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A co-immobilized mediator and microorganism mediated method combined pretreatment by TiO_2 nanotubes used for BOD measurement

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ABSTRACT

In this paper, we proposed a method by using co-immobilized *Escherichia coli* (*E. coli*) as a biocatalyst and neutral red (NR) as an artificial electronic acceptor to modify glassy carbon electrode (GCE) for biochemical oxygen demand (BOD) measurement. Two different modification approaches of GCE were utilized and compared. In one approach, NR was electropolymerized on the surface of GCE, and *E. coli* cells were mixed with grafting copolymer PVA-g-PVP (briefly gPVP) and covered on NR polymer film to obtain a (gPVP/*E. coli*)/PNR/GCE. In the second approach, both NR and *E. coli* cells were mixed with the copolymer gPVP and modified GCE, after drying, which was electrochemically treated similar as above for obtaining a (gPVP/*E. coli*/NR)p/GCE. Based on the electrochemical evaluation, the performance of the latter was better, which may be caused by that the NR deposited on the surface of *E. coli* resulting in a good electron transport and permeability of cells membrane. To develop the results obtained at (gPVP/*E. coli*/NR)p/GCE further, the pretreatment by TiO_2 nanotubes arrays (TNTs) was employed, and different effects on samples of GGA, OECD, urea and real wastewater were evaluated. These results suggest that the present method holds a potential application for rapid BOD biosensor.

1. Introduction

Biochemical oxygen demand (BOD) is one of the most frequently used parameters in environment monitoring [1]. The conventional BOD measuring method (5-days BOD, BOD_5) was restricted by its time-consuming and cannot timely reflect the effect of pollution [2]. So many rapid BOD measuring methods were developed over three decades, but were developed slowly in recent years. One important barrier is that those rapid BOD methods were based on monitoring the change of oxygen in sample, and some limitations as the low concentration of dissolved oxygen and its variations upon environmental conditions were exposed. For overcoming those limitations, the artificial electron acceptor, also known as mediator, has been induced for BOD measurement, which is called BOD mediator (BOD_{Med}) method [3].

Since Roller et al. reported the redox-mediator used in microbial fuel cell, some relative development has been reported, and among those the BOD_{Med} method was developed successfully during the past decades [4,5]. The BOD_{Med} method has the potential to be used in practice for environment detection, and the relative woks were continuous and series [6–8]. However, BOD_{Med} method was also restricted by some difficulties. On one hand, microorganisms are suspended in solution and culturing microorganisms was required each time before BOD measurement. On the other hand, the mediator was dissolved in solution, which then could cause a wasteful of reagent and a secondary environmental contamination. In 2010, our group reported a method of immobilizing microorganism cultured from native resource wastewater (called native biofilm, NBF) for BOD measurement [9]. Based on the advantages of the NBF, a BOD bioreactor has been developed, but the mediator was still suspended in solution and unfavorable for practical application.

In this work, we established a method by using co-immobilized neutral red (NR) and *E. coli* for BOD measurement. NR was proved to be an attractive mediator to harvest microbial metabolic electrons due to its excellent electrochemical reversibility and compatible redox potential (−0.33 V) to the major metabolic electron carriers (e.g., −0.32 V of $NADH/NAD^+$) [10]. The phenazine dye NR is one of azines, and poly(neutral red) (PNR) as well was often been used as a redox mediator [11]. In addition, previous studies suggested that in the valence band organic compounds could be degraded to the compounds of lower molecular weight by TiO_2 [12,13], and lower molecular compounds would be faster assimilated to microorganism cells. This theory offered an interesting combination of material science with environment monitoring. TiO_2 nano-material has been utilized for increasing of the sensitivity of biosensors

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[14–16]. Based on this, using TiO_2 nanotube arrays (TNTs) for pretreatment approach was introduced in the present work for BOD monitoring, which offered a better means for practical application as compared to previous BOD_{Med} method.

