Rapid and highly sensitive detection of pyocyanin biomarker in different *Pseudomonas aeruginosa* infections using gold nanoparticles modified sensor

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Abstract

Successful antibiotic treatment of infections relies on accurate and rapid identification of the infectious agents. *Pseudomonas aeruginosa* is implicated in a wide range of human infections that almost complicated and become life threating especially in immunocompromised and critically ill patients. Conventional microbiological methods take more than 3 days to obtain accurate results. Pyocyanin is a distinctive electroactive biomarker for *Pseudomonas aeruginosa*. Here, we have developed a rapid diagnostic (polyaniline) PANI gold nanoparticles (Au NPs) modified indium tin oxide (ITO) electrode that showed 100% sensitivity for pyocyanin in culture of *Pseudomonas aeruginosa* clinical isolates and high selectivity for pyocyanin at low concentration when measured in the presence of other substances like ascorbic acid, uric acid, and glucose as interferences. The constructed electrode was characterized using scanning electron microscopy and cyclic voltammetry. The determined linear range for pyocyanin detection was from 238 µM to 1.9 µM with a detection limit of 500 nM. Compared to the screen-printed electrode used before, the constructed electrode showed a 4-fold enhanced performance.

***Keywords*:** Pseudomonas aeruginosa, Biosensor, Pyocyanin, cyclic voltammetry, Biomarker

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a prevalent and opportunistic pathogen that is considered one of the most annoying bacteria causing deadly infections in critically ill patients [1-4]. It commonly produces infections in patients with surgical wounds, burn wound or cystic fibrosis. Infections caused by *P. aeruginosa* have high morbidity and mortality rates, particularly among immunocompromised patients such as cancer patients and premature infants [5-7]. *P. aeruginosa* may acquire multidrug resistance, making its eradication with antibiotics challenging [8]. The increasing resistance of bacteria is partially due to the late diagnosis and the misuse of antibiotics [9]. Hence, the early and fast detection of this serious pathogen is essential for a more targeted antibiotic prescription that will hasten recovery and reduce the emergence of antibiotic resistance [10, 11]. Typically, *P. aeruginosa* infections are identified in clinics using selective plate culturing techniques, which take about 24 h or more to provide results. Polymerase chain reaction (PCR) identification is available in a growing number of clinics. However, PCR identification requires extensive sample preparations, uses expensive reagents, and takes several hours to complete [12]. Therefore, developing a sensitive, specific and rapid identification methods for pathogen detection in cost and time competent manners have found broad attention in the last few years [13]. Pyocyanin is one of the virulence factors exclusively secreted by *P. aeruginosa*. It is a unique, quorum-sensing molecule that is linked to biofilm formation, induces inflammation and causes apoptosis of neutrophils [14-16].

Until lately, pyocyanin was measured by chromatography or spectrophotometric techniques that are time-consuming and needs purification from bacterial cultures [15]. Electrochemical sensors are endorsed robust tools for the detection of environmental and disease-related biomarkers [13]. They are easy to use with low detection limits, high sensitivity, good stability

together with cost and time effectiveness [17, 18]. The redox-active nature of pyocyanin molecules permits its rapid detection by electrochemical sensors in two minutes [19]. It is still a challenge to find new and more sensitive methods to provide rapid and accurate information about *P. aeruginosa* that can aid treatment decisions as early as possible when bacteria are still responsive to antibiotics.

Gold (Au) nanostructures are currently used to modify electrodes of biosensors because of its excellent optical and electrical properties and affinity to bind with biomolecules [20-25]. Moreover, the use of Au modified indium tin oxide (ITO) electrode has led to a fast and ultrasensitive detection of some multidrug-resistant bacteria such as *E. coli* and *S. aureus* [26].

Conducting polymers [27] have much interest in the recent researchers because of their good conductivity, stability, ease of preparation. In general, the electronic and electrochemical properties of [28-31] conducting polymers made them have many applications in photovoltaic cells, organic light emitting diode, and biosensors. Polyaniline has received much attention in the research work. This is mainly due to the fact that PANI and its derivatives or composites with other materials are easy to synthesize chemically or electrochemically [32].

Therefore, conducting polymers/metal or metal oxides hybrid materials possess the unique combination of the conducting polymers properties (biocompatibility, direct electrochemical synthesis) and the characteristics of nanomaterials e.g. large surface area, size, flexibility for the immobilization of biomolecules and quantum effect [33]. In our previous work, we have reported on the fabrication of poly(4–aminothiophenol) nanostructures decorated gold nanodots patterned ITO electrode, which demonstrated a highly electrochemical sensitivity and selectivity towards a mixture of two DNA bases (adenine and guanine) [24].

Hybrids organic and inorganic nanocomposites not only possess the sum of their individual components, but the role of the inner interfaces could be predominant; thus hybrids organic/inorganic nanocomposites have used in a wide range of applications including biosensors [22, 34-38] and sensors [39].

The present work aims to assess the efficacy of using PANI/Au nanostructures modified ITO sensor for early detection and quantification of pyocyanin in *P. aeruginosa* cultures of clinical isolates based on cyclic voltammetry (CV) technique. The developed sensor showed high sensitivity towards pyocyanin over a wide range of concentrations from 238 µM to 1.9 µM with a limit of detection (LOD) of about 500 nM. Also, this sensor showed high selectivity towards detection of pyocyanin in the presence of several interferences such as urea, glucose and vitamin c. Furthermore, the applicability of this sensor has been confirmed by direct detection of pyocyanin in *P. aeruginosa* culture of clinical isolates obtained from cases of *P. aeruginosa* infections.

2. Methods

2.1. Materials

Pyocyanin (P0046-5MG), gold (III) chloride hydrate and ITO coated glass slide square were obtained from Sigma Aldrich. Deionized water (DIW) with a resistivity of 18.2 MΩ.cm that was purified with a Purite purification system (UK) was used for all preparations. The buffer system used in this work was phosphate buffer saline (PBS) (0.01 mol/L) at pH 7.4 that was prepared by dissolving PBS powder in 1 L of DIW. Luria-Bertani (LB) broth (Oxoid, UK) was utilized in this study.

2.2. Equipment

All electrochemical measurements were performed using the Autolab potentiostat instrument (Netherlands) connected to a three-electrode cell; Metrohm Model 663VA stand was controlled by Nova software at room temperature. The three-electrode system consists of a platinum wire as a counter electrode, Ag/AgCl as the reference electrode, and PANI/Au modified ITO electrode as a working electrode.

2.3. Clinical isolates of Pseudomonas aeruginosa

P. aeruginosa culture was made from clinical isolates obtained from the department of Medical Microbiology and Imunology that was isolated from clinical cases of *P. aeruginosa* infections admitted to Assiut University hospital as pneumonia , corneal ulcers, urinary tract infections and wound infections. These clinical isolates of *P. aeruginosa* were confirmed by VITEK 2 automated microbiology system. The study protocol was approved by the local ethical Committee of the faculty of Medicine Assiut University and an informed written consent was taken from all the participants in the study.

2.4. Methods

2.4.1. Preparation of gold nanostructures ITO electrode

The ITO-coated glass substrates with a geometrical size of 25 mm X 12.5 mm X 1.1 mm was cleaned *via* sonication for 15 min each in 1% Triton X-100, DIW and then in ethanol. The substrates were immersed in a basic piranha solution (H_2O_2: NH_3: H_2O ratio, 1:1:5) for 30 min at 80 °C. Finally, the substrates were cleaned again with DIW and ethanol and dried under nitrogen

gas. The Au NPs modified ITO electrodes were prepared according to our previously reported method [21] in which an aqueous solution of 1 mM $HAuCl_4$ was added into the electrochemical cell and we have issued the deposition process of Au NPs on the ITO substrates by using CV technique within potential window from 1.5 V to -1 V for 5 cycles at scan rate of 50 mV/sec against Ag/AgCl as a reference electrode. The surface morphology of the modified electrodes was analyzed by SEM (JOEL-JSM-5400LV).

2.4.2. Preparation of gold modified ITO electrode

Polyaniline hydrochloride (PANI) salt was prepared according to the previously published work [40]. Typically, 0.5 g of aniline hydrochloride was dissolved in 20 mL of DIW and stirred in ice bath for 1h (first solution). In another conical flask, a solution of 0.5 g of ammonium persulphate in 20 mL DIW was stirred in ice bath for 1h and then added to the first solution and keep stirring for further 4h. The dark green precipitates were filtrated and dried in an oven at 80 °C [41]. To fabricate a layer of PANI on the surface of Au NPs/ITO electrode, Au NPs/ITO electrode was immersed in a solution of PANI in NMP (0.001 gm/mL) for 8 hrs and then rinsed with DIW to remove the PANI from the non-conductive side and dried under N_2 gas [40, 42].

2.4.3. Electrochemical Measurements of pyocyanin

The electrochemical measurements were carried out by immersing the working electrode together with the reference and counter electrodes in the presence of different concentrations of pyocyanin ranging from 238 µM to 1.9 µM; the solutions were prepared in PBS (10 mmol/L, pH 7.4).

2.4.4. Selectivity of the Developed pyocyanin Sensor

The selectivity of the prepared electrodes towards the pyocyanin was studied by using a mixture of pyocyanin solution with glucose, vitamin c, and urea as interferences, which are commonly present in clinical samples.

2.4.5. Electrochemical detection of pyocyanin in Pseudomonas aeruginosa

Under complete sterile conditions a colony (or more) of *P. aeruginosa* were added in 10 ml of LB broth in 14 ml tube and placed on a shaker (200 rpm) at 37 °C overnight. 1ml of this suspension was removed and added to 9 ml fresh LB broth in 14 ml tube and placed again in a shaker at 37 °C for 24 hrs. The OD at 600 nm (OD600) was measured to quantify the density of the bacteria in each culture sample. During the 24 hours, 3 samples were collected at different time-points (after 2, 10 and 24 hours). The pyocyanin concentration was measured using the PANI/Au NPs modified ITO electrode and the OD600 was detected to confirm the increasing bacterial number.

