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Development of a Mediator-Type Bioelectrochemical Sensor Based on Polypyrrole Immobilized Ferricyanide and Microorganisms for Biochemical Oxygen Demand Fast Detection

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A mediator-type bioelectrochemical sensor was developed by using polypyrrole (PPy) immobilized ferricyanide (FC) as mediator and immobilized *Pseudomonas aeruginosa* (*P. aeruginosa*) as a biosensing film for biochemical oxygen demand (BOD) fast detection. The sensor chip consists of a three-electrode system, with Au working electrode (WE), Pt counter electrode (CE) and Pt pseudo-reference electrode (RE) compactly integrated as a disposable using micro-electro-mechanism system (MEMS) technology. The FC mediator and *P. Aeruginosa* microorganisms have been embedded in PPy matrix on gold microelectrode surface during the electropolymerization of pyrrole monomer using electrochemical cyclic voltammetry (CV) method. This bioelectrochemical sensor responds to BOD due to yielded ferrocyanide during catalytic reduction by metabolic reactions of microorganisms. A good linear correlation with chemically determined BOD values was obtained from 5 to 100 mg/L with fast response time. The proposed sensor in this paper is significant for BOD fast detection.

Keywords: mediator-type bioelectrochemical sensor, polypyrrole, immobilized ferricyanide and microorganisms, biochemical oxygen demand fast detection,

1. INTRODUCTION

Biochemical oxygen demand (BOD) is an indicator of the amount of biodegradable organic compounds and is one of the most important parameter in water quality monitoring. The main disadvantage of conventional 5-days BOD measuring method (BOD_5) is that it is time-consuming and

can not reflect the water pollution status in time[1]. So the rapid BOD biosensor is attracting more and more attention. However, there is a limited factor for the commercially available biosensors, that is those rapid BOD biosensors were based on oxygen as terminal electronic acceptor and its low solubility (8.7mg/L at 25°C) in water always caused weak and fluctuating signals[2]. In recent years, a rapid method was developed by using mediator (artificial electron acceptor) instead of oxygen for BOD detection. With the high solubility of mediator, the mediator-type BOD bioelectrochemical sensor overcame the problem of the limitation from oxygen[3].

Since Pasco et al.[4] reported the ferricyanide-mediated (FC-mediated) microbial reaction for BOD measurement in 2000, the FC-mediated BOD method gained great attention and some different FC based mediator-type BOD bioelectrochemical sensor were succesfully developed during the past decades[5]. However, there are also some limitations for these FC based mediator-type BOD bioelectrochemical sensor. On one hand, the FC mediator was dissolved in samples, which is not only laborious in sample preparation, but also troublesome in a secondary environmental contamination. On the other hand, microorgnasims are usually physically adsorbed and immobilized in alginate[6], agarose[7], or some non-conductive polymers (such as polyurethane, Al_2O_3 sol-gel, PCS, ormosil-PVA etc.)[8-16] as a biomembrane attached on the surface of working electrode, which could cause poor reproducibility due to cell losing during mutiple measurements, and is not suitable for microelectrode surface owing to its low controllability. In our previous study, we reported a FC mediator-type BOD bioelectrochemical sensor[17], in which the immobilized *Yeast* microorganisms and dissolved ferricyanide were incubated in a bioreactor. Owing to the advantage of the bioreactor and IDA microelectrode, high responses were obtained, but 1h measuring time was still slow and unfavorable for BOD fast detection.

Herein, we developed a new mediator-type BOD bioelectrochemical sensor by using polypyrrole (PPy) immobilized FC and *P. Aeruginosa* for BOD fast detection. PPy is an conducting polymer and compatible with biomolecules and materials (i.e., proteins, DNA, enzymes, organelles, and amino acids), thus offering huge potential for their application to biosensors[18-21]. Recently, Tokonami et.al.[22] discovered that baciliform bacteria could be doped into PPy film by electrochemical deposition owing to the negative charges accumulated on their outer membranes, and PPy played an important role in maintaining the living condition of the *P. Aeruginosa* cells. This discovery offered an high controllable method for immobilization of living cells on electrode, especially on microelectrode surface. Based on this, *P. Aeruginosa* living cells were doped in PPy and electrochemical immobilized on disk microelectrode surface, which offered a favorable means for BOD fast detection.

