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Development of Penicillin G biosensor based on Penicillinase enzymes immobilized onto bio-chips

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Abstract Penicillin G (Pen-G) biosensor was developed by immobilizing penicillinase enzymes (Pen-X) onto tiny bio-chips using thioglycolic acid self-assembled monolayer (TGA-SAM). The selective pen-G biosensor was investigated by ferri/ferrocyanide couple using electrochemical method for catalytic hydrolysis of Pen-G/Pen-X in a very sensible approach. Pen-G was detected with modified bio-chip (Gold electrode of bio-chip/Thioglycolic acid/Penicillinase enzyme, in shortly AuE/TGA/Pen-X) by reliable cyclic voltammetric method at pH 7.1 in room conditions. The AuE/TGA/Pen-X modified bio-chip sensor demonstrates good linearity (50.0 nM to 5.0 mM; R=0.9987), low-detection limit (~1.26 nM, SNR~3), and higher sensitivity (~4.97 μA.μM^{-1}.cm^{-2}), lowest-small sample volume (<70.0 μL), good stability, selectivity, and reproducibility. To the best of our knowledge, this is the first statement in which such a very high sensitivity, low-detection limit, and low-sample volume are required for Pen-G biosensor using AuE/TGA/Pen-X bio-chips assembly. The results of these studies have introduced for biomedical applications with interesting assembly (AuE/TGA/Pen-X) towards the development of selective Pen-G biosensors. The AuE/TGA-SAM system was implemented a facile approach to the integration of Pen-X/Pen-G fabricated bio-chips, which can offer analytical access to a large group of enzymes for wide range of biomedical applications in health-care fields.

Keywords Penicillin G · TGA self-assembled monolayer · Cyclic voltammetry · Bio-chips · Sensitivity · Penicillinase

1 Introduction

Penicillin G (benzylpenicillin) is a parenterally administered form of penicillin, a pioneer in β-lactam antibiotics. Although having a wide-scale spectrum it is more effective against gram-negative micro-organisms. Though it is one of the oldest antibiotics available to myelodysplastic syndromes, it is still one of the first choices in the treatment of several pathologies, like syphilis (Gonçalves et al. 2014; Schoot and Bergveld 1987; Seki et al. 1998; Rahman 2011). The efficacy of penicillin (β-lactam) is threatened by naturally occurring bacterial β-lactamases (Abraham and Chain 1940; Osa 1993), enzymes that obliterate β-lactam stopping them from destroying the bacteria's cell-wall. Furthermore, penicillinase with time becomes more active and efficient to perform this activity. Thus, this leads to a real-time monitoring by bio-medical scientists along with an attentive result using antibiotics. It can be followed in two ways, whether to grow new antibiotics to which resistance have not been established or conjugate the therapeutical administration with β-lactamases' inhibitors like clavulanic acid. When treating a new patient with a potentially severe bacterial infection, the doctor's will start experimental antibiotic treatment, i.e., a choice of antibiotics thinking of the most probable infectious agent taking into account besides the patient's signs and symptoms all epidemiological information available including age, gender, previous health condition, among several others factors. Nevertheless, the clinician often starts developing bacterial cultures to set-up resistances and susceptibilities of that microorganism. Additionally, the broad use of these antibiotics, largely with veterinary approaches,

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has led to environmental contamination that, besides aggravating the emergence of antibiotic-resistant bacterial strains, has generated many problems from disturbing eco-systems to polluting natural water sources among many other not fully understood issues.