2.4.6. Electrochemical testing of bacterial cultures

Each strain of *P. aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae and Streptococcus pneumoniae* was cultivated in LB broth at 37 °C overnight. The cyclic voltammetry of each strain culture was measured using the PANI/Au NPs modified ITO electrode.

2.4.7. Electrochemical detection of P. aeruginosa isolated from clinical samples

Sterile swabs were used to collect different *P. aeruginosa* clinical isolates for electrochemical testing. Swab samples were inoculated into LB broth and incubated at 37 °C overnight. CV of each sample was measured using the PANI/Au NPs modified ITO electrode.

3. Results and discussion

3.1 Preparation and Structural Features of the Au NPs/ITO and PANI/Au NPs/ITO Electrodes

One of the most critical advantages of using Au NPs for the preparation of biosensors is their ability to give a stable immobilization of biomolecules, thereby retaining their bioactivity. Moreover, Au NPs can allow electrons to be transported without using an electron transfer mediator by encouraging the electron transfer between redox proteins and electrode materials. Electron transfer is facilitated because Au NPs possess attractive characteristics, such as high surface-to-volume ratio, high surface energy, the ability to decrease protein-metal particle distance and to function as electron-conducting pathways [43].

In this work, Au NPs modified ITO electrode was prepared based on electrochemical deposition of Au onto the ITO surface by using CV technique. Figure 1 showed the cyclic voltammograms corresponding to the electrochemical deposition process within a potential window from 1.5 V to -1.0 V to allow a complete reduction of Au^{3+} ions into Au^{0} under this potential window range after 5 cycles [20]. **Figure 1** demonstrated a reduction peak at -0.5, -0.13 and 0.1 V, anodic peak at -0.1 and 0.84 V during the first cycle of Au nanoparticles deposition,

which is shifted to -0.35 and 0.41 V and anodic peaks at 0.061 and 0.84 V during the deposition process in the remaining 4 cycles. The shifting in the reduction peaks is related to the reduction process of Au^{3+} to form metallic Au nanostructures on the ITO electrode surface [20].

The nanostructured surface morphology of the Au NPs modified ITO electrode could also have a significant effect on the sensitivity of electrochemical detection, which was further explored by SEM characterization. As shown in **Figure 2a**, the SEM image of the Au nanostructured modified ITO electrode illustrated a nice coverage of Au NPs on the surface of the ITO electrode with the formation of polydispersed Au NPs. The particle size was analyzed by using SPIP (version 6.7.7) program (**Figure 2b**), which demonstrated that the mean particle size was found to be about 42.5 nm in diameter with a standard deviation of about 101 nm.

3.2. The Electrochemical Behavior of pyocyanin by using Cyclic Voltammetry

Figure 3a showed the CV behavior of 50 µM of pyocyanin in PBS at the bare ITO electrode, which demonstrated a very weak redox peaks. So, the CV response of higher concentration was investigated (**Figure 3b**), which showed an anodic peak at -0.203 V and a cathodic peak at -0.305 V. Thus, the bare ITO electrode is unsuitable for detection low concentrations of pyocyanin. In order to develop an electrode that could sense the pyocyanin; we have modified the ITO electrode with Au NPs and used it to study the electrochemical behavior of pyocyanin based on CV technique. **Figure 3c** showed the cyclic voltammograms of three different concentrations of pyocyanin at Au NPs modified ITO electrode, which illustrated a quasirevisable response with an oxidation peak at -0.21 V and a reduction peak at about -0.3 V. These results indicate the capability of the Au NPs modified ITO electrode to detect the pyocyanin; this capability is attributed to the signal amplification of Au NPs that enhanced the

electron transfer characterization [21]. However, the Au NPs modified ITO electrode didn't show any response to pyocyanin solution with concentrations lower than 36 µM. To enhance the sensitivity of the developed electrode, we have modified the Au NPs/ITO electrode with a layer of PANI and used to study it to detect the pyocyanin marker. **Figure 2c** showed the SEM image of the PANI/Au NPs/ITO, which showed the formation of a thin layer of PANI with large diameter. The cyclic voltammogram of 50 µM pyocyanin at PANI/Au NPs/ITO electrode was represented in **Figure 3d**, which showed an increase in the oxidation peak at -0.23 V and reduction peak at -0.3 V. Furthermore, it is intersted to note that the redox current peak is higher than that in either case of using ITO or Au NPs/ITO electrodes, I addtion the revisabilty of the redox behavior was increased with electrode modification. So that PANI/Au NPs/ ITO electrode is more sensitive to pyocyanin than either bare ITO electrode or Au NPs/ITO electrode.

Figure 4a showed the effect of different scan rate within a range from 0.01 V/s to 0.12 V/s on the maximum peak current of pyocyanin, which illustrated an increase in the redox peaks with the increase of the scan rate. **Figure 4b** showed the relationship between the value of the scan rate versus the maximum peak current of pyocyanin at Au NPs/ITO electrode, which demonstrated a linear relationship over a wide range of scan rate from 0.01 V/s to 0.12 V/s.

3.3. The sensitivity of the developed sensor towards pyocyanin marker

To evaluate the sensitivity of the PANI/Au NPs modified ITO electrode towards pyocyanin, a wide range of pyocyanin concentrations from 238 µM to 1.9 µM in PBS was used and their CVs response at PANI/Au NPs modified ITO electrode was investigated. **Figure 5a** showed the CVs bahvior of different concentrations of pyocyanin at PANI/Au NPs modified ITO electrode, which demonstrated an increase in the redox current peak with increasing the pyocyanin

concentration. **Figure 5b** represented the relationship between the oxidation current peak and the pyocyanin concentration at PANI/Au NPs modified ITO electrode, which illustrated a linear response between the anodic current peaks and the concentration of pyocyanin. The LOD of the PANI/Au NPs modified ITO electrode was calculated according to the equation (LOD =3.3*(STEYX/Slope of calibration curve)), and it was found to be 500 nM. This result is better than the results obtained by Alatraktchi et al., who detected pyocyanin by a disposable screen-printed electrode [44]. The results in **Figure 5** showed the high sensitivity for detection of pyocyanin using the Au modified ITO, which attributed to the use of PANI/Au NPs modified ITO as a working electrode that achieved the precise, rapid and sensitive measurement of pyocyanin with low cost. **Table 1** showed the LOD of our modified electrode in comparison with the LOD of the previously reported of some other electrodes for the electrochemical determination of pyocyanin that revealed that our electrode possessed lower LOD in comparison with most of the previously reported electrodes [44-50].

3.4. Selectivity of the developed sensor towards pyocyanin in the presence of different interferences

One of the concerns raised about the utility of the PANI/Au NPs modified ITO electrode for investigating the presence of *P. aeruginosa* in human samples is that there may be other molecules, which may interfere with electrode performance. It is always of great importance to achieve the highest selectivity and sensitivity towards pyocyanin in the presence of different interferences in clinical samples. Our study is focused on the selective detection of pyocyanin in presence of vitamin c, glucose and urea, which may present in clinical samples. **Figure 6** represented the CV of pyocyanin and interferences; there are no peaks of interferences in the

potential window of pyocyanin. According to the obtained results, the selective detection of pyocyanin is applicable with high sensitivity. The results reported (**Figure 6**) showed a clear electrochemical fingerprint of pyocyanin, which was clearly observed when pyocyanin is measured among other redox-active compounds such as vitamin C, urea and glucose.

3.5. Electrochemical detection of pyocyanin in Pseudomonas aeruginosa culture

In this study, samples from the *Pseudomonas aeruginosa* cultures were collected during log and stationary phase. The pyocyanin was released from *P. aeruginosa* culture and detected electrochemically by PANI/Au NPs modified ITO after 2,10 and 24 hours. The results in (**Figure 7**) show that no pyocyanin was initially produced in the culture after 2 hours of incubation as the OD at 600 nm was 0.1 so the culture was in the log phase. Pyocyanin could be detected after 10 hours as the culture reached the stationary phase. This result is consistent with the results obtained by Cabeen 2014 who reported that pyocyanin release is controlled by the quorum sensing system, which is not present in the early stage of a growing culture [**51**].

3.6. Electrochemical testing of bacterial cultures

The concern in this application was whether other bacterial pathogens produce redox-active molecules would interfere with the sensor's response in the potential window of pyocyanin. To address this concern, a range of clinically-relevant bacterial pathogens (listed in **Figure 8**) were electrochemically measured after 24 hours of growth using the PANI/Au NPs modified ITO. The results in (**Figure 8**) indicated that the sensor demonstrated high selectivity towards pyocyanin. A notable oxidation peak at -0.23 V was originated from *P. aeruginosa* strain and there were no other bacteria that produce a redox-active peak in this potential window.The lack of a detectable

peak from the other pathogens in the potential window further confirms that *P.aeruginosa* is the only bacterium producing redox-active molecules among the species tested and that the possibility of a false positive identification of *P.aeruginosa* using this method is unlikely.

3.7. Electrochemical measurements of clinical P. aeruginosa strains

Liquid cultures of clinical *P. aeruginosa* isolates were incubated at 37 °C for 24 hours with electrochemical measurements taken after incubation. All *P. aeruginosa* isolates were having a positive test result of electrochemical detection by PANI/Au NPs modified ITO electrode . The observed electrochemical peak (shown in **Figure 9**), due to pyocyanin oxidation at -0.20V, indicates the presence of *P.aeruginosa* in the sample. The negative control is LB broth growth media, which lacks the redox-active oxidation peak.

4. Conclusions

In this work, we have fabricated a PANI/Au NPs modified ITO electrode based on the electrodeposition of Au NPs onto the ITO surface by using CV technique, followed by covering the surface with a layer of PANI. The prepared electrode showed 100% sensitivity, selectivity and a low detection limit for pyocyanin . The capability of the PANI/Au NPs modified ITO sensor to detect pyocyanin released in *P. aeruginosa* culture will aid in the fast precise detection of pyocyanin biomarker and diagnosis of *P. aeruginosa* infections specially in critically ill patients. Cosequently, this will achieve a rapid appropriate treatment and reduce the emergence of resistance made by empirical treatment.