2. EXPERIMENT

2.1 Reagents and instruments

The BOD^{198} standard GGA solution (150mg/L glucose and 150 mg/L glutamic acid) was prepared according to procedures described in the APHA standard methods[23]. Solution with other

concentrations was prepared by appropriate dilution of the BOD^{198} standard solution with deionized water. PPy, $K_3Fe(CN)_6$, Na_2HPO_4, K_2HPO_4, H_2SO_4, K_2SO_4 and Nutrient broth (NB) broth were obtained from Sinopharm Chemical Reagent Beijing Co. Ltd (Beijing, China). Dried bacterial cells of *P. Aeruginosa* were acquired from China General Microbiolgical Culture Collection Center. Unless otherwise stated, all chemical solutions were of analytical grade and prepared with deionized water. Electrochemical measurements were carried out with the Reference 600 workstation (Gamry Instruments, USA), and the data were analyzed through Gamry Echem Analyst software (Gamry Echem Analyst Version5.50).

2.2 Bacterial cultivation

P. Aeruginosa were cultured in the NB broth and incubated at 37°C for 12h with shaking at 150rpm. The bacterial suspensions were centrifuged at 7000 rcf for 20min, and the supernatant was discarded. The bacterial cells were repeatedly washed thrice in PBS (0.1M, pH 7.0) to obtain a purified bacterial suspensions. The cell concentrations were measured at 600nm (OD_{600}) using the spectrometer.

2.3 Microelectrode chip fabrication

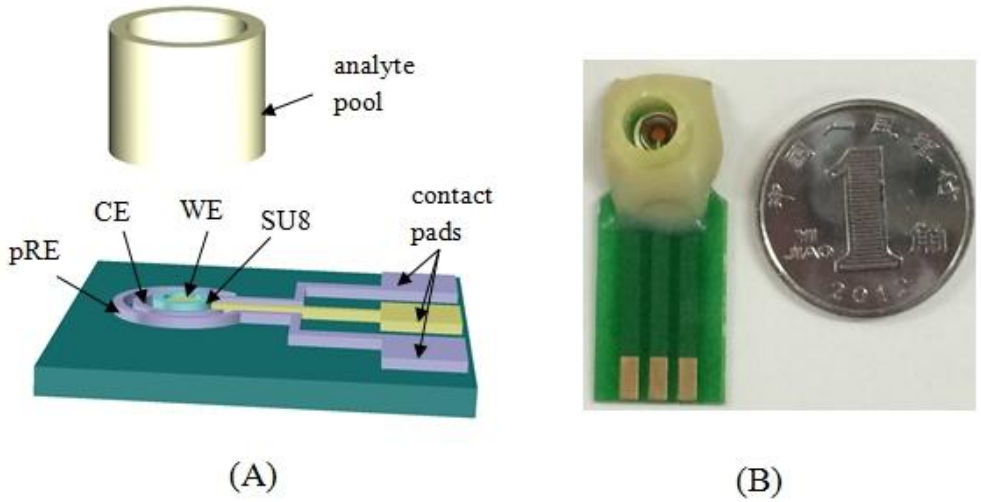


Figure 1. Schematic of the structure of microelectrode chip (A) and photography of the packaged microelectrode chip

