

Gold-black micropillar electrodes for microfluidic ELISA of bone metabolic markers

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

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10

11 **Summary**

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

15

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

1 and portability, miniaturized electrochemical sensor chips facilitate bedside diagnosis
2 and assays at home, such as self-monitoring of blood glucose in diabetic patients¹.
3 However, one of the major problems with detection using miniaturized systems is the
4 low detection current. A typical miniaturized system consists of two-dimensional (2D)
5 planar electrodes placed on the bottom of a microchannel. Although electrochemical
6 sensing using a microchannel is generally considered to be highly efficient, the
7 reactions are in fact limited to the vicinity of the planar electrodes and, despite the small
8 size of the channel, a large proportion of the analyte molecules may pass over the
9 electrodes without being used for the analysis². In this study, we performed a
10 preliminary evaluation of the efficiency of a 2D planar working electrode using a
11 typical electrochemical microfluidic system. A planar working electrode (500 μm \times
12 500 μm) was formed in a microchannel (width: 500 μm and height: 55 μm), and a
13 solution of 1 mM L-ascorbic acid, as a model electroactive analyte, was passed over the
14 electrode at a flow rate of 10 $\mu\text{l}/\text{min}$. A simple calculation made using the obtained
15 results revealed that only $\sim 10\%$ of the analyte was consumed by the 2D electrode under
16 this condition. If we assume that the flow mode is 2D Poiseuille flow, 10% of the
17 analyte is equivalent to the number of molecules that flow within ~ 10 μm above the
18 surface of the electrode. Fabrication of a narrow microchannel with a 10- μm height

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

4 Micro/nano fabrication technologies such as nano electrode arrays³, platinum-black
5 electrode arrays⁴, and electrodes modified with nanotubes⁵ and nanoparticles⁶ have been
6 used to prepare lab-on-a-chip biosensors. Although the use of some of these
7 technologies enlarges the surface area of the electrode and improves its detection
8 current and sensitivity, these modifications have been applied to inherently 2D planar
9 electrodes, and are not necessarily able to collect a considerable amount of analyte
10 molecules passing through the region that is at a distance of more than 10 μm from the
11 electrode surface in a microchannel. Meanwhile, electrodes with protruding
12 three-dimensional (3D) micro-scale structures, which may be instrumental in collecting
13 analytes that pass over the electrode in a microchannel, have scarcely been used in
14 lab-on-a-chip biosensors. In a few previous studies^{7,8}, the potential application of 3D
15 electrodes in ELISA had not been investigated; nevertheless, their results clearly
16 indicate the feasibility of using protruding 3D electrodes in microfluidic biosensors.
17 Needless to say, it is expected that the detection current of microfluidic biosensors can
18 be increased by increasing the aspect ratio of the 3D microstructures. However, the

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

6 A PDMS substrate with a microchannel was placed on top of a glass substrate
7 with electrodes (Fig. 1). A detailed description of its fabrication procedure is provided
8 in the Electronic Supplementary Information. Briefly, a planar three-electrode system
9 was patterned using the conventional photolithography techniques, including
10 sputter-deposition of metals, photoresist patterning, and chemical etching. 3D
11 micropillars with a height of 50 μm and diameter of 30 μm were fabricated using a thick
12 film photoresist SU-8 on the active area of a gold working electrode. A new gold layer
13 was sputter-deposited on the micropillars of the working electrode through a stainless
14 steel mask with a square hole of 500 $\mu\text{m} \times 500 \mu\text{m}$. The surface of the micropillars
15 was modified with gold black at a current density of $-60 \mu\text{A}/\text{mm}^2$ for 5 min in a
16 vigorously stirred solution containing 83 mM hydrogen tetrachloroaurate(III)
17 tetrahydrate and 1.58 mM lead(II) acetate⁹. Scanning electron microscopy was used to
18 reveal the formation of the nanoporous gold black layer on the gold surface of the 3D

1 micropillar electrode (Fig. 1b).

