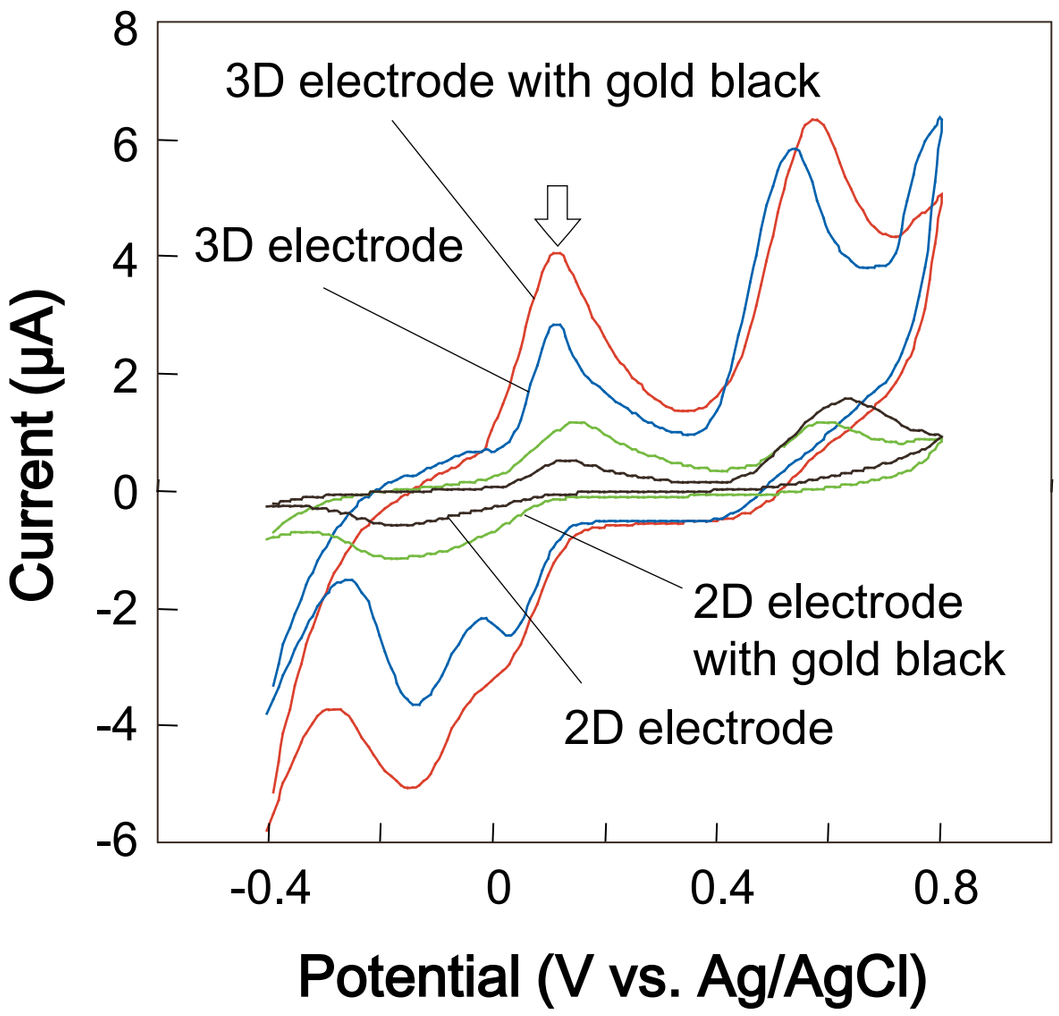
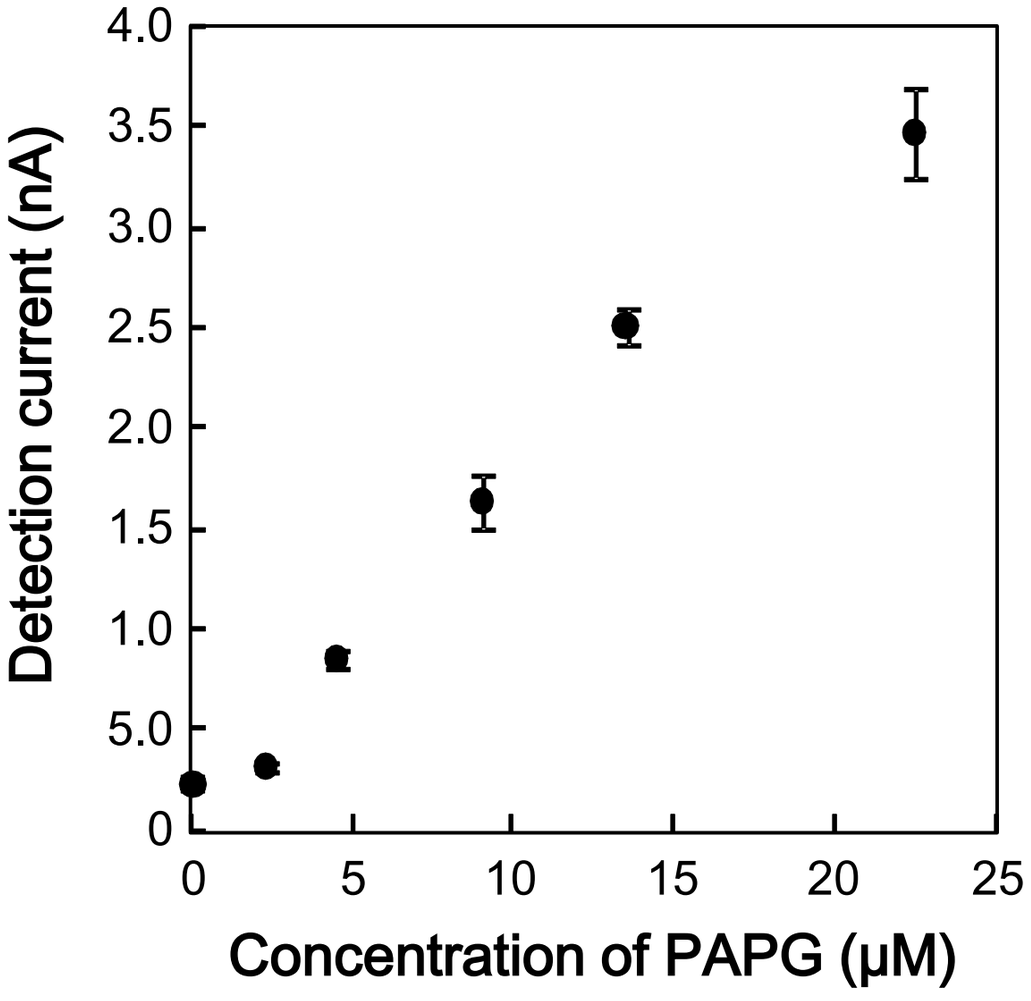


Fig. 3 Sonthaya Nui et al.

(a)



(b)