A microelectrode chip with ring type 3-electrode system was obtained by fabricating the Au working electrode (WE), Pt counter eletrode (CE), and Pt pseeudo-reference (pRE) electrode using micro electro-mechanism system (MEMS) technology. First, a layer of 30nm Cr / 200nm Pt was deposited on a glass substrate and patterned by lift-off process to form the CE and pRE. Then the second layer of 30nm Cr / 200nm Au was deposited and patterned by the same process to form the WE. Then a SU8 negative photo resist was spin-coated and patterned around the WE to control the

active area. The on-chip reference electrode is Ag/AgCl electrode was made by coating Ag/AgCl ink on Pt electrode and then annealed at 90°C for 1h. Then a plastic pipe was fixed on the microelectrode by epoxide-resin glue as the analyte pool. Fig. 1A shows the schematic of the microelectrode chip and partial electrode structure. Finally the microelectrode was wire-bonded and encapsulated on print circuit board strips. The photograph of the packaged microelectrode chip was shown in Fig. 1B. The sensitive area of the WE is 1 mm^2.

2.4 Immobilization of mediator and microorganisms

Before immobilization, the WE was washed and cleaned in 0.01M H_2SO_4 by CV scan from 0 to 1.5V for 10 cycles until a reproducible voltammogram was obtained. FC mediator was immobilized by cyclic voltammetry (CV) scan from -0.3V to 0.9V in N_2 saturated 0.1M K_2SO_4 and 0.1M H_2SO_4 solution containing 0.01M pyrrole and FC mixed solution (pyrrole-FC deposition solution). *P. Aeruginosa* microorganisms were immobilized at the potential of -0.9V (*versus* CRE) for 300s in N_2 saturated 0.1M PBS solution (pH=5.0) containing 0.01M pyrrole and *P. Aeruginosa* (pyrrole-*P. Aeruginosa* deposition solution) .

2.5 Detection procedures

The electrochemical characteristics of immobilized FC mediator on Au microelectrode ((FC/PPy)/AuME) was investigated by CV between -0.3V and 0.6V in 0.1M K_2SO_4 and 0.1M H_2SO_4 mixed solution. The electrochemical characterisition of *P. Aeruginosa* microorganisms modified microelectrodes ((*P. Aeruginosa*/PPy)/(FC/PPy)/AuME) were carried out with CV between 0 and 0.9V (vs CRE) in 0.1M PBS solution and 0.1M PBS and 200 mg/L BOD mixed solution respectively. Chronoamperometry method was used for the detection of BOD, and the applied potential was 0.45V for 300s. Before electrochemical detection, the biosensor was incubated with samples at 37°C for 10 min. The biosensor was stored at 4°C when not in use.

3. RESULTS AND DISCUSSION

3.1 Characterization of modified microelectrode

In the present work, the FC mediator and *P. Aeruginosa* microorganisms were immobilized on working electrode by two steps using electrochemical method. Firstly, FC mediator was immobilized by CV method. As shown in Fig.2a, the CV curve at the modified (FC/PPy)/AuME in 0.1M K_2SO_4 and 0.1M H_2SO_4 mixed solution was revealed. The well-defined oxidation and reduction peaks of (I) and (II) appeared at 0.328 and 0.317V respectively, representing the redox potential of $Fe(CN)_6^{3-}$ /$Fe(CN)_6^{4-}$ system. The two obvious redox couples of FC suggested that the FC doped into PPy layer had a good electrochemical activity, indicating that FC had been immobilized on the Au microelectrode successfully[24]. Fig.1b showed that the peak current value was linearly related with

the CV scan rate from 10 to 100 mV/s, illustrating that the electrode process was controlled by the surface adsorption, which further demonstrated that FC had been immobilized on working electrode surface and remained good electrochemical redox characteristic. Then CV scan of PBS blank solution and BOD solution were recorded at the completed modified (*P. Aeruginosa*/PPy)/(FC/PPy)/AuME. After incubated with organic substrate, as shown in Fig.2b, the peak (I) current increased as some of the immobilized FC was reduced by microorganisms, suggesting that the *P. Aeruginosa* living cells were immobilized on working electrode successfully.