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

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

10 In the above experiments, we demonstrated the applicability of a 3D electrode
11 for a single assay by directly performing the ELISA on the surface of the micropillars.
12 Then, we used this 3D-electrode system to fabricate a more versatile ELISA device.
13 Multiple samples can be measured using a single sensing electrode by separating the
14 ELISA reaction site from the electrode and switching the analyte solution flowing into
15 the electrode. Microbeads trapped in a microchannel have been typically used as a
16 reaction site¹⁴. However, a possible challenge in this approach is to uniformly pack the
17 microbeads to form a dam region in the microchannel for preventing biased flow and
18 thus acquiring reproducible data. In this study, the reactions were performed on the

1 surface of PDMS micropillars uniformly fabricated in a serpentine channel (Fig. 1).
2 The enzymatically converted analyte was detected downstream by amperometry. The
3 SU-8 master for the PDMS mold was fabricated using a mask having the desired shape.
4 The serpentine channel was 55 μm in height, 500 μm in width, and 50 mm in length.
5 The PDMS micropillars were 55 μm in height and 50 μm in diameter. The inter-pillar
6 distance (edge to edge) was 50 μm , and the number of PDMS micropillars was
7 approximately 6000. As a preliminary experiment to test this microfluidic detection
8 system, we directly immobilized β -galactosidase onto the PDMS micropillars via
9 covalent binding with glutaraldehyde¹⁵. After activating the surface of the micropillars,
10 0.5 U/ml β -galactosidase solution was introduced into the microchannel at a flow rate of
11 1 $\mu\text{l}/\text{min}$ for 15 min. Following this, PAPG with six different concentrations was
12 introduced into the microchannel at 1 $\mu\text{l}/\text{min}$ and enzymatically converted PAP was
13 oxidized downstream at the planar working electrode. The potential of the working
14 electrode was set to +0.3 V (vs. Ag/AgCl), and the steady oxidation current was
15 recorded using the potentiostat. In this amperometric assay, a clear correlation was
16 observed between the detection current and the concentration of PAPG (Fig. 3a).
17 Although the enzyme may be denatured during the binding step, our results indicate that
18 at least 4.5 μM PAPG is required for ELISA in this setup to obtain an enough detection

1 current.

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

1 with gold black and the 2D planar electrode without gold black. In both the cases, a
2 linear relationship was observed (Fig. 3b). Because the average TRACP-5b activity in
3 normal women is 2.83 ± 1.1 U/L and it increases with age, it is important to detect any
4 abnormal increase in the activity at an early stage¹⁶. The detection current of the
5 micropillar electrode modified with gold black increased considerably by ~10 times as
6 compared to that of the 2D electrode. On the other hand, the higher surface electrodes
7 increase noise in general. If the signal-to-noise (s/n) ratio is defined such that the
8 obtained current represents the signal and the corresponding standard deviation
9 represents the noise, then, the s/n ratios, calculated using the data plotted in Fig. 3(b),
10 are 12.5 ± 4.7 for the 2D electrode and 12.1 ± 5.2 for the 3D electrode. As such, there
11 is no significant difference between the s/n ratios of the 2D and 3D electrodes. The 3D
12 electrode with a larger output current should be beneficial because it enables the use of
13 a portable and cheap electrochemical instrument, without requiring specially designed
14 noise-reduction circuits.

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

1 performing electrochemical ELISA of bone metabolic marker proteins. In this study,
2 we focused on the applicability of the 3D electrodes and performed ELISA off chip. In
3 the future, we will use the electrodes in conjunction with lab-on-a-chip components
4 such as valves for manipulating multiple samples and solutions for ELISA on a chip.
5 Furthermore, because there are several bone metabolic marker proteins, and it is
6 desirable to simultaneously detect them on an electrode, our future work aims to achieve
7 the simultaneous detection of multiple analytes in serum using the designed electrodes¹⁷,
8 ¹⁸. Our versatile and efficient 3D micropillar electrodes could potentially be a
9 fundamental tool for the development of more sophisticated on-site diagnosis systems.