Biosensors based on field-effect principle in semiconductor structures have been extensively studied within the recent years (Caras and Janata 1980, 1985; Lee et al. 2009a, b; Hafeman et al. 1988). In this method, the enzyme molecules are immobilized on the surface of a semiconductor structure which converts the respective substrate to turn out a charged product. The product is significantly detected by an ion-sensitive surface layer of the sensor device and the resulting surface charge modules the space charge region at the insulator–semiconductor interfaces. Previously charge transfer type pH sensor based on Charge Coupled Device technique was developed successfully by Sawada group (Sawada et al. 1999, 2004). The ISFET type penicillin sensor is widely used (Poghossian et al. 2003). Penicillinase can catalyze the hydrolysis of penicillin to penicillinic acid, resulting in a decrease of solution pH. This forms the basis of many reported penicillin biosensors, in which the pH change is usually monitored by potentiometry (Rusling et al. 1976; Kumaran et al. 1991; Rahman et al. 2011a; Meier et al. 1992), conductometry (Nishizawa et al. 1992), colorimetry (Healey and Walt 1995), or fluorescence (Xie et al. 1992). The potentiometric modes have been employed most frequently; they are simple in configurations but have drawbacks of slow response speeds and high detection limits (0.08–1.5 mM). The conductometric and optical modes are advantageous to the potentiometric ones in response speeds, but they still have relatively high detection limits (0.05–0.25 mM). Consequently any new tools to monitor the efficacy of penicillin (β-lactamases) and to determine β-lactams in all kinds of environmental samples are welcome. β-Lactamase catalyzes the hydrolysis of pen-G to penicillinoic acid. This changes the pH and can be measured in varied forms (Rusling 1976; Siqueira et al. 2009; Rahman 2010). Conversely, an amperometric approach to determine penicillin enzymatic consumption is hardly found in literature. Nevertheless, one can find from the previous study such as a hematein based biosensor on a platinum electrode surface (Stred'anský et al. 2000), with a dot-blot genosensor (Pividori et al. 2001), with co-immobilization of carbon nanotubes and hematein on a glassy carbon electrode (Chen et al. 2010a, b), and using a screen-printed carbon electrode (Gamella et al. 2013). In this report, a highly sensitive penicillin G biosensors based on TGA-SAM assembly on tiny bio-chips at 7.1 pH is developed. Here, it is also measured the lower sample volume and lower-detective pen-G sensor using bio-chips, which is designed and fabricated successfully by photolithographic methods. The most significant characteristics of the developed Pen-G biosensor using bio-chips are highly sensitivity, large linear dynamic range, most selective, very low detection limit, small sample volume required, highly stable, and reproducible.

2 Experimental section

2.1 Materials and methods

Penicillin G (Pen-G), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), potassium ferricyanide [$K_3Fe(CN)_6$], Thioglycolic acid (TGA), Monosodium phosphate, Disodium phosphate, and Penicillinase (Pen-X, EC 3526 Becillus cereus, specific activity: 1650 U/mg protein) were purchased from Sigma-Aldrich company (www.sigmaaldrich.com). All other chemicals were analytical-grade and used without further experimental purification. 0.1 M phosphate buffer solution (pH ~7.1) is prepared by mixing the uni-molar proportion of 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4. All solutions were prepared with de-ionized distilled water, which obtained from a water purifying apparatus (12.0 MΩ.cm) (AQUA MEDIA; www.aquamediadirect.com). Pen-X enzyme solution was freshly prepared daily before the fabrication of the bio-chips by dissolving the Pen-X in the PBS buffer system. The 0.1 M Pen-X solution (50.0 μL) was immobilized on the sensing area of bio-chip using the simple adsorption method. Then it was dried for 30.0 min at room temperature. Various concentration of Pen-G solution (50.0 nM, 100.0 nM, 1.0 μM, 10.0 μM, 100.0 μM, 500.0 μM, 1.0 mM, 5.0 mM, 10.0 mM, and 50.0 mM) were prepared prior to conducting the measurements in PBS/$K_3Fe(CN)_6$ system. For developing the bio-chip type pen-G biosensors, all experiments were carried out into a cell containing 50.0 μL (pH 7.1) in test and target solution at room temperature (25.0 °C). In the measurement, the Pen-G sample was introduced into the bio-chip of each sensor and allowed to remain for 3.0 min for the enzymatic reaction to take place. Finally, the output current was measured within the next 10 s. The electrochemical experiments were measured using a votammetric-analyzer (CV-50W, BAS, USA; http://www.basinc.com). All investigations were carried out on tiny bio-chip (5.0 mm×5.0 mm), which sensing area is close to 0.02218 cm^2. The total electrochemical investigation were performed with Pen-X modified bio-chips composed as working, Pt-layer as counter, and Ag/AgCl (saturated KCl) as reference electrodes. Cyclic voltammetry (or, CV) is a type of potentiodynamic-electrochemical measurement. In a cyclic voltammetry experiment the working electrode potential is ramped linearly versus time like linear sweep voltammetry. Cyclic voltammetry takes the experiment a step further than linear sweep voltammetry which ends when it reaches a set potential. When cyclic voltammetry reaches a set potential, the working electrode's potential ramp is inverted. This inversion can happen multiple times during a single experiment.