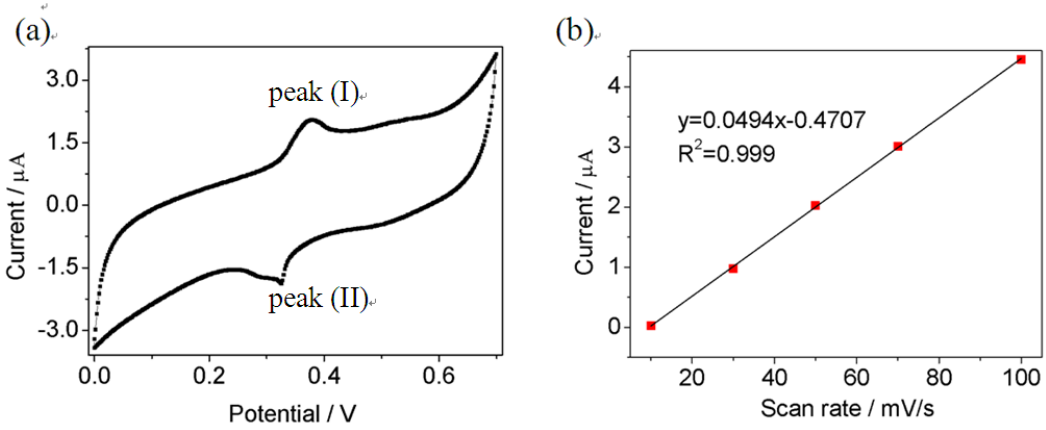


Figure 2. CV curve at (FC/PPy)/AuME in 0.1M K_2SO_4 and 0.1M H_2SO_4 mixed solution (a), and linear relationship between peak (I) values and scan rates.

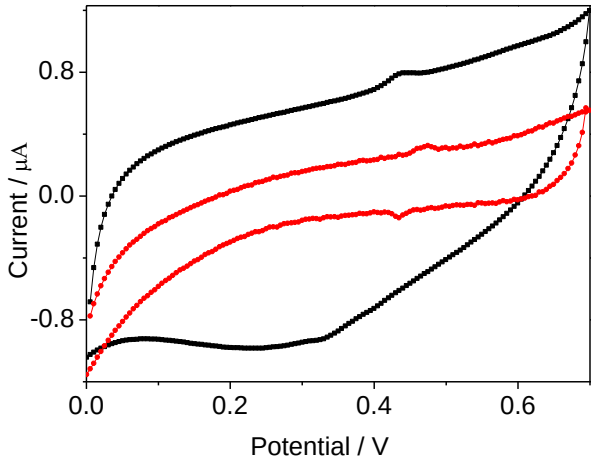


Figure 2. CV curves in 0.1M PBS blank solution (red) and 200 mg/L BOD solution (black) at (*P. Aeruginosa*/PPy)/(FC/PPy)/AuME

3.2 Influence of FC concentration and bacteria concentration

The influence of FC concentration in pyrrole-FC deposition solution on the biosensor response was examined. The deposition solutions with different FC concentrations and constant pyrrole

concentration of 0.01M were adopted for FC immobilization on microelectrode surface. As shown in Fig.3, the anodic peak current response in 0.1M PBS increased with the increased FC concentration from 5 to 20mM. When the FC concentration was continued to increase, however, the current response was decreased and the characteristic redox peak of FC was not obvious. The reason was presumed that excessive FC embedded in PPy matrix hindered the electron-transfer ability of PPy layer and the redox active sites of FC were decreased. So 20mM FC was selected for the pyrrole-FC deposition solution.