Conflicts of interest

There are no conflicts to declare.

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References

1. Koh AY, Priebe GP, Pier GB (2005) Virulence of Pseudomonas aeruginosa in a murine model of gastrointestinal colonization and dissemination in neutropenia. *Infection and Immunity*, 73(4): 2262-72.

2. Tran Cindy S, Eran Y, Ruch Travis R, Bryant David M, Datta A, Brakeman P, et al. (2014) Host Cell Polarity Proteins Participate in Innate Immunity to Pseudomonas aeruginosa Infection. *Cell Host & Microbe*. 15(5): 636-43.

3. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M (2014) Requirements for Pseudomonas aeruginosa Acute Burn and Chronic Surgical Wound Infection. *PLOS Genetics*. 10(7): e1004518.

4. Boucher HW, Scheld M, Bartlett J, Talbot GH, Bradley JS, Spellberg B, et al. (2009) Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*. 48(1): 1-12.

5. Abu EA, Su S, Sallans L, Boissy RE, Greatens A, Heineman WR, et al. (2013) Cyclic voltammetric, fluorescence and biological analysis of purified aeruginosin A, a secreted red pigment of Pseudomonas aeruginosa PAO1. *Microbiology*. 159(8): 1736-47.

6. Miller LC, O'Loughlin CT, Zhang Z, Siryaporn A, Silpe JE, Bassler BL, et al. (2015) Development of Potent Inhibitors of Pyocyanin Production in Pseudomonas aeruginosa. *Journal of Medicinal Chemistry*. 58(3): 1298-306.

7. Damkiær S, Yang L, Molin S, Jelsbak L. (2013) Evolutionary remodeling of global regulatory networks during long-term bacterial adaptation to human hosts. *Proceedings of the National Academy of Sciences*. 110(19): 7766-71.

8. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, et al. (2012) Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. *Nature Reviews Microbiology*. 10: 841.

9. Llor C, Bjerrum L. (2014) Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. *Therapeutic Advances in Drug Safety*. 5(6): 229-41.

10. Bren L. (2002) Battle of the bugs: fighting antibiotic resistance. *FDA Consum*. 36(4): 28-34.

11. O'Neill JJTRoAR, Wellcome Trust, Governent H. Rapid diagnostics: stopping unnecessary use of antibiotics. 2015. 2017.

12. Webster TA, Sismaet HJ, Conte JL, Chan IpJ, Goluch ED (2014) Electrochemical detection of Pseudomonas aeruginosa in human fluid samples via pyocyanin. *Biosensors & Bioelectronics*. 60: 265-70.

13. Amiri M, Bezaatpour A, Jafari H, Boukherroub R, Szunerits S. (2018) Electrochemical Methodologies for the Detection of Pathogens. *ACS Sensors*. 3(6): 1069-86.

14. Michel-Briand Y, Baysse C. (2002) The pyocins of Pseudomonas aeruginosa. Biochimie. 84(5-6): 499-510.

15. Jayaseelan S, Ramaswamy D, Dharmaraj S. (2014) Pyocyanin: production, applications, challenges and new insights. *World Journal of Microbiology and Biotechnology*. 30(4): 1159-68.

16. Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK. (2006) The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of Pseudomonas aeruginosa. *Molecular Microbiology*. 61(5): 1308-21.

17. Amlil A, Koffi O, Bile B, Bengourram J, Najih R, Latrache H. (2016) Electrochemical Biosensor for the Immediate Detection of Bacteria. *Biosens & Bioelectron*. 7(197): 2.

18. Del Pozo M, Alonso C, Pariente F, Lorenzo E (2005) Electrochemical DNA sensing using osmium complexes as hybridization indicators. *Biosensors & Bioelectronics*. 20(8): 1549-58.

19. Sismaet HJ, Banerjee A, McNish S, Choi Y, Torralba M, Lucas S, et al. (2016) Electrochemical detection of Pseudomonas in wound exudate samples from patients with chronic wounds. *Wound Repair Regeneration*. 24(2): 366-72.

20. El-Said WA, Abd El-Hameed K, Abo El-Maali N, Sayyed HG (2017) Label-free Electrochemical Sensor for Ex-vivo Monitoring of Alzheimer's Disease Biomarker. *Electroanalysi*s. 29(3): 748-55.

21. Kim TH, El-Said WA, An JH, Choi JW (2013) ITO/gold nanoparticle/RGD peptide composites to enhance electrochemical signals and proliferation of human neural stem cells. *Nanomedicine:Nanotechnology, Biology and Medicine.* 9(3): 336-44.

22. Jung M, El-Said WA, Choi J-W (2011) Fabrication of gold nanodot arrays on a transparent substrate as a nanobioplatform for label-free visualization of living cells. Nanotechnology. 22(23): 235304.

23. El-Said WA, Lee J-H, Oh B-K, Choi J-W (2010) 3-D nanoporous gold thin film for the simultaneous electrochemical determination of dopamine and ascorbic acid. *Electrochemistry Communications*. 12(12): 1756-9.

24. El-Said WA, Choi J-W (2014) Electrochemical Biosensor consisted of conducting polymer layer on gold nanodots patterned Indium Tin Oxide electrode for rapid and simultaneous determination of purine bases. *Electrochimica Acta*. 123: 51-7.

25. Lv X, Ge W, Li Q, Wu Y, Jiang H, Wang X. (2014) Rapid and ultrasensitive electrochemical detection of multidrug-resistant bacteria based on nanostructured gold coated ITO electrode. *ACS Appl Mater Interfaces*. 6(14): 11025-31.

26. Lv X, Ge W, Li Q, Wu Y, Jiang H, Wang X (2014) Rapid and Ultrasensitive Electrochemical Detection of Multidrug-Resistant Bacteria Based on Nanostructured Gold Coated ITO Electrode. *ACS Applied Materials & Interfaces*. 6(14): 11025-31.

27. Wei D, Ivaska A. (2006) Electrochemical biosensors based on polyaniline. *Chemia analityczna*. 51: 839-52.

28. Das G, Yoon HH. (2015) Amperometric urea biosensors based on sulfonated graphene/polyaniline nanocomposite. Int J Nanomedicine. 10 Spec Iss(Spec Iss): 55-66.

29. Ruecha N, Rodthongkum N, Cate DM, Volckens J, Chailapakul O, Henry CS. (2015) Sensitive electrochemical sensor using a graphene–polyaniline nanocomposite for simultaneous detection of Zn (II), Cd (II), and Pb (II). *Analytica Chimica Acta*. 874: 40-8.

30. Wu W, Pan D, Li Y, Zhao G, Jing L, Chen S. (2015) Facile fabrication of polyaniline nanotubes using the self-assembly behavior based on the hydrogen bonding: a mechanistic study and application in high-performance electrochemical supercapacitor electrode. *Electrochimica Acta*. 152: 126-34.

31. Mohamad FS, Mat Zaid MH, Abdullah J, Zawawi RM, Lim HN, Sulaiman Y, et al. (2017) Synthesis and characterization of polyaniline/graphene composite nanofiber and its application as an electrochemical DNA biosensor for the detection of mycobacterium tuberculosis. *Sensors*. 17(12): 2789.

32. Huang J. (2006) Syntheses and applications of conducting polymer polyaniline nanofibers. *Pure and Applied Chemistry*. 78(1): 15-27.

33. Dhand C DN, Mishra S, Solanki P, Mayandi V, Beuerman RW, Ramakrishna S, Lakshminarayanan R, Malhotra B. (2015) Polyaniline-based biosensors. *Dove press*. 4: 25-46.

34. Srivastava M, Srivastava S, Nirala N, Prakash R. (2014) A chitosan-based polyaniline–Au nanocomposite biosensor for determination of cholesterol. *Analytical Methods*. 6(3): 817-24.

35. H. Zheng MW, J. Chen, M. Liu, Y. Ye, Z. Yan. (2018) Improved Sensitivity and Selectivity Glucose Biosensor Based on PANI-GRA Nanocomposite Film Decorated with Pt Nanoparticles. *International Journal of Electrochemical Science*. 13: 6272-85.

36. Khan AL, Jain RJ. (2018) Polypyrrole/titanium dioxide nanocomposite sensor for the electrocatalytic quantification of sulfamoxole. *Ionics*. 24(8): 2473-88.

37. Sattarahmady N, Heli H, Moosavi-Movahedi AA. (2010) An electrochemical acetylcholine biosensor based on nanoshells of hollow nickel microspheres-carbon microparticles-Nafion nanocomposite. *Biosens & Bioelectron*. 25(10): 2329-35.

38. Ghadimi H, Nasiri-Tabrizi B, Nia PM, Basirun WJ, Tehrani RMA, Lorestani F. (2015) Nanocomposites of nitrogen-doped graphene decorated with a palladium silver bimetallic alloy for use as a biosensor for methotrexate detection. *RSC Advances*. 5(120): 99555-65.

39. Gong J, Zhou T, Song D, Zhang L, Hu X. (2010) Stripping voltammetric detection of mercury(II) based on a bimetallic Au-Pt inorganic-organic hybrid nanocomposite modified glassy carbon electrode. *Anal Chem*. 82(2): 567-73.

40. El-Said W, Yea C-H, Choi J-W, Kwon IK. (2009) Ultrathin polyaniline film coated on an indium–tin oxide cell-based chip for study of anticancer effect. 661-7 p.

41. El-Said WA, Yea C-H, Choi J-W, Kwon I-K. (2009) Ultrathin polyaniline film coated on an indium–tin oxide cell-based chip for study of anticancer effect. *Thin Solid Films*. 518(2): 661-7.

42. El-Said WA, Abdel-Shakour M, Abd-Elnaiem AM. (2018) An efficient and low-cost photoanode for backside illuminated dye-sensitized solar cell using 3D porous alumina. *Materials Letters*. 222: 126-30.

43. Abdulbari HA, Basheer EAM. (2017) Electrochemical Biosensors: Electrode Development, Materials, Design, and Fabrication. *CBEN*. 4(2): 92-105.