2. Experimental

2.1. Materials and instrumentation

NR was purchased from Ajax. The poly (vinyl alcohol) grafted 4-vinylpyridine (briefly gPVP) was prepared according to the previous report [17]. Glucose-glutamic acid (GGA) solution contains 3400 mg/L glucose and 3400 mg/L glutamic acid with a BOD_5 value calculated as 5000 mg O/L [18]. The GGA solution with other concentrations was prepared by appropriate dilution of this BOD standard solution with phosphate buffer solution (PBS, 0.06 M Na_2HPO_4/0.04 M K_2HPO_4, pH 7). The composition of organization for economic corporation and development (OECD) synthetic wastewater was as follows (g/L): peptone, 16; meat extract, 11; urea, 3; NaCl, 0.7; $CaCl_2{\cdot}2H_2O$, 0.4; $MgSO_4{\cdot}7H_2O$, 0.2; K_2HPO_4, 2.8. The BOD_5 value of this solution is defined as 14,000 mg O/L [19]. All chemicals used in this study are of analytical reagent grade and all solutions were prepared with deionized water being sterilized. Real wastewater was obtained from a local wastewater treatment plant (Gold Coast, Australia), and its BOD_5 value was ~140 mg O/L.

2.2. Modify the electrode

The glassy carbon electrode (GCE) was modified via two parallel ways. One is the (gPVP/*E. coli*)/PNR/GCE method. In detail, the GCE was pretreated by polishing in 1 μm alumina slurry on a polishing cloth, washed and cleaned in 5 mM H_2SO_4 by cyclic voltammetry (CV) at −0.2 V to 0.9 V for 30 cycles. And then, the GCE was put in NR solution for electrochemical polymerization of NR (PNR) at −1.4 V to 1.8 V for 10 cycles, and at −0.8 V to 0.8 V for 10 cycles after initial activity polymerization of a higher potential. According to the previous report, at a high potential, the amino functionality of NR makes it amenable to electropolymerization. After electrochemical modification, the PNR/GCE was obtained and washed by PBS thoroughly. A biomatrix prepared by mixing *E. coli* cells and gPVP. Based on our previous work, the concentration of microbe 1.5 g/mL was adopted [20]. 100 μL of 1.5 g/mL *E. coli* cells were mixed adequately with 200 μL grafting copolymer gPVP to obtain a mixture. And then 20 μL of the mixture was covered on the surface of PNR/GCE. After drying at 4 °C, a (gPVP/*E. coli*)/PNR/GCE was obtained. Another method was using NR mixed into mixture of *E. coli* cells and gPVP. A full capacity of NR was used for obtaining the maximum electrochemical signal. The final mixture was dropped on the surface of GCE. After drying at 4 °C, the (gPVP/*E. coli*/NR)/GCE were treated in PBS by CV method similar as above and then the (gPVP/*E. coli*/NR)p/GCE was obtained.

The morphology of microorganisms was characterized by confocal laser scanning microscopy (CLSM) system Leica TCS SP2 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). The microorganisms were peeled off from the (gPVP/*E. coli*/NR)p/GCE. Before imaging with CLSM, the microorganisms were washed thoroughly by PBS. The excitation wavelength was 543 nm for NR. A 50× objective was used for all imaging.

2.3. Preparation of TNTs

The TNTs were prepared by an electrochemical anodization process. Briefly, the anodization process was carried out in a two-electrode compartment, where Pt mesh was used as a cathode, and the cleaned Ti foil as an anode. The electrolyte solution was prepared by dissolving NH_4F (1.0 wt.%) and H_2O (15 vol.%) in 1,2,3-propanetriol. The anodization potential was controlled at 25 V for 2 h with a ramp rate of 500 mV s^{-1}. The crystallization of the as-prepared TNTs was achieved by annealing in a tubular furnace at 450 °C for 4 h with a heating rate of 5 °C/min in air. The resulting samples are designated as TNTs.

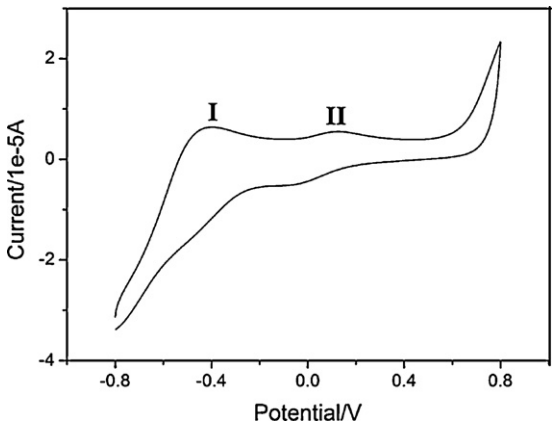

Fig. 1. CV curve at (gPVP/*E. coli*)/PNR/GCE in PBS.