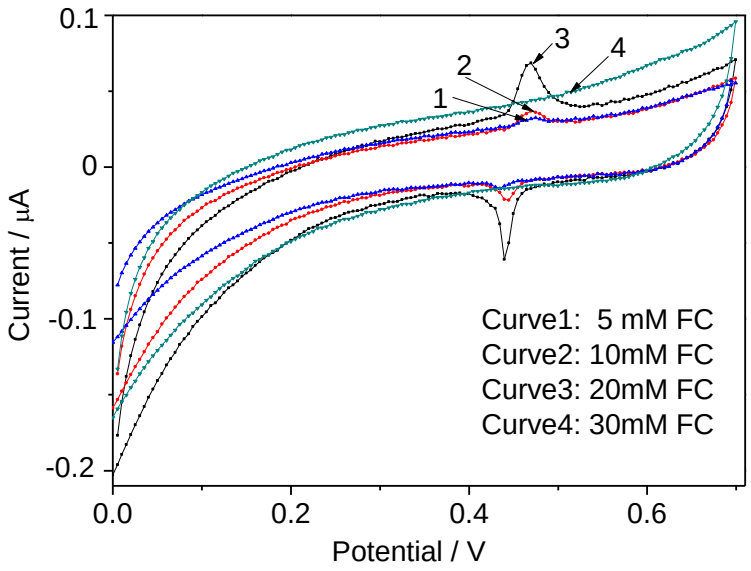


Figure 3. CV curves recorded at different (FC/PPy)/AuMEs in 0.1M PBS solution (pH=7.0). The (FC/PPy)/AuMEs were obtained in pyrrole-FC deposition solutions with different FC concentration from 0 to 30mM.

The microorganism amount is a key factor of the respiratory activity of a microbiofilm. So an optimal concentrations of *P. Aeruginosa* bacteria in pyrrole-*P. Aeruginosa* deposition solution was investigated. It seems that the higher response would be obtained with the more cells were immobilized on electrode due to the more FC was to be reduced. However, the diffusion rates of substrates were proportional to the thickness of the biofilm[25]. The higher bacteria concentration in pyrrole solution means that more cells would be immobilized in PPy layer. So the thick biofilm with more cells would decrease the PPy conductivity and hinders substrates diffusion for the biochemical reaction with FC. Different concentrations of *P. Aeruginosa* bacteria were prepared for immobilization in this study. As shown in Fig. 4, it is observed that the highest response was at the concentration of 2.0×10^5 CFU/cm^2. So 2.0×10^5 CFU/cm^2 *P. Aeruginosa* was added into 0.01M pyrrole as pyrrole- *P. Aeruginosa* deposition solution