44. Alatraktchi F, Breum Andersen S, Krogh Johansen H, Molin S, Svendsen WJS. (2016) Fast selective detection of pyocyanin using cyclic voltammetry. *Sensors*. 16(3): 408.

45. Alatraktchi FA, Johansen HK, Molin S, Svendsen WE. (2016) Electrochemical sensing of biomarker for diagnostics of bacteria-specific infections. Nanomedicine (Lond). 11(16): 2185-95.

46. Ciui B, Tertiş M, Cernat A, Săndulescu R, Wang J, Cristea C. (2018) Finger-Based Printed Sensors Integrated on a Glove for On-Site Screening Of Pseudomonas aeruginosa Virulence Factors. *Analytical Chemistry*. 90(12): 7761-8.

47. Sharp D, Gladstone P, Smith RB, Forsythe S, Davis J. (2010) Approaching intelligent infection diagnostics: Carbon fibre sensor for electrochemical pyocyanin detection. *Bioelectrochemistry*. 77(2): 114-9.

48. Alatraktchi FAa, Noori JS, Tanev GP, Mortensen J, Dimaki M, Johansen HK, et al. (2018) Paper-based sensors for rapid detection of virulence factor produced by Pseudomonas aeruginosa. *PLOS ONE*. 13(3): e0194157.

49. Buzid A, Shang F, Reen FJ, Muimhneacháin EÓ, Clarke SL, Zhou L, et al. (2016) Molecular Signature of Pseudomonas aeruginosa with Simultaneous Nanomolar Detection of Quorum Sensing Signaling Molecules at a Boron-Doped Diamond Electrode. *Scientific Reports*. 6: 30001.

50. Elliott J, Simoska O, Karasik S, Shear JB, Stevenson KJ (2017) Transparent Carbon Ultramicroelectrode Arrays for the Electrochemical Detection of a Bacterial Warfare Toxin, Pyocyanin. *Analytical Chemistry*. 89(12): 6285-9.

51. Cabeen MT. (2014) Stationary phase-specific virulence factor overproduction by a lasR mutant of Pseudomonas aeruginosa. *PLoS One*. 9(2): e88743.

Table caption

Table 1. Comparison between the sensitivity of our sensor with the previous work

Sensor	Electrochemical technique	LOD	References
Screen-printed electrode (gold working electrode)	CV	2 µM	**(44)**
Screen-printed electrode (gold working electrode)	Amperometry	0.125 µM	**(45)**
Screen printed sensing glove (carbon ink)	SWV	0.003 µM	**(46)**
Three electrode configurations consisting of a Carbon Fibre tow laminate working electrode	SWV	0.030 µM	**(47)**
Paper-based sensor (carbon electrode)	SWV	0.095 µM	**(48)**
Boron-doped diamond (BDD) thin-film electrode	DPV	0.05 µM	**(49)**
-T-Macro -1.54T-CUA -CS/GNP 1.54T-CUA	SWV	0.51 µM 1.0 µM 1.6 µM	**(50)**
PANI/Au NPs/ITO	CV	0.5 µM	**The present work**

Figures captions

Figure 1. Electrochemical deposition of Au NPs onto ITO substrate based on CV technique within potential window from 1.5 V to -0.1 V. Scan rate is 50 mV/sec.

Figure 2. (a) SEM image of Au NPs modifed ITO electrode prepared after 5 cycles, (b) SPIP analysis of the SEM image of the Au NPs modifed ITO electrode prepared after 5 cycles and (c) SEM image of PANI/ Au NPs/ITO electrode.

Figure 3. Cyclic voltammetry behavior of (a) 50 µM pyocyanin in PBS buffer at bare ITO, (b) three different concentrations of pyocyanin in PBS buffer at bare ITO, (c) three different concentrations of pyocyanin in PBS buffer at Au NPs modified ITO and (d) 50 µM pyocyanin in PBS buffer at PANI/Au NPs modified ITO. The scan rate was 50 mV/sec.

Figure 4. a) CV of pyocyanin 50 µM at different scan rate from 0.01 V/s to 0.12 V/s and b) scan rate versus the oxidation peak current of pyocyanin.

Figure 5. (a) Cyclic voltammograms of different concentrations of pyocyanin from 238 μM to 1.9 μM at scan rate 50 mV/sec, (b) relationship between the anodic current peaks and the pyocyanin concentration, and (c) linear relation between current peak and pyocyanin concentrations from 25.62 μM to 1.9 μM.

Figure 6. (a) CV of pyocyanin, vitamin c, glucose and urea, and (b) CV of pyocyanin and a mixture of pyocyanin, vitamin c, glucose and urea.

interference of vitamin c, urea and glucose with the electrochemical detection of pyocyanin

Figure 7. Electrochemical detection of pyocyanin in *P.aeruginosa* culture at 37 °C after 2, 10 and 24 hours of incubation

Figure 8. Cyclic voltammetry of different bacterial cultures after one day of growth at 37 ºC.

Figure 9. Cyclic voltammetry of a clinical isolate of *P. aeruginosa.*

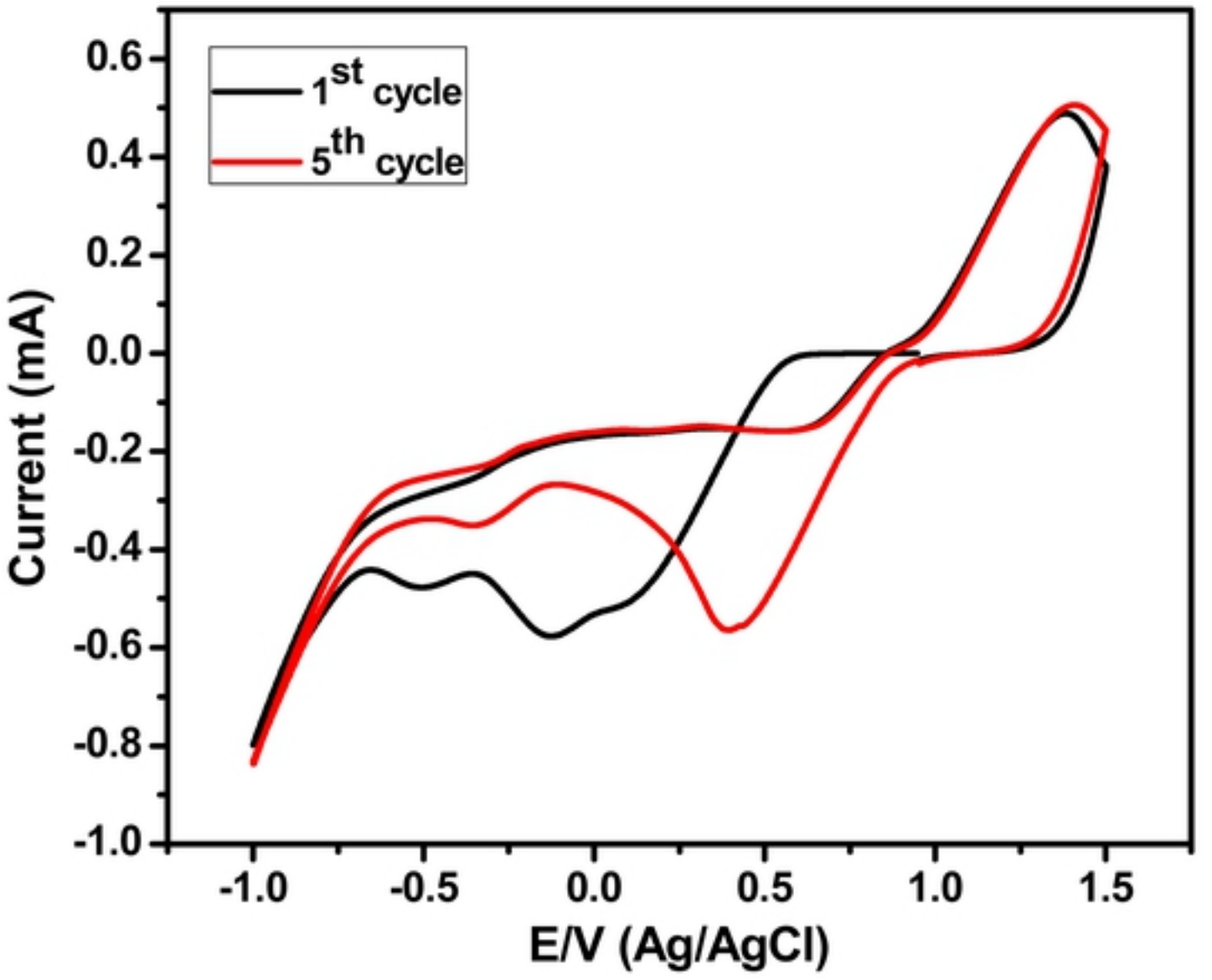
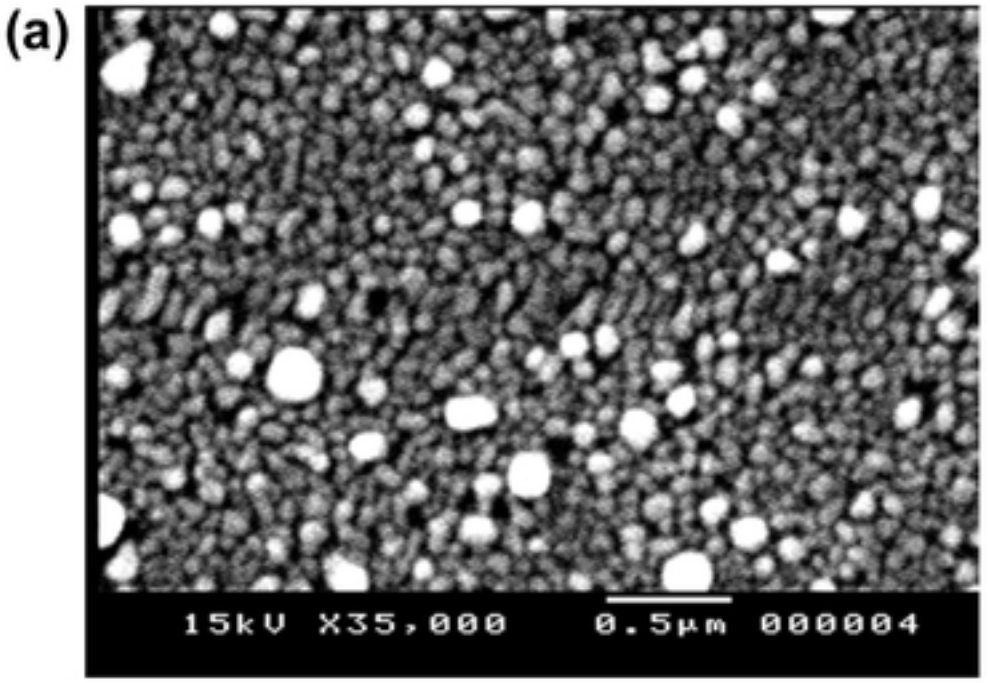
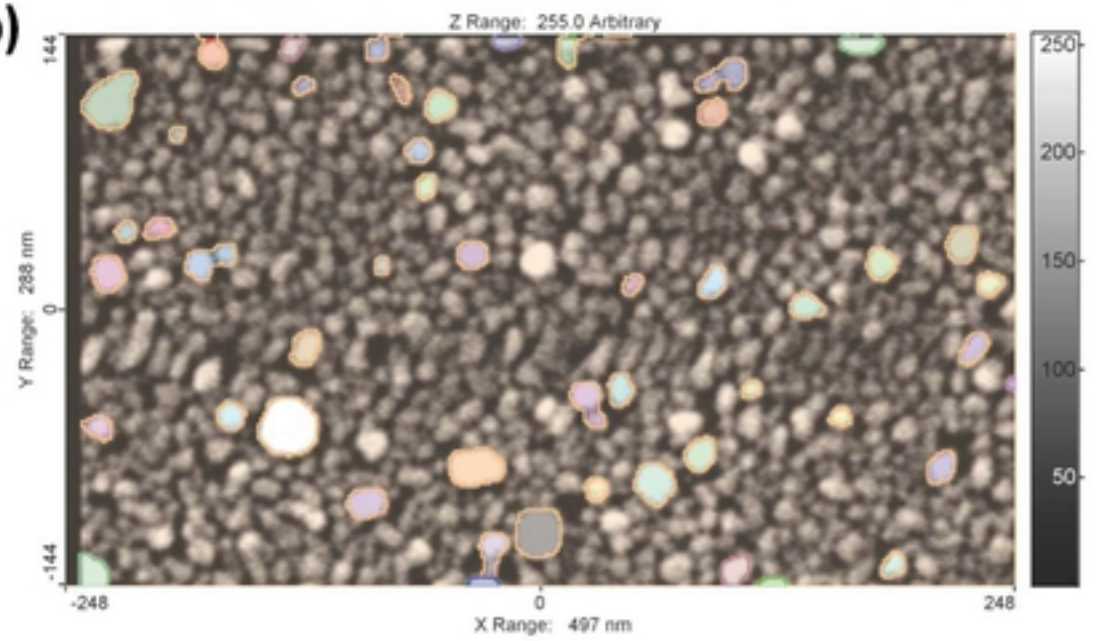