The current at the working electrode is plotted versus the applied voltage to give the cyclic voltammogram trace. Cyclic voltammetry is generally used to study the electrochemical properties of an analyte in solution. CV was recorded at Pen-X/TGA/AuE electrode from −0.1 to +0.5 V (versus Ag/AgCl) in a 0.1 M PBS/ $K_3Fe(CN)_6$ solution (pH 7.1) at 100.0 mV/s scan rates. The total experimental set-up is presented with camera-view photographs for tiny bio-chips (Fig. 1). Here, the potential analyzer (CV-50W) is controlled and interfaced with SOTECH-PC, which is directly connected with the lab-set electronic system composed of tiny biochips, where (A) real-image of bio-chips, (B) magnified view of bio-chips, and (C) magnified view of immobilization of Pen-X enzyme onto bio-chips. Calibration curve is prepared by using the current value in CV curve, respect to each concentration during in CV measurement.

2.2 Fabrication of bio-chips

Preparation of bio-chips by photolithographic method is explained in the electronic supplementary materials sections [presented in ESM, Ψ]. The semiconductor bio-chips were made on silicon wafer. Aluminum was sputtered to fabricate as wiring and bonding pads. Pt/Ti/TiN was sputtered on heated silicon oxide and patterned using photolithography to prepare counter electrode (CE). Ti/TiN layers were used for strong adhesion. Au/Ti was sputtered and lithographed, which prepared circular working-electrode (WE) with a diameter of 1.6 mm in the chip-center. After electrodes fabrication, parylene layer was fabricated using evaporation technique as a passivation layer. The wafer was diced to 5.0×5.0 mm^2 chips. This chip was bonded to a package by silver paste. Aluminum pads were connected to the package by gold wire. Finally, adhesive (Araldite, Hantsman, Japan) was pasted on the periphery of the chips to protect the target solution from contacting pads, which is presented in Fig. 2a. The magnified construction view of internal chip-center composition (sensing area) is shown in the Fig. 2b. A cross section of the total sensor chip and sensing area is presented in Fig. 2c and d respective.

3 Results and discussion

The sensing protocol using the AuE/TGA/Pen-X modified bio-chips is presented in Fig. 3. It is used for covalent bond formation to immobilize the Pen-X enzyme on the TGA-SAM via peptide conjugation in presence of activating agent (EDC). First, the self-assembled monolayer of TGA is formed onto bio-chips (dropping methods) for 2 h. Then Pen-X enzyme is immobilized on the TGA-SAM by amide-bond formation between the terminal-unbound carboxylic acids (−COOH) groups on the TGA film and the amine groups ($-NH_2$) of the Pen-X enzymes. For the stable attachment of Pen-X onto TGA-SAM after activated by EDC, the bio-chip was placed into the refrigerator at 4.0 °C for 2.0 h. The enzymatic reactions were performed on the bio-sensing system in chip for the efficient detection of Pen-G as follows the Fig. 3. Catalytic reaction is depended on Pen-G concentration in the reaction medium. On the bio-chips, Pen-G is hydrolyzed to form penicilloic acid and hydrogen ions (by releasing electron) to produce electrons in presence of Pen-X. This current is directly proportional to the target Pen-G concentration in the solution system in this enzymatic reaction (Fig. 3).