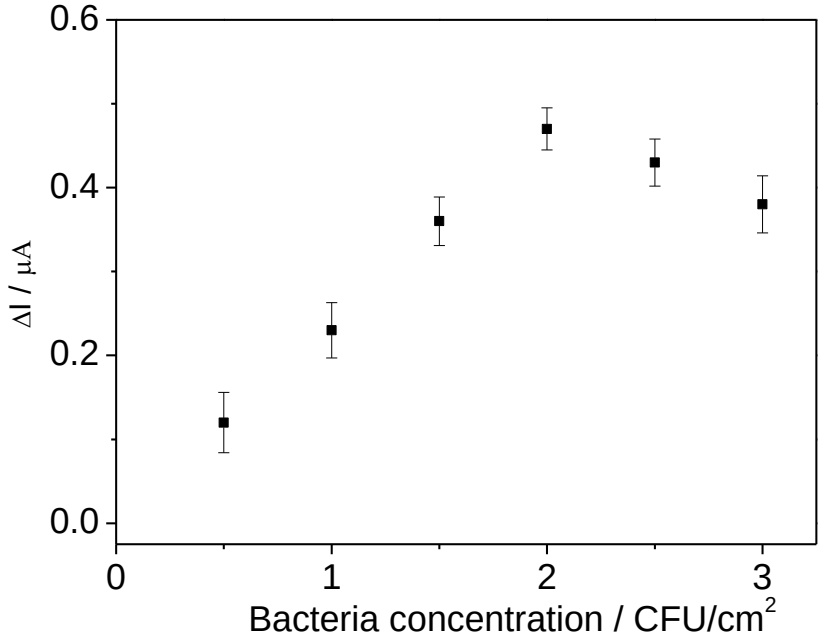


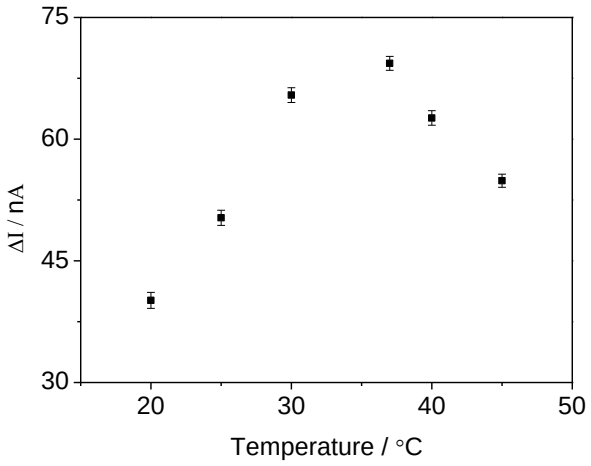
Figure 4. The anodic peak current values of 200mg/L BOD (the background current was subtracted) with the modified electrodes obtained in different bacteria concentration solutions.

3.3 Optimization of detection parameters

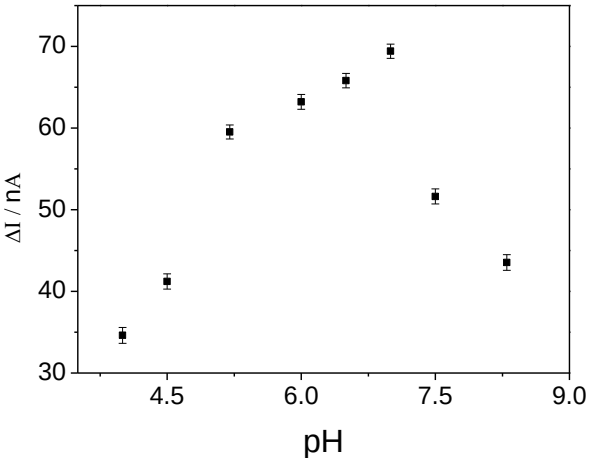
The effect of temperature on the microbial biosensor was investigated by monitoring current response to a constant BOD concentration of 100mg/L. As illustrated in Fig. 5a, there are high current responses in the range of 30-37°C. Lower responses appeared when the temperature was above 40°C or below 25°C. These results confirmed that the microorganism viability in the (*P. Aeruginosa*/PPy)/(FC/PPy)/AuME was temperature-dependent.

The effect of pH on the microbial biosensor was also studied by electrochemical detection of 100mg/L BOD at 37°C with the pH varied from 4.0 to 8.2. As shown in Fig. 5b, the current response increased with the rise of the pH, and reached high level from pH 5.2 to pH 7.0, whereas decreased slightly when continue increasing the pH. It implied that the respiratory activity of *P. Aeruginosa* microorgnisms had certain toleration to the pH change.

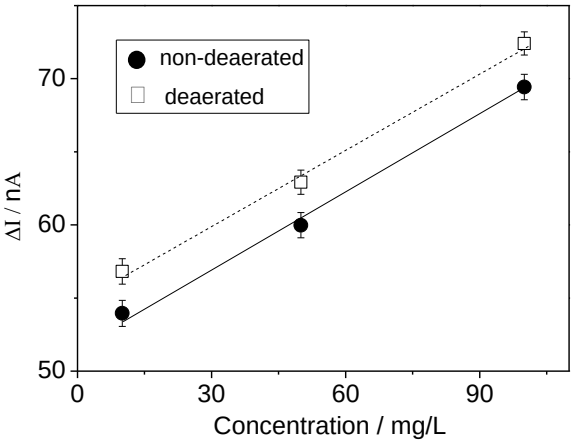
The effect of dissolved oxygen on current responses of the biosensor was examied at pH 7.0. Sample solutions with three BOD concentrations of 10, 50 and 100mg/L were oxygen saturated and deaerated by oxygen and nitrogen injection for 10min, respectively. As described in Fig. 5c, no significant difference was found between the response obtained from oxygen saturated solution and that obtained from the deaerated solution. This result was agreement with other reported mediator-type BOD sensors[26,27]. So, compared with oxygen, FC is a good electron acceptor in the experimental condition.