0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Current (mA)
1st cycle
5th cycle
-1.0
-0.5
0.0
0.5
1.0
1.5
E/V (Ag/AgCl)

Figure 1


(a)
15kV X35,000 0.5µm 000004


(b)
Z Range: 255.0 Arbitrary
144
0
-144
Y Range: 288 nm
-248
0
248
X Range: 497 nm
250
200
150
100
50

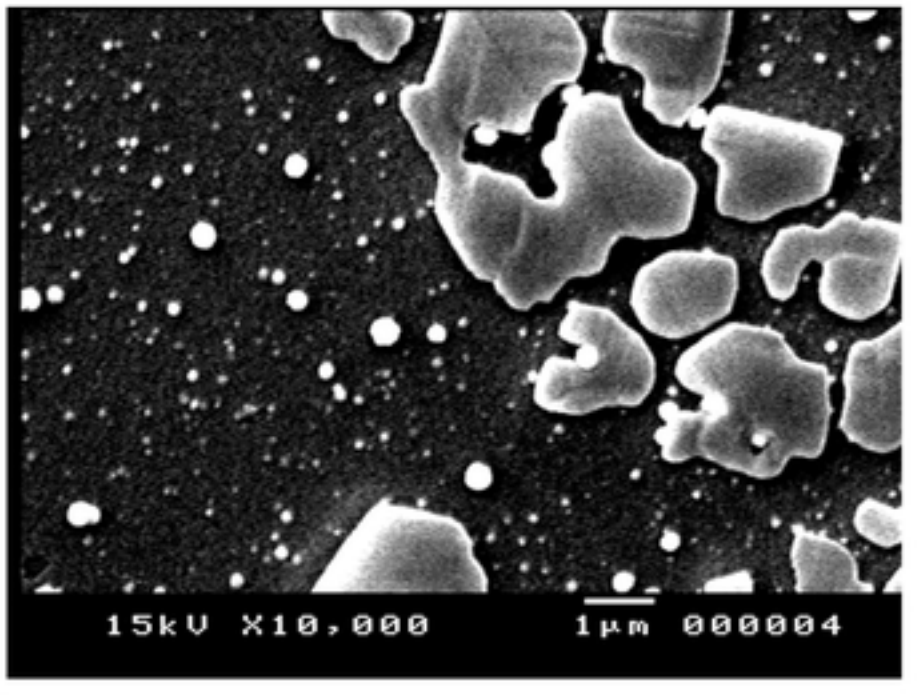

(c)
15kV X10,000 1µm 000004

Figure 2

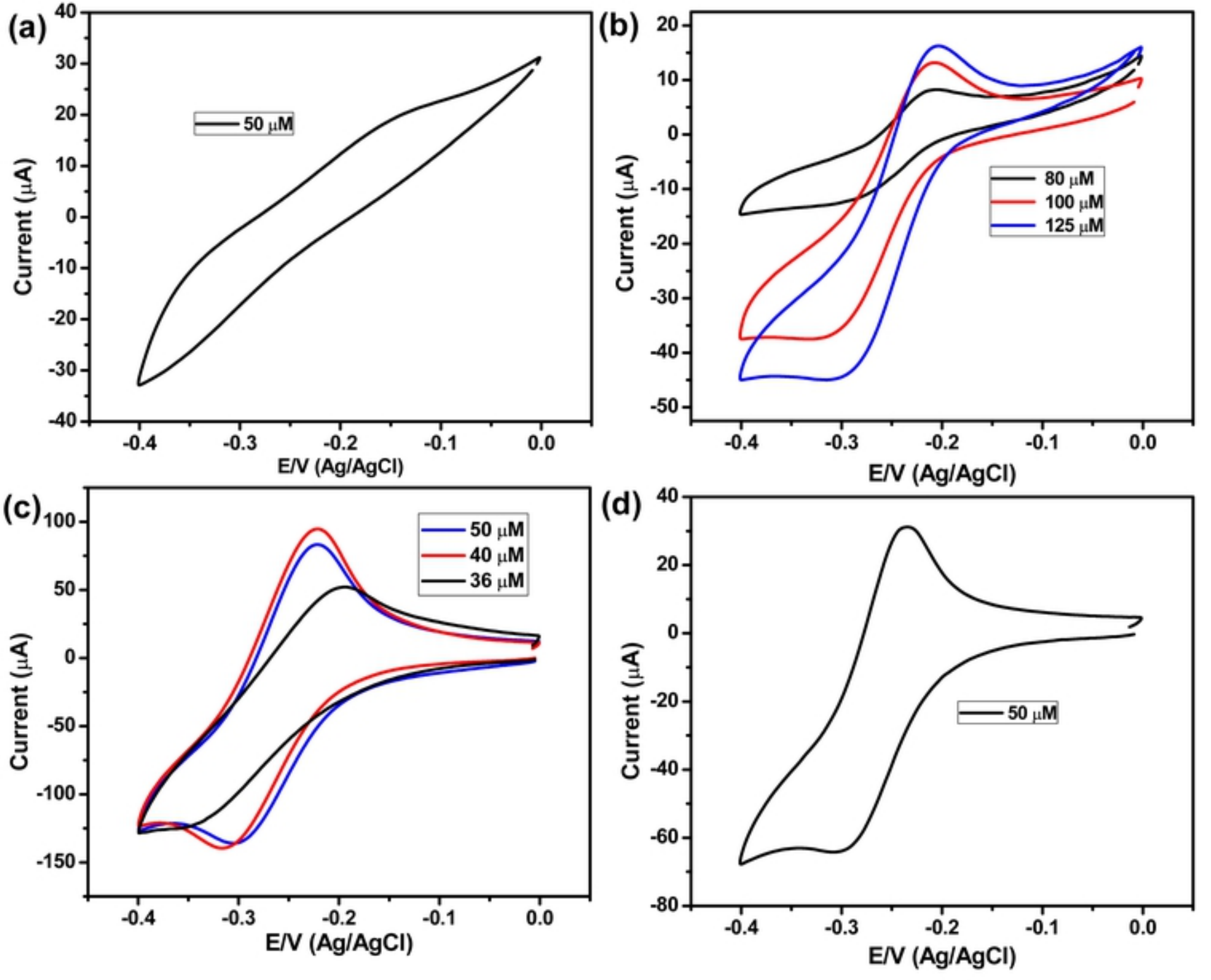

(a)
Current (μA)
E/V (Ag/AgCl)
50 μM
(b)
Current (μA)
E/V (Ag/AgCl)
80 μM
100 μM
125 μM
(c)
Current (μA)
E/V (Ag/AgCl)
50 μM
40 μM
36 μM
(d)
Current (μA)
E/V (Ag/AgCl)
50 μM