2.4. Pretreatment by TNTs

The samples were pretreated by TNTs with a XPA-II photochemical reactor (Nanjing Xujiang Machine Electronic Plant). The 1 cm^2 TNTs were immersed in 10 mL quartz test tubes containing 4 mL different samples, respectively. After that, the UV–vis light pretreatment experiment was conducted for different times.

2.5. Electrochemical measurement

Electrochemical responses were measured with a CHI 660 electrochemical analyzer (CHI Co., Shanghai, China). Prior to the surface modification, the GCE was polished carefully with 1.0, 0.3 and 0.05 μm alumina slurry, respectively, and rinsed with doubly distilled water, followed by sonication in acetone and doubly distilled water successively. Then, the electrode was allowed to dry under nitrogen. After the surface modification, the (gPVP/*E. coli*)/PNR/GCE or (gPVP/*E. coli*/NR)p/GCE were used as working electrode. A piece of Pt was used as the counter electrode, and an Ag/AgCl (sat. KCl) as reference electrode. Before electrochemical measurement, the solutions were incubated in 35 °C for 20 min for stable. For the detection of BOD, the amperometric *i–t* curve experiments were carried out with the scan rate of 100 mV s^{-1} and the applied potential −0.3 V. Magnetic stirring solutions were used mildly, while a water bath for keeping 35 °C throughout measurements. The sensor was stored under 4 °C when not in use.

3. Results and discussion

3.1. Characterization of (gPVP/E. coli)/PNR/GCE and (gPVP/E. coli/NR)p/GCE

In the present work, two methods, (gPVP/*E. coli*)/PNR/GCE and (gPVP/*E. coli*/NR)p/GCE, were compared for modifying electrode. As shown in Fig. 1, the CV curve at completed modified (gPVP/*E. coli*)/PNR/GCE in PBS was revealed. The peaks (I) and (II) represent the two redox couples of PNR, suggesting the PNR has been modified on the GC electrode successfully. And the PNR remained electrochemical response of the NR redox-active sites and ensured a long-term stability [11]. To compare with this method, another was that the NR and microbe were mixed into