A



B



C

Figure 5. Biosensor current responses (the background current was subtracted) to different detection parameters. (a) temperature from 20 to 45°C; (b) pH from 4.0 to 8.3; (c) in deaerated and non-deaerated conditions.

3.4 Analytical performance of BOD biosensor

A calibration curve was obtained by measuring different concentrations of the standard BOD solutions. Before the determination, 100 μL 0.1M PBS at pH 7.0 was injected into the micro analyte pool and incubated for 10min utill a stable current was obtained. Then the standard BOD solutions were added in to the pool, and the corresponding current responses were recorded at the potential of 0.45V for 5min after 10min incubation. The current response increased with the increase of BOD concentration. As shown in Fig. 6, an apparent linear correlationship of the current response and BOD concentration was presented in the range of 5-100mg/L. The detection limit was calculated as 2mg/L (S/N≥3). The repeatability was calculated to be 3.6% according to 5 replicate measurement of 20mg/L BOD using one single biosensor. The reproducibility was performed using three biosensors, and was less than 10% at the same concentration of BOD as shown in Table 1.

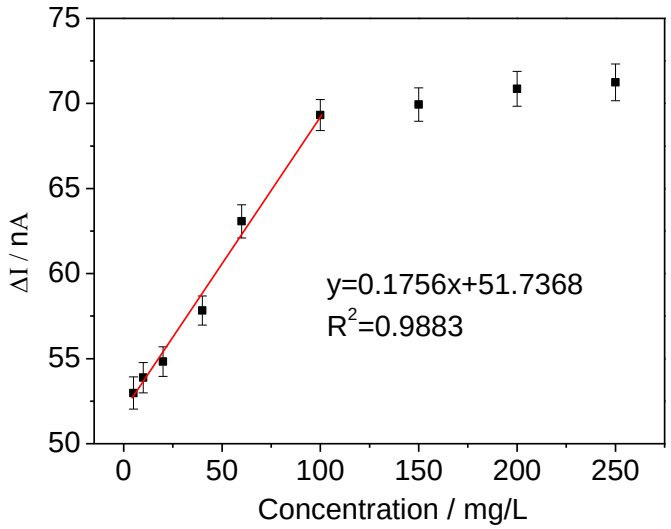


Figure 6. The calibration curve of BOD microsensor (the background current was subtracted).

Table 1. Reproducibility of three biosensors

C / mg/L	1# / nA	2# / nA	3# / nA	RSD / %
5	56.03	52.08	48.73	5.71
10	57.94	53.01	49.66	6.35
20	58.88	54.03	50.78	6.10
40	60.93	56.87	52.65	5.95
60	66.63	62.05	58.01	5.69
100	72.89	68.47	64.28	5.13

4. CONCLUSION

In this study, we constructed a novel mediator-type bioelectrochemical sensor by immobilizing *P. Aeruginosa* into PPy by electrochemical deposition, which is suitable for microelectrode

modification. The mediator, FC, was also immobilized in the PPy layer via electropolymerization. The immobilization process and electrochemical characteristics of the modified microelectrode were investigated by cyclic voltammetry method. The experiment results showed that the PPy immobilized FC and *P. Aeruginosa* biofilm had a good biodegradation ability to BOD. The biosensor is favorable for BOD fast detection due to its short measurement time of 10min. A calibration curve ranging from 5 to 100mg/L BOD was obtained, and the limit of detection was 2mg/L. More importantly, a robust BOD biosensor can be expected using this new immobilization method on ultramicroelectrode arrays.

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