Figure 3

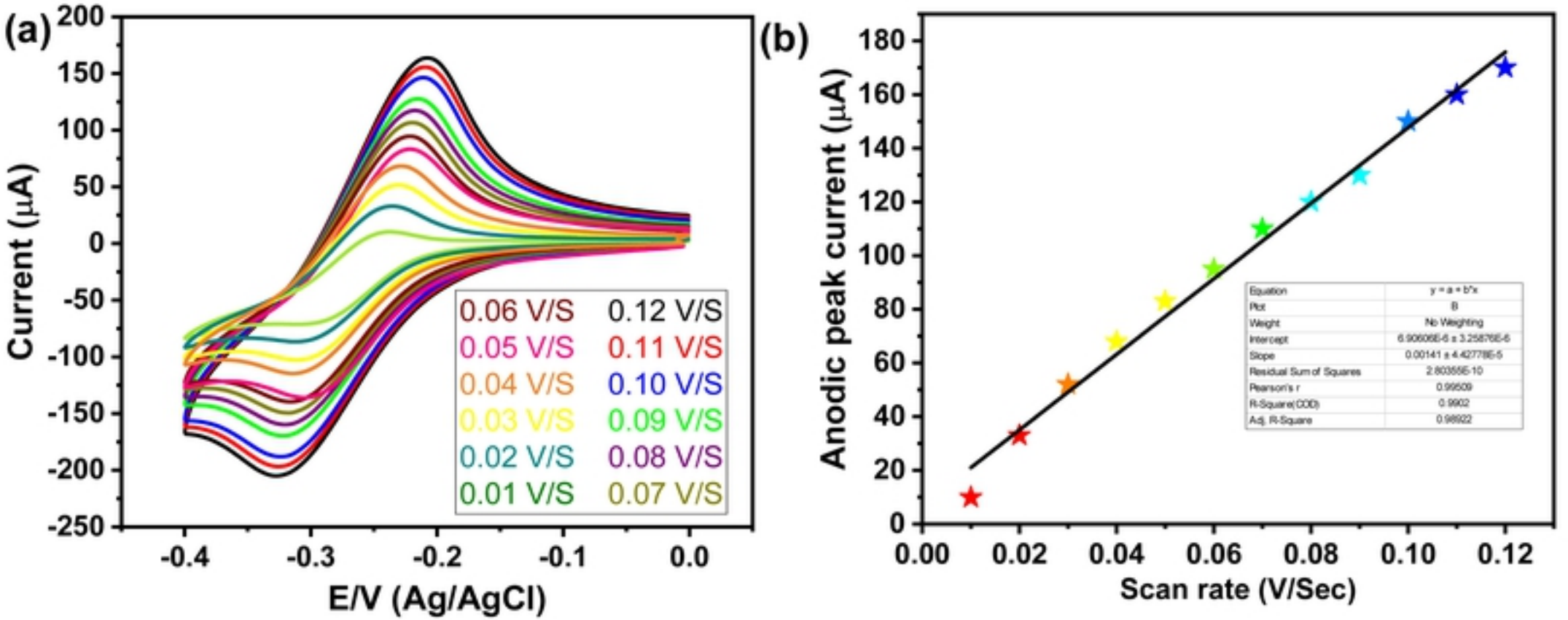
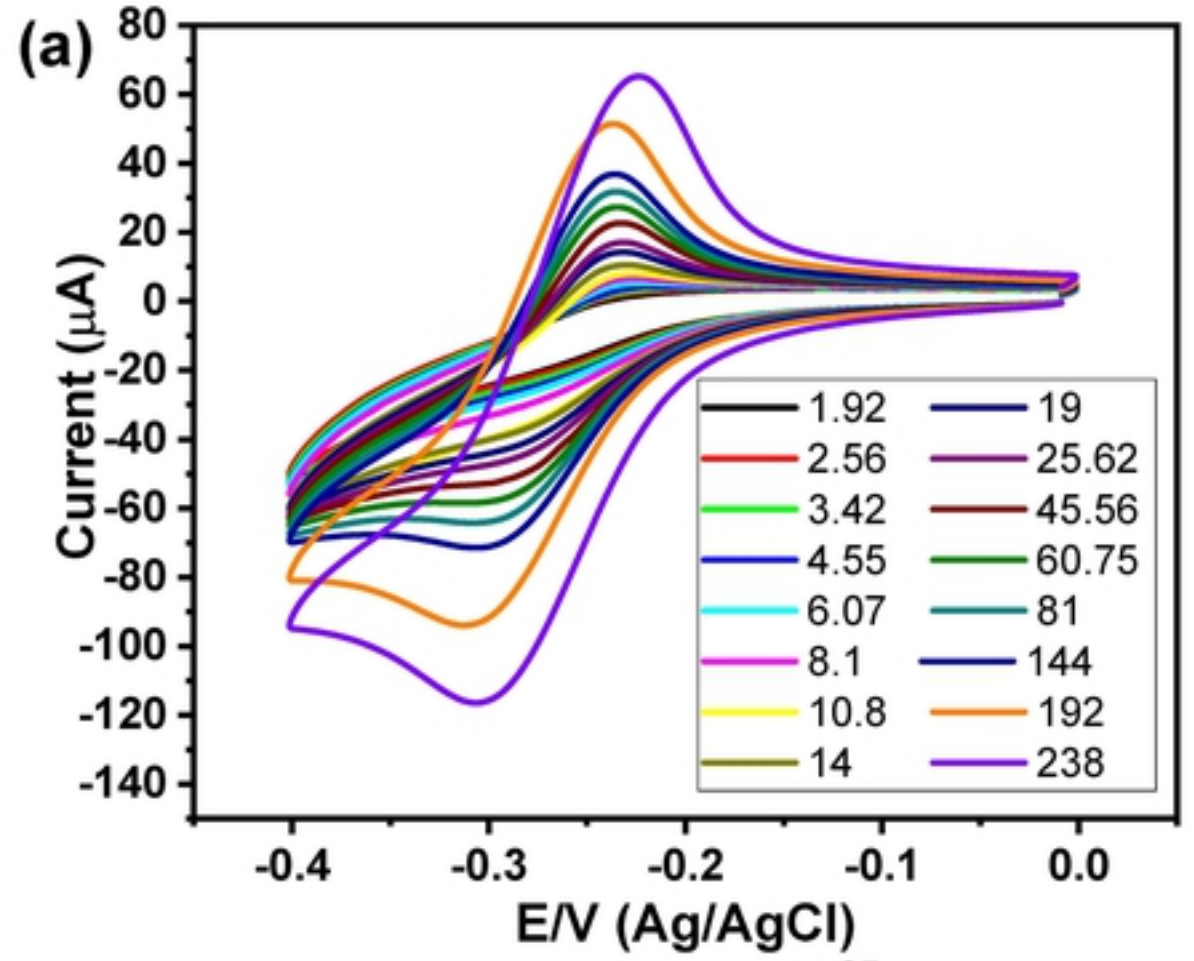
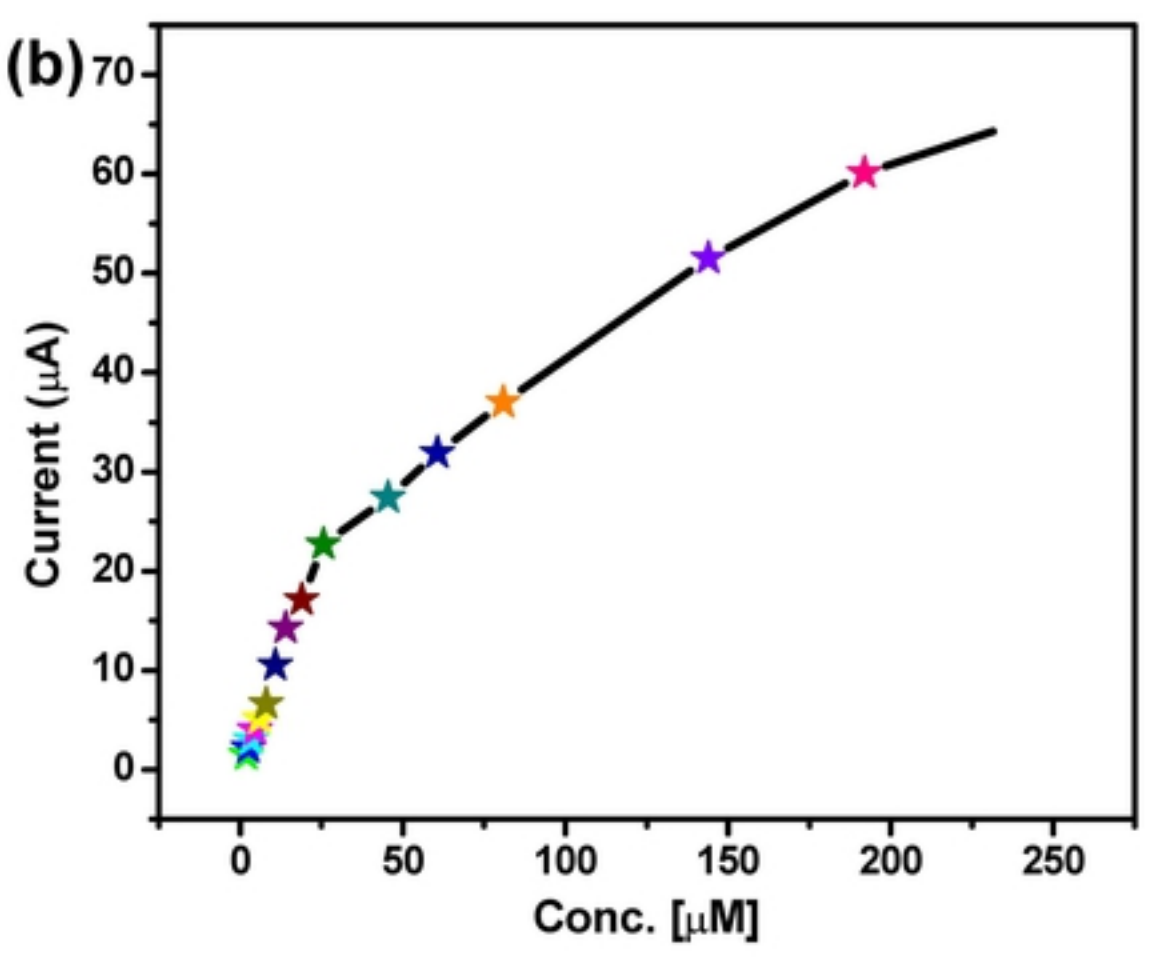