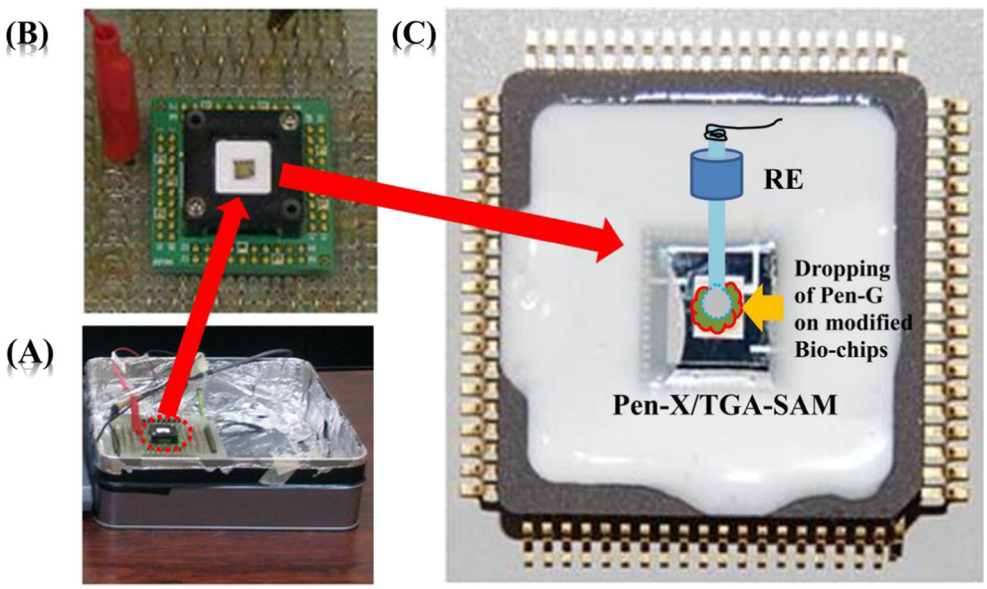

Fig. 1 Experimental set-up of bio-chip. (**a**) real-image, (**b**) magnified view, and (**c**) magnified view of immobilization of Pen-X onto bio-chips

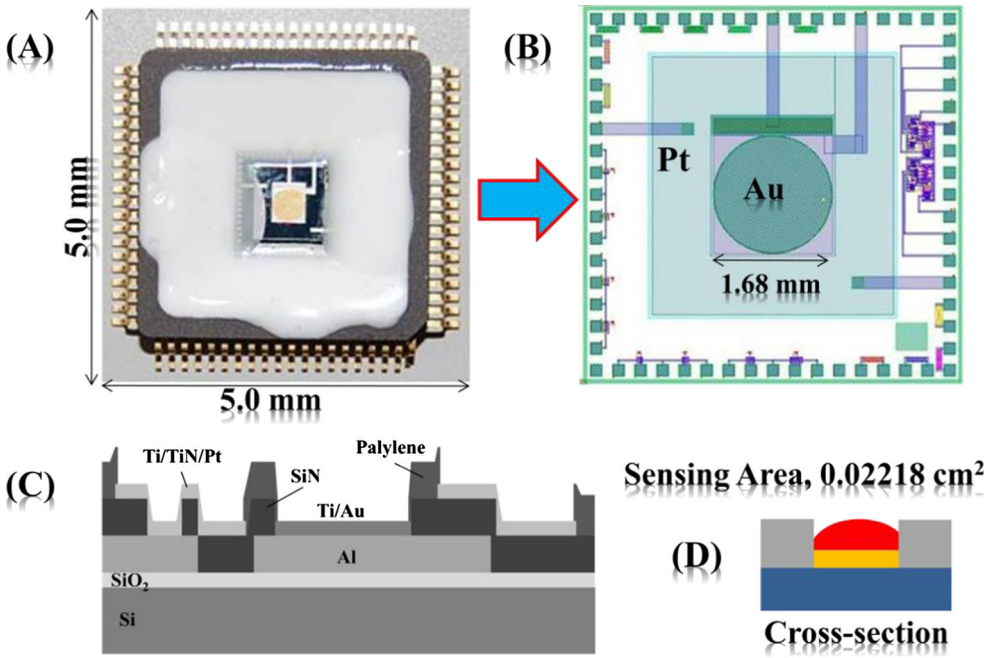

Fig. 2 Schematic diagram of bio-chip fabrication assembly. (**a**) top camera-view, (**b**) magnified view of sensing area, (**c**) total cross-sectional view of bio-chip, and (**d**) cross-section of sensing area of bio-chips

Successful fabrication of Pen-G biosensor using Pen-X on the sensing area of bio-chip was confirmed based on TGA-SAMs by cyclic voltammetric methods. Conventional electrochemical method is the most versatile electro-analytical method for the study with bio-active materials and species, which was extensively used in industrial applications and academic research for research and development (R&D) approaches (Rahman et al. 2011b, c). CV is also a significant method to assess the blocking property of the monolayer-coated electrodes using diffusion controlled redox couples. Bio-chip surface was cleaned by Piranha solution [H_2SO_4:H_2O_2 (3:1)] and washed with pure water, then dried adequately by nitrogen gas. TGA was dissolved in ethanol to make 10.0 mM solution. TGA solution was dropped on a sensing area of bio-chip, and