10

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14

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15

16 **Figure captions**

17 Figure 1

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

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

8

9 Figure 2

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

13

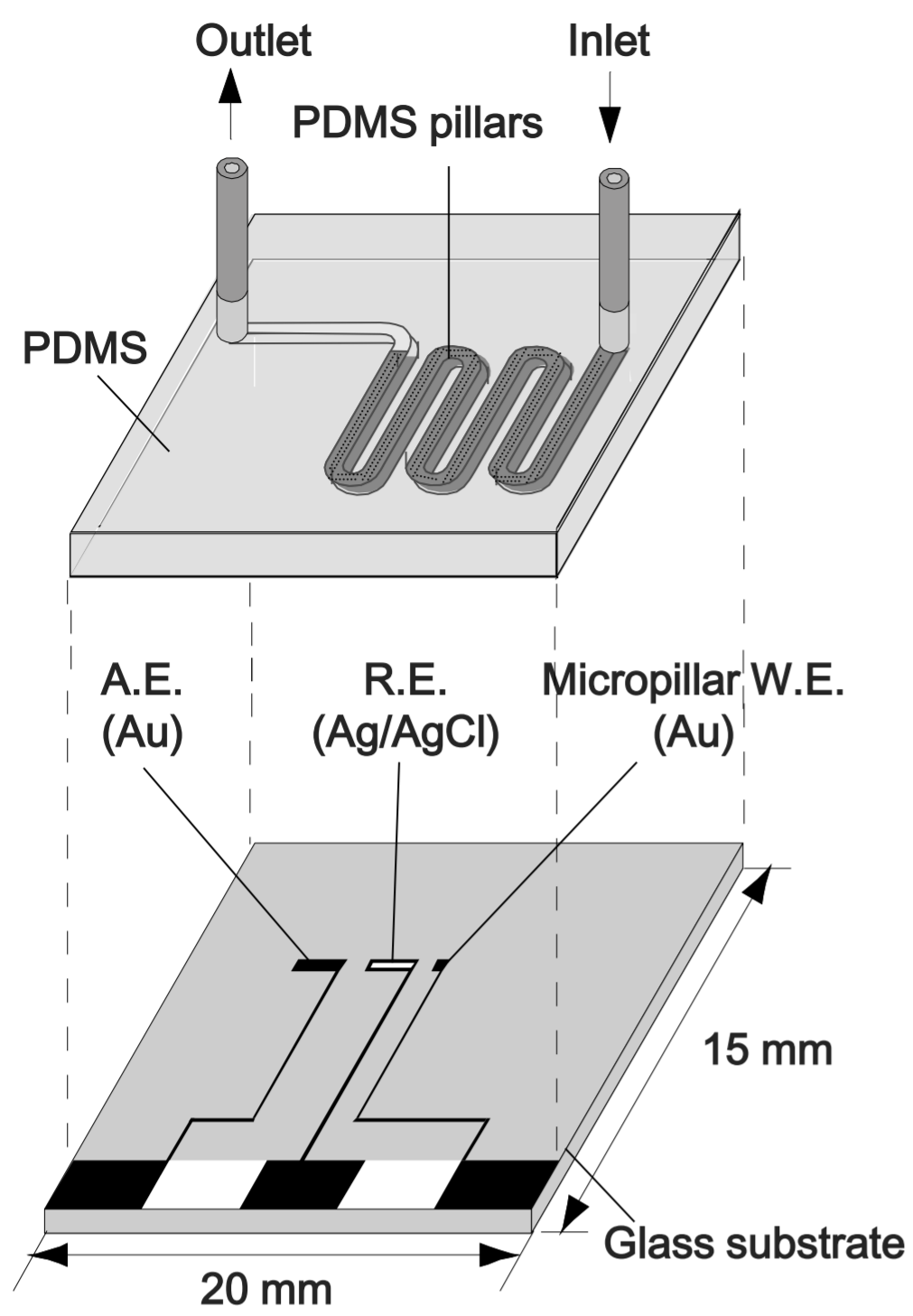
14 Figure 3

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

1 current on concentration of TRACP-5b. ELISA was performed on the PDMS
2 micropillars, and PAP converted from PAPG was detected downstream at the 3D
3 micropillar electrode with gold black and the 2D electrode.
4
5

Fig. 1 Sonthaya Nui et al.

(a)



(b)

Micropillars modified with gold black
(for detection electrode)

PDMS pillars
(for ELISA reaction site)