(a)
Current (μA)
E/V (Ag/AgCl)
0.06 V/S
0.05 V/S
0.04 V/S
0.03 V/S
0.02 V/S
0.01 V/S
0.12 V/S
0.11 V/S
0.10 V/S
0.09 V/S
0.08 V/S
0.07 V/S
(b)
Anodic peak current (μA)
Scan rate (V/Sec)
Equation
Plot
Weight
Intercept
Slope
Residual Sum of Squares
Pearson's r
R-Square(COD)
Adj. R-Square

Figure 4





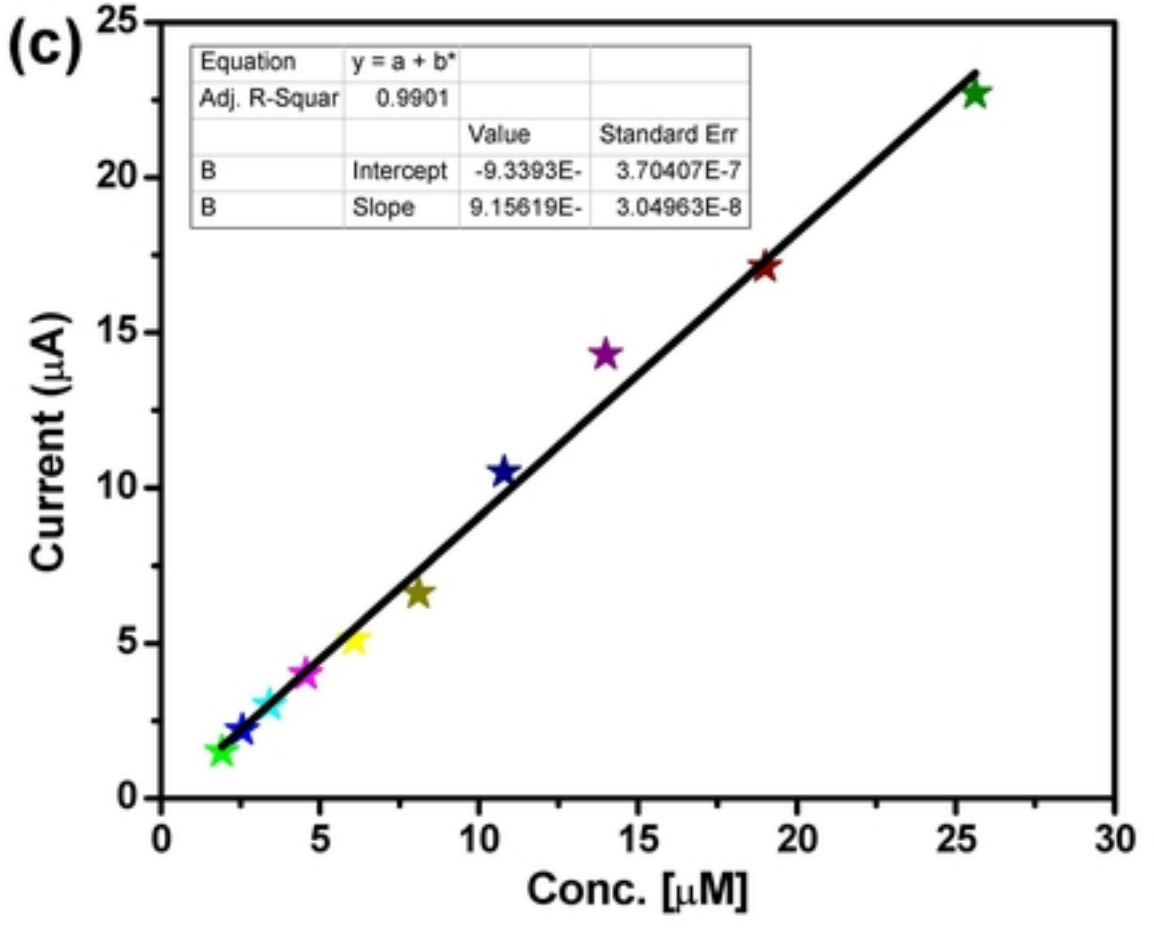


Figure 5

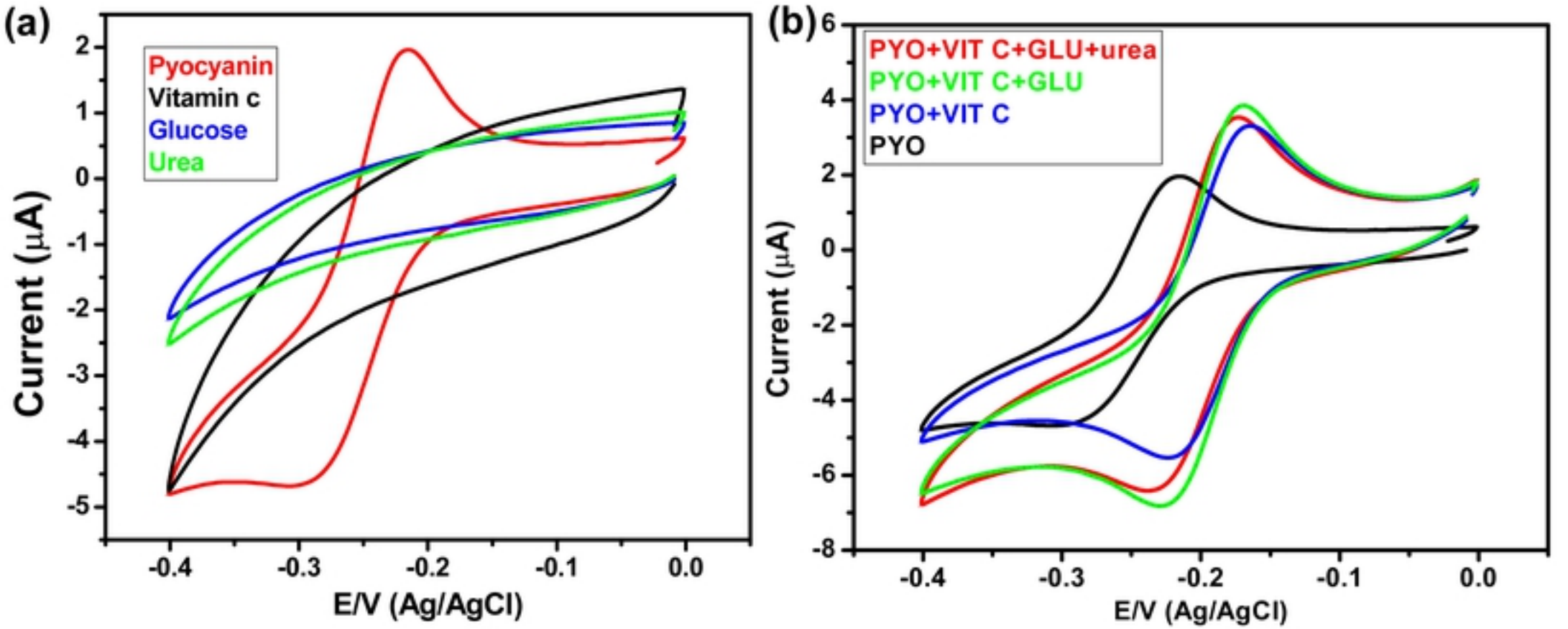

(a)
Pyocyanin
Vitamin c
Glucose
Urea
Current (μA)
E/V (Ag/AgCl)
(b)
PYO+VIT C+GLU+urea
PYO+VIT C+GLU
PYO+VIT C
PYO
Current (μA)
E/V (Ag/AgCl)