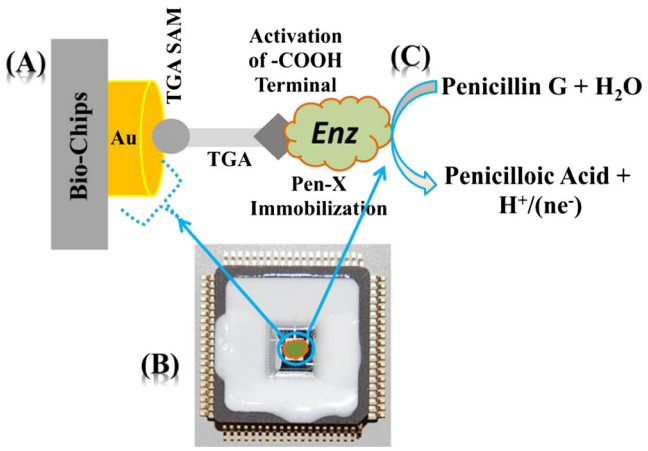

Fig. 3 Schematic representation of fabrication procedure by Pen-G biosensor using bio-chips. (*A*) Sensitive and active surface contact of bio-chips, (*B*) Real-image of immobilized bio-chips, (*C*) Reaction mechanism of Pen-G in presence of selective Pen-X enzymes

then kept for 2 h at room conditions. Figure 4 shows the CVs of un-modified and TGA-SAM modified bio-chip electrodes in 5.0 mM $K_3Fe(CN)_6$ with 0.1 M PBS as the supporting electrolyte at 0.1 V/s scan rates. It can be seen from the Fig. 4a, that the bio-chip electrode (black-line) shows a reversible voltammogram for the redox couple indicating that the electron transfer reaction is completely diffusion controlled. In contrast, the absence of any peak formation in the CVs of the TGA monolayer modified bio-chip electrodes (blue-line) shows the redox reaction is inhibited. The CVs for TGA indicated a good blocking behavior for the electron transfer reaction, which means that a highly ordered, compact monolayer is formed on the sensing surface of the bio-chips (Rahman et al. 2009). Pen-X was embedded onto the TGA-SAM modified surface by the phenomena of peptide conjugation. First 10.0 mM EDC in 0.1 M phosphate buffer solution was put onto the TGA-SAM bio-chips and then it was kept at 4.0 °C in the refrigerator to activate terminal carboxylic group of TGA for 2 h. Then EDC-treated electrode was washed slowly with 0.1 M PBS to eliminate excess EDC. Then Pen-X solution was dropped onto the sensing area (center) of bio-chip and incubated in the refrigerator at 4.0 °C for 24 h. Pen-X was effectively immobilized onto TGA SAM via covalent attachment, which was confirmed by the current change in Fig. 4b. It showed that the CVs recorded for the AuE/TGA/Pen-X (red-line), and 0.1 mM Pen-G solution (black-line) of fabricated bio-chips in a 0.1 M PBS/5.0 mM $K_3Fe(CN)_6$ at 0.1 V/s scan rates. A small current change was observed at approximately +0.32 V versus Ag/AgCl (sat. KCl) for 0.1 mM Pen-G solution and this was due to the current of enzymatic reaction with Pen-G in presence of Pen-X on the sensing surface of the bio-chips. The enzymatic current approximately 0.29 μA was