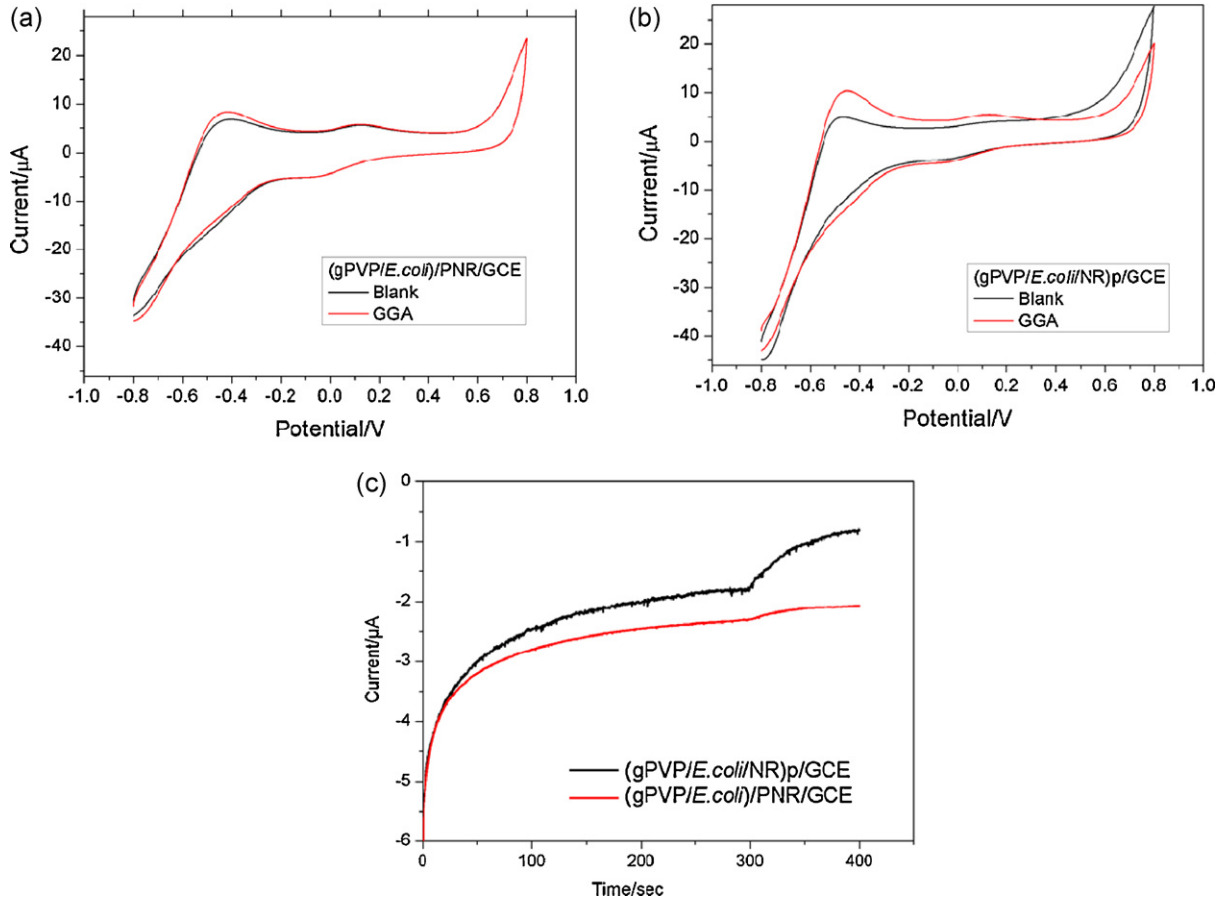

Fig. 2. CV curves in GGA solution (blank) and PBS (red) at (a) (gPVP/*E. coli*)/PNR/GCE and (b) (gPVP/*E. coli*/NR)p/GCE. (c) Chronoamperometry curves at (gPVP/*E. coli*)/PNR/GCE (red) and (gPVP/*E. coli*/NR)p/GCE (blank). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gPVP all together, and the mixture was then dropped on the surface of electrode. After drying, the (gPVP/*E. coli*/NR)/GCE was subjected to similar electrochemical treatment (CV curves were not shown), and then a (gPVP/*E. coli*/NR)p/GCE was obtained. Furthermore, the electrochemical analysis of the two modified electrodes was studied as follows.

3.2. Performances of modified GCEs

The GGA solution has been extensively used as a standard to calibrate BOD biosensors. Here, we also selected the GGA solution for checking whether the overall methodology is appropriate. As shown in Fig. 2a and b, the CV curves of GGA and blank solution were recorded at (gPVP/*E. coli*)/PNR/GCE and (gPVP/*E. coli*/NR)p/GCE, respectively. The exchange of peak (I) (Fig. 1) representing the electrochemical reduction of PNR (or NR). After reacted with organic substrate, the anodic peak current increased as some of the PNRs were reduced by microorganisms. It is obvious that the current increasing at (gPVP/*E. coli*/NR)p/GCE is more obvious than at (gPVP/*E. coli*)/PNR/GCE. Fig. 2c compares the signals in chronoamperometry obtained at the modified GCEs. The signal at (gPVP/*E. coli*/NR)p/GCE was enhanced by about 3-fold than that of (gPVP/*E. coli*)/PNR/GCE.