Figure 6

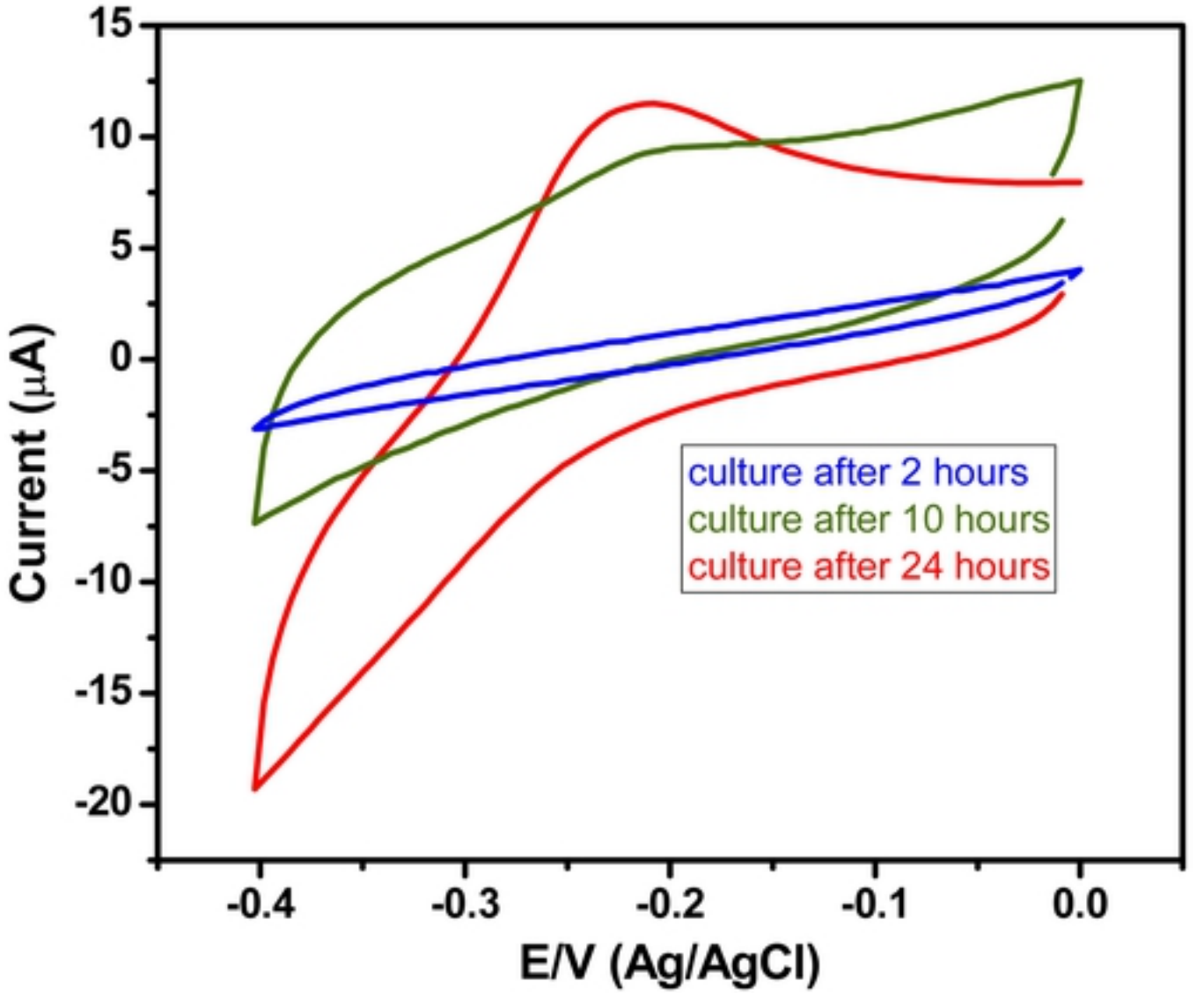

15
10
5
0
-5
-10
-15
-20
Current (μA)
-0.4
-0.3
-0.2
-0.1
0.0
E/V (Ag/AgCl)
culture after 2 hours
culture after 10 hours
culture after 24 hours

Figure 7

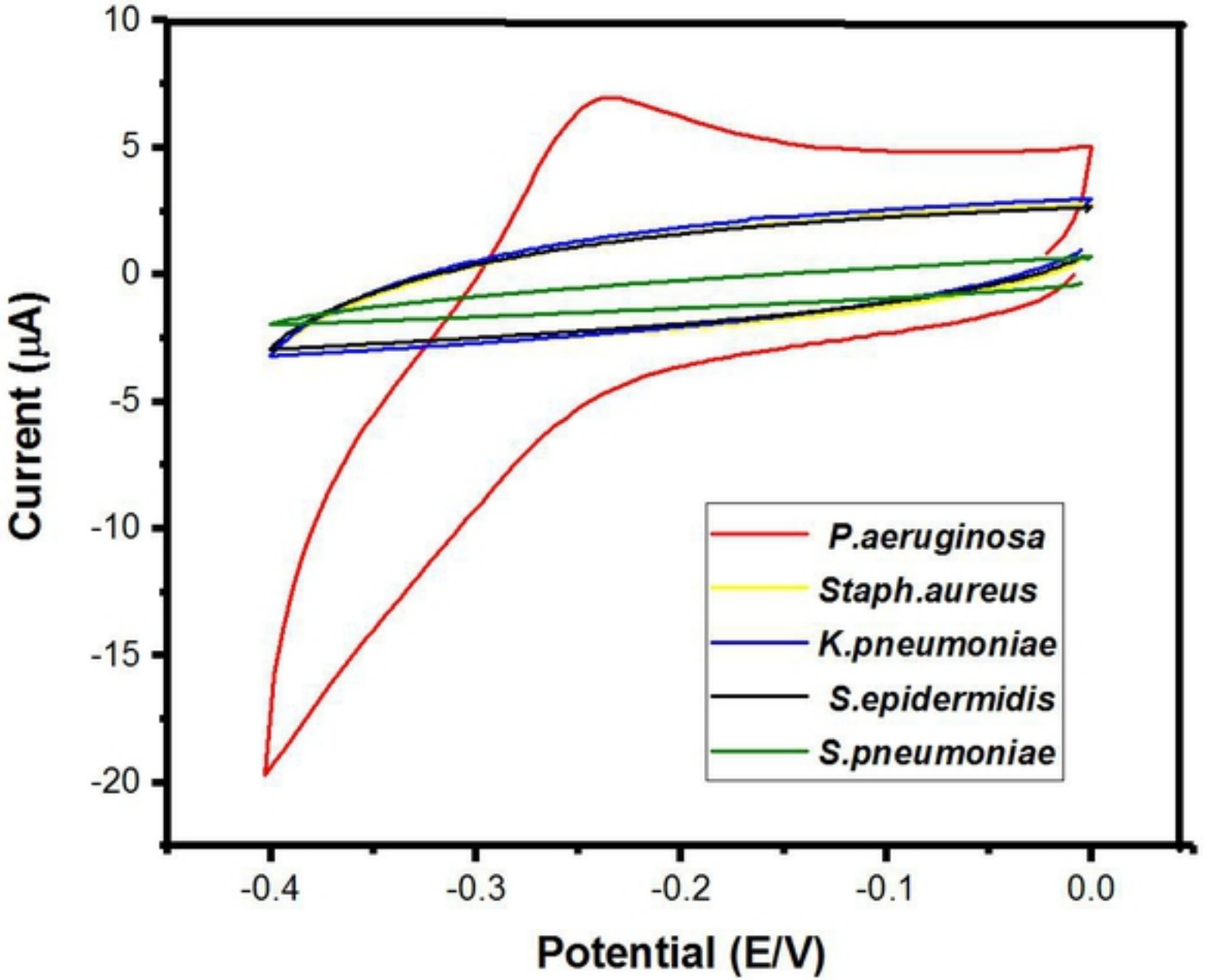

10
5
0
-5
-10
-15
-20
Current (μA)
-0.4
-0.3
-0.2
-0.1
0.0
Potential (E/V)
P.aeruginosa
Staph.aureus
K.pneumoniae
S.epidermidis
S.pneumoniae

Figure 8

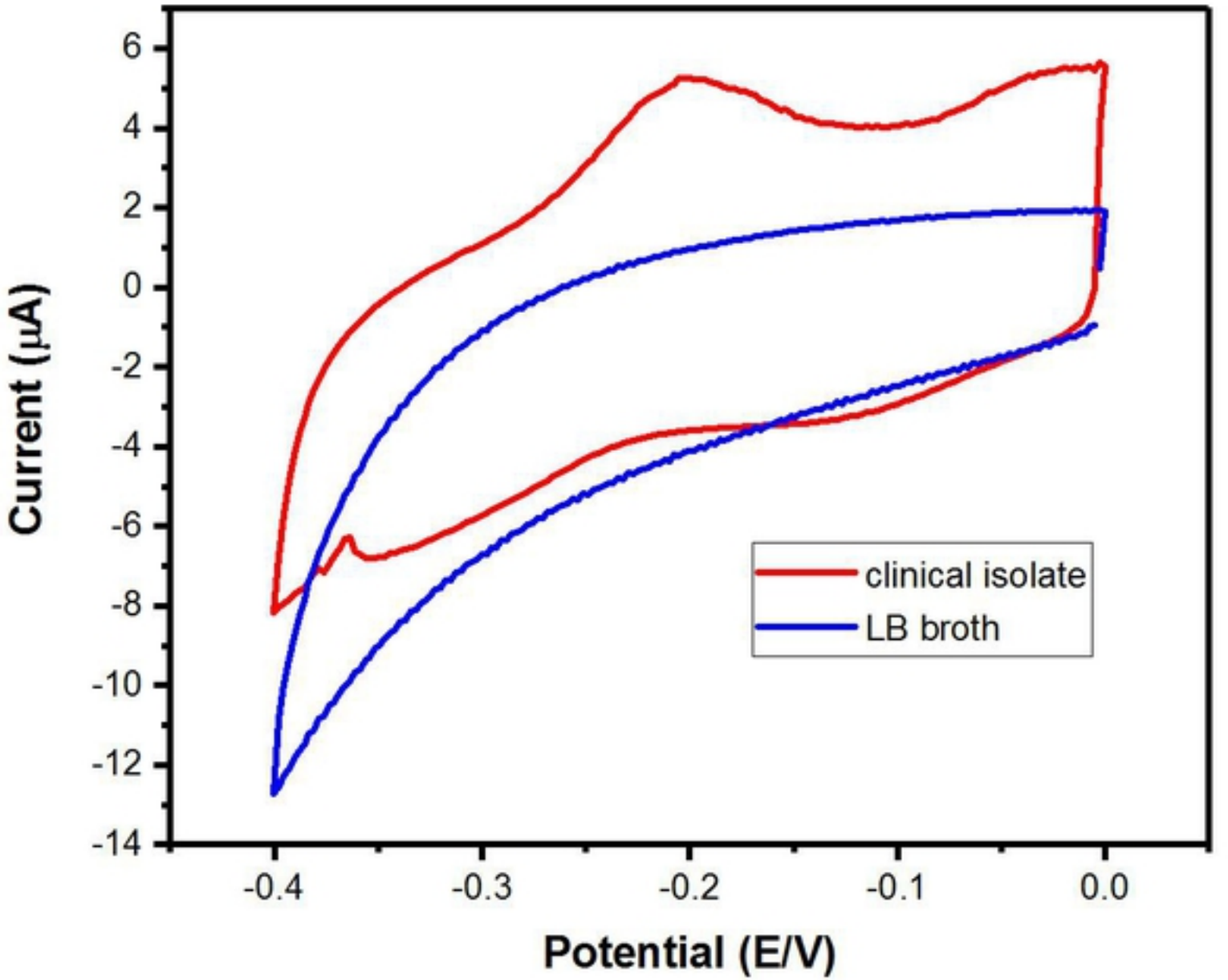

Current (μA)
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-0.4
-0.3
-0.2
-0.1
0.0
Potential (E/V)
clinical isolate
LB broth

Figure 9