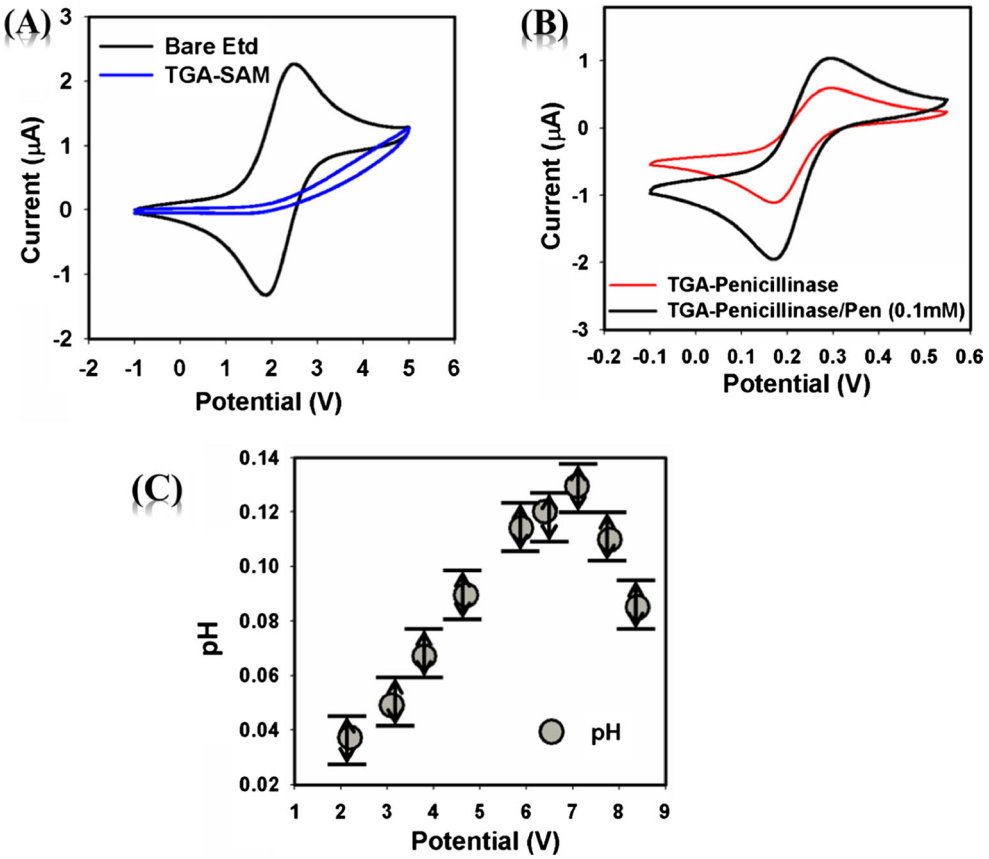

Fig. 4 Optimization of fabricated Pen-G biosensors. (**a**) CVs of 5.0 mM $K_3Fe(CN)_6$/PBS (0.1 M) for bare (*black-line*) and TGA-SAM (*blue-line*) modified bio-chip electrodes; (**b**) CVs recorded in 0.1 M phosphate buffer (with 5.0 mM $K_3Fe(CN)_6$) solution of bare bio-chip (*red-line*), Pen-X modified bio-chip (*black-line*) in presence of 0.1 mM Pen G solution; (**c**) Effect of pH of AuE/TGA/Pen-X electrode in 0.1 mM Pen-G solution with the bio-chips. Scan rate: 0.1 V/s; Ref. Etd: Ag/AgCl (saturated KCl); Supporting electrolytes: 0.1 M phosphate buffer solution

executed to be increased on the increasing Pen-G concentration in the PBS/ $K_3Fe(CN)_6$ solution. The experimental conditions affecting the performances (detection limit, sensitivity, and response time) of the biosensors were optimized in term of pH and presented in Fig. 4(c). The pH of the buffer shows a strong effect on the activity of the sensing layer on Pen-X enzyme fabricated bio-chips. The effect of the pH of the buffer on the current change is studied over the pH range of 2.0 to 8.5. Figure 4(c) shows the CV peak currents measured at various pH values for 0.1 mM Pen-G in 0.1 M phosphate buffer solution system. The peak height is increased from pH 5.0 to 7.1 and then decreased above pH 7.1 until 8.5. The peak current decreases above pH 7.1 might have been owing to the poor Pen-X enzyme activity at higher basic medium. Therefore, the pH of the phosphate buffer solution system is kept constant at 7.1 throughout the experiments.

Cyclic voltammetric was performed to confirm the detection of Pen-G concentration with mediators in simple phosphate buffer solution system. 50.0 μL of each Pen-G solution with mediator ($K_3Fe(CN)_6$/PBS) was dropped on the sensing area of the bio-chips and measured the sensing oxidation current. Figure 5(a) exhibits a typical CV (current–voltage) plot

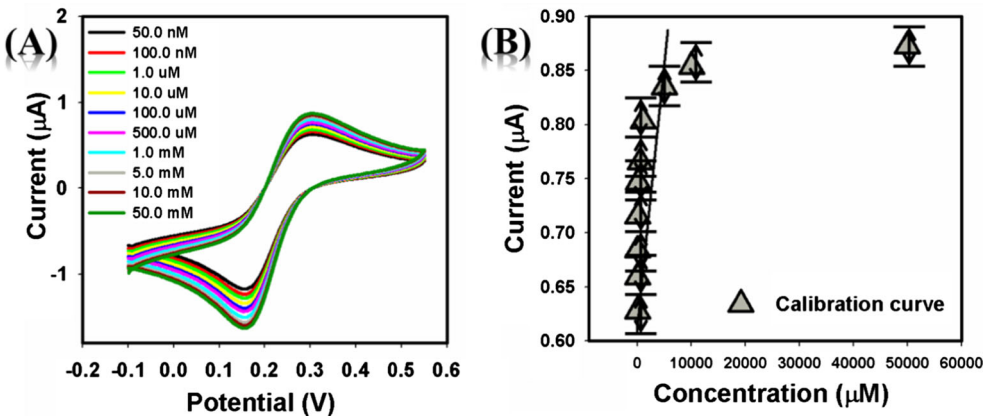

Fig. 5 Cyclic voltammogram study of Pen-G biosensors. Electrochemical biosensor responses of (**a**) variation of Pen-G concentrations (50.0 nM to 50.0 mM), (**b**) calibration curve of developed on bio-chip at room conditions