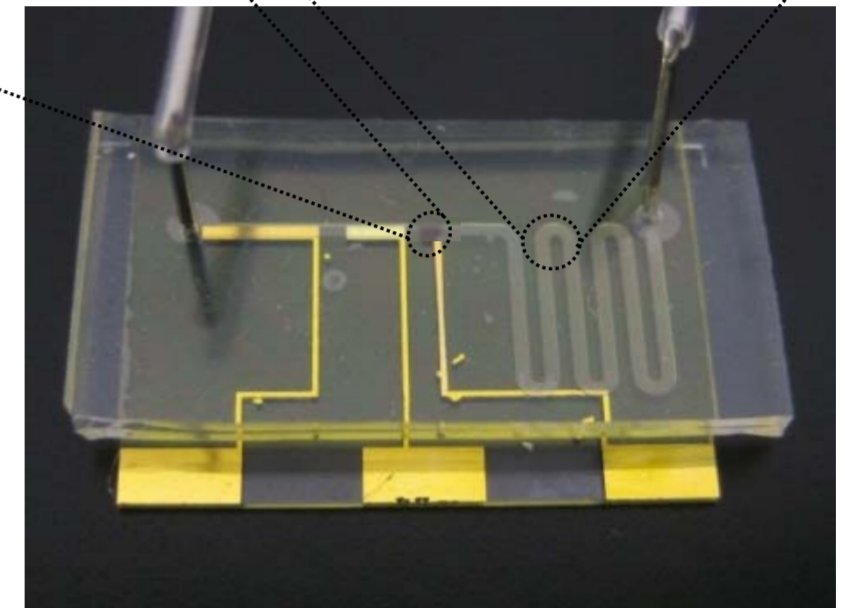
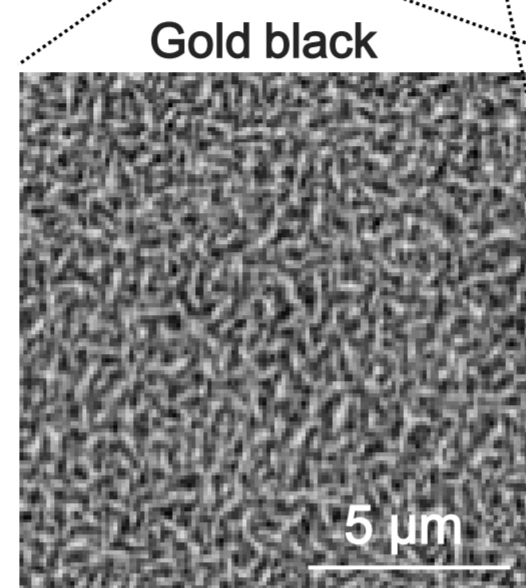
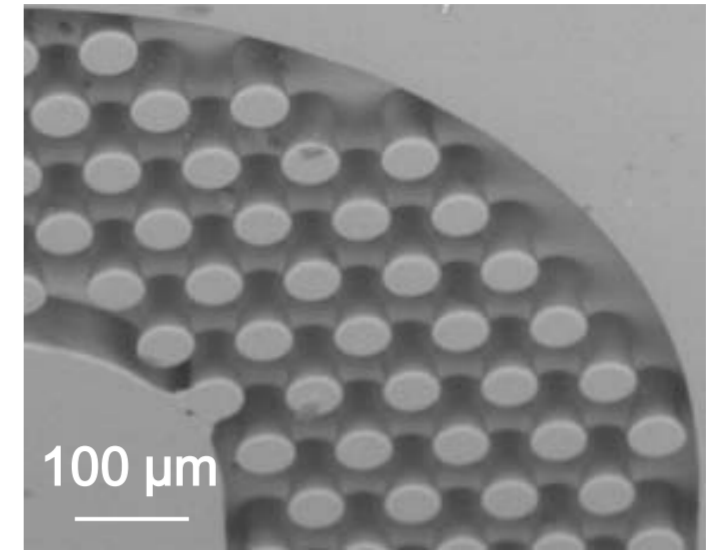
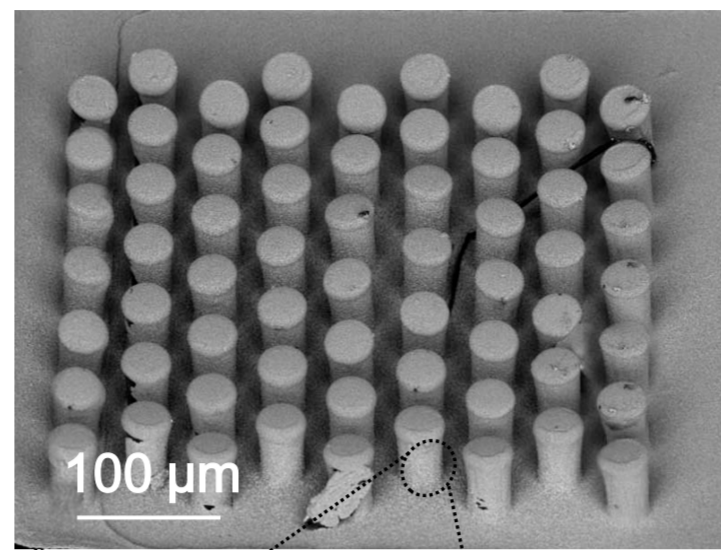


Fig. 2 Sonthaya Nui et al.