Table 1 Evaluation of the AuE/TGA/Pen-X modified Pen-G biosensor performances compared with various enzyme-material conjugated sensors

Electrode materials	Sensitivity	Detection limit	Sample volume (μL)	Response time (s)	Linear dynamic range (LDR)	Linearity (R)	Ref[n]
ZnO NRs/Pen-G	121.0 mV/decade	–	–	5 s	100.0 μM to 100.0 mM	–	(Ibupoto et al. 2011)
CNT/Hematein/Pen	–	24.0 μM	–	–	24.0 μM to 0.11 mM	–	(Chen et al. 2010a, b)
SWNTs/ PAMAM/LBL	55.0 mV/pH	5.0–25.0 μM	–	0.5–3 min	250.0 μM to 25.0 mM	–	(Siqueira et al. 2009)
Si3N4/POx/ISFET	6.56 mV/mM	0. 1 mM	100.0 μL	0.5–3	0–12.5 mM	0.9875	(Lee et al. 2009a, b)
Biochip/TGA/Pen-X	~4.97 $\mu A \mu M^{-1} cm^{-2}$	~1.26 nM	<70.0 μL	10 s	50.0 μM~5.0 mM	0.9987	Current work

for the addition of varying amounts of target Pen-G in a 0.1 M phosphate buffer solution (pH 7.1) solution. The currents are increased steadily with increasing the concentration of Pen-G (50.0 nM to 50.0 mM) in stable and saturated value. The AuE/TGA/Pen-X modified bio-chip electrode is achieved 95.0 % of steady state currents with in 10.0 s. The increase of oxidation current is presented due to the Pen G hydrolyzed in presence of Pen-X and the current change leads to the higher current value. Figure 5(b) shows the calibration plots for the Pen-G found from the current–voltage responses with fabricated bio-chips. Under the optimized conditions, the steady-state currents are exhibited a linear relationship with the target Pen-G concentration in the range from 50.0 nM to 5.0 mM.

The linear dependence of the Pen-G concentration acquiesced with a correlation coefficient of 0.9987. The detection limit of the bioassay was defined as the lowest concentration that can be determined to be statistically different (standard deviations) from the average response of Pen-G samples. The detection limit for Pen-G was executed to be approximately ~1.26 nM, based on signal to noise ratio ($^{3N}/_{S}$). The Pen-G biosensor also exhibited higher sensitivity, which was calculated as ~4.97 $\mu A.\mu M^{-1}.cm^{-2}$. The sensitivity is much higher than the previously reported Pen-G biosensors, where the total comparative performances are included in Table 1. For comparing, the biosensor performances of the Pen-X fabricated bio-chip is compared with the previously reported Pen-G biosensors based on the enzymes conjugated materials and it was confirmed that the AuE/TGA/Pen-X bio-chip penicillin biosensor performances exhibited an excellent results.

A series of successive measurements of Pen G in 0.1 M phosphate buffer solution yielded a good reproducibility signal at AuE/TGA/Pen-X biosensor with a relative standard deviation (RSD) 3.4 %. The sensor-to-sensor and run-to-run reproducibility for 0.1 mM Pen-G detection were found to be 1.4 and 1.1 % respectively. To study the long-term storage stabilities, the response of the AuE/TGA/Pen-X biosensor was done with the respect to the storage time. After each experiment, the biosensor was washed with the buffer solution and stored in 0.1 M phosphate buffer solution at 4.0 °C until use. The long-term storage stability of the biosensor was tested every 3 days. The recovery (R) was determined as the ratio between measured concentration (Cm) and added concentration (Ca): $R(\%)=(C_m/C_a)\times 100$. The sensitivity retained 84.0 % of initial sensitivity up to 1 month. After 1 month,

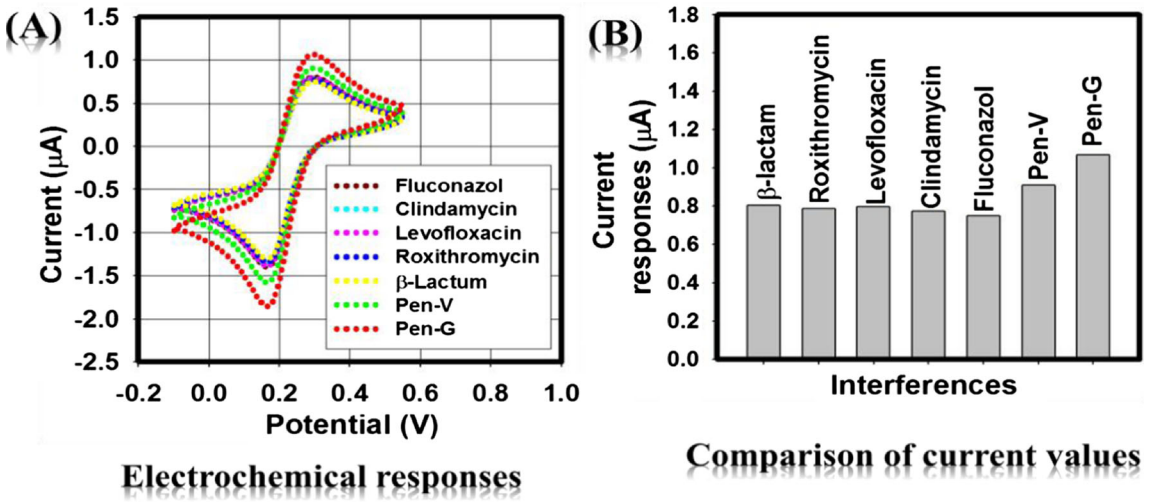

Fig. 6 Selectivity study. The selectivity of the AuE/TGA/Pen-X assembly towards Pen-G biosensor was evaluated in the presence of common interfering biological compounds. ([Analytes]: 0.1 mM; PBS: 0.1 M)

the response slowly decreased, probably due to the loss of the enzyme activity on the biosensor bio-chips. The above results clearly indicated that the bio-chip of Pen-G biosensor can be used for a month without any significant loss in sensitivity. In order to checked the accuracy of the developed methods, analytical recovery of added Pen-G in five samples was investigated. The analytical performance of Pen-G in target sample is much higher than conventional electrode by other electroanalytical methods (Hassen et al. 2007; Gustavsson et al. 2002). The simple fabrication method of the biosensor has several advantages over conventional technique such as ease of fabrication, enhanced electro-catalysis, and efficiently preserving the activity of bio-molecules. The selectivity of the AuE/TGA/Pen-X biosensor was evaluated in the presence of other electro-active compounds such as penicillin V, β-lactam, roxithromycin, levofloxacin, clindamycin, and fluconazole, which is presented in Fig. 6. No significant current-response was found when 0.1 mM penicillin V, β-lactam, roxithromycin, levofloxacin, clindamycin, and fluconazol were injected into the 0.1 M phosphate buffer system. But when 0.1 mM Pen-G solution was added to the electrolyte solution, a clear response was observed, indicating the selective detection of Pen-G with the AuE/TGA/Pen-X sensor layer. Extensively at this concentration level (penicillin V, β-lactam, roxithromycin, levofloxacin, clindamycin, and fluconazol), these were not showed any interfere in 0.1 mM Pen-G detection using tiny bio-chips. Thus, the selectivity of the AuE/TGA/Pen-X bio-chip biosensor is acceptable for Pen-G detection in the presence of the common interfering compounds in normal physiological levels.

4 Conclusion

Successful fabrication of highly sensitive Pen-G biosensor based on the immobilization of Pen-X on gold-fabricated bio-chip sensor has been investigated in $[K_3Fe(CN)_6]$\PBS system. Pen-X was successfully attached to tiny bio-chips by carboxylic terminal TGA SAM, being possible to obtain in this way of Pen-G biosensors development. This experimental setup cannot only be further used to study the efficiency of Pen-X inhibitors and also resistances towards selective Pen-G antibiotics, but can also be used for analytical purposes since a higher sensitivity and lower LOD was obtained. The selective Pen-G biosensors were exhibited the higher sensitivity (~4.97 $\mu A.\mu M^{-1}.cm^{-2}$), low-detection limit (~1.26 nM) with satisfactory stability, large linear dynamic range (50.0 nM to 5.0 mM), and reproducibility (the experiment was repeated for 5 times to check for reproducibility). Finally, this is the first report in which such a high-sensitivity and low-detection-limit has been achieved with ultra-sample volume for the Pen-G detection by AuE/TGA/Pen-X bio-chips assembly at room conditions.

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