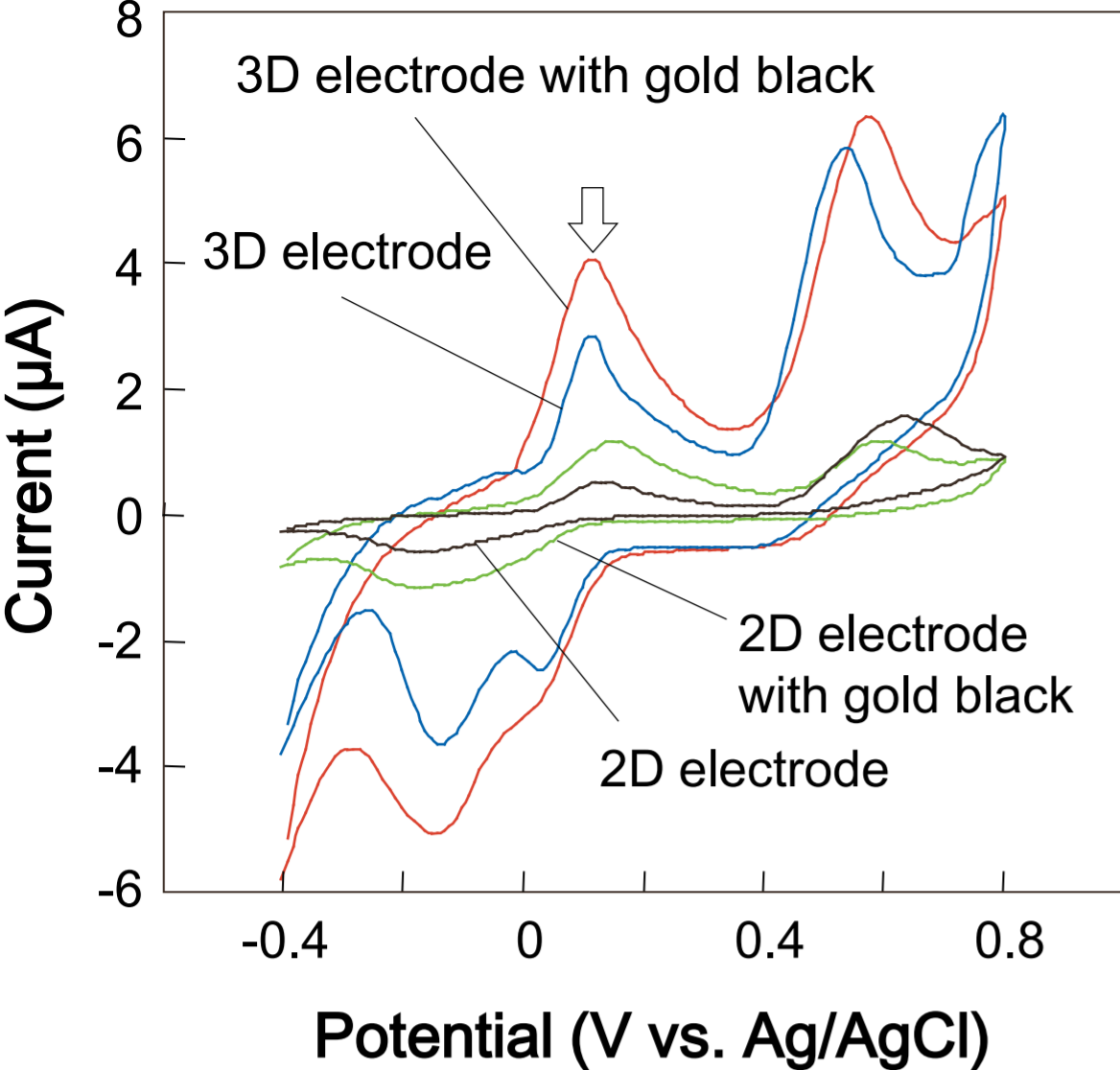


Fig. 3 Sonthaya Nui et al.