3.3. Optimizing measurement condition and calibration of GGA

The different experimental conditions to affect results were pH, temperature and time. For the detection of BOD, the amperometric *i–t* curve experiments were carried out and the time for measuring was terminated at the trend of curve was gently. Based on the demand of activity of the microbe, the pH 7 was adopted and phosphate buffer solution (PBS) was used as buffer system. The effect of measurement temperature on the response at the (gPVP/*E. coli*/NR)p/GCE was then evaluated by using BOD1000 GGA solution. Fig. 3a shows that the analytical signal reached a high response when the temperature was in the range of 30–35 °C. The responses decreased when the incubation temperature was below 25 °C or above 40 °C. The results suggested that the microorganism viability in the (gPVP/*E. coli*/NR)p/GCE was temperature-dependent. The reason of the electrochemical signal at (gPVP/*E. coli*/NR)p/GCE and (gPVP/*E. coli*)/PNR/GCE was different had been analyzed. As shown in Fig. 3b, the CLSM images of the microbe peeled off from modified (gPVP/*E. coli*/NR)p/GCE were revealed. To compare the fluorescence and corresponding bright field (in superposition), it is obvious that the NR was covered on the surface of microbe. The primary difference between the two modified electrodes was the surface of microbes with or without NR, and it showed the significant difference in characterization of their performance. Furthermore, the reproducibility of the signal at the present (gPVP/*E. coli*/NR)p/GCE was good, and the relative standard deviation (RSD) was 2.8% for four repeated measurements. As shown in Fig. 4, a linear correlation from 50 to 1000 mg O/L was obtained. So the (gPVP/*E. coli*/NR)p/GCE was used for following measurement.

3.4. Pretreatment by TNTs

The above results showed that the electrochemical signal obtained at the (gPVP/*E. coli*/NR)p/GCE was not enough for its application. One of the reasons was mainly due to the short incubation

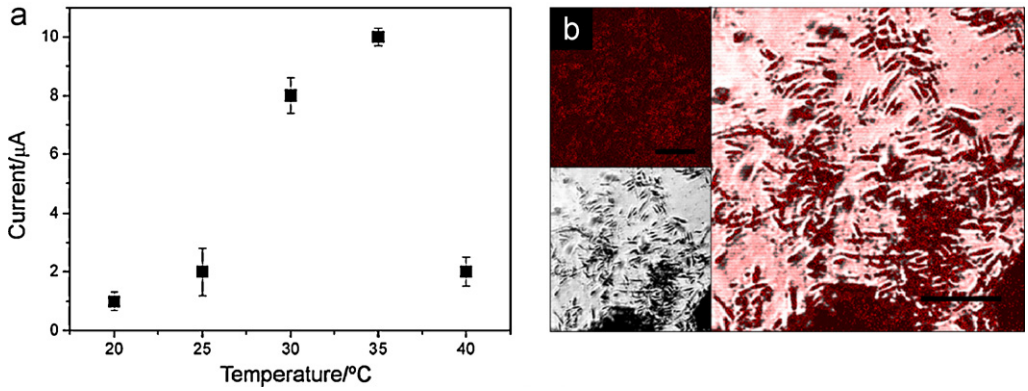

Fig. 3. (a) Effect of temperature on the response at the (gPVP/*E. coli*/NR)p/GCE. (b) CLSM images of the microbes from (gPVP/*E. coli*/NR)p/GCE, fluorescence (above in left), corresponding bright field (below in left) and superposition (right). The bars represented 12 μm.

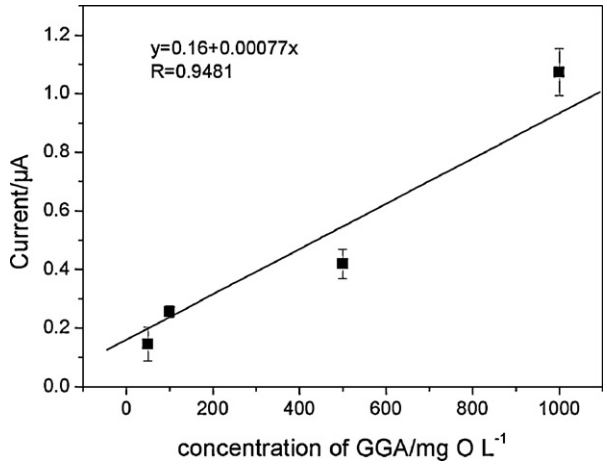

Fig. 4. Linear curve of GGA solution with concentrations in the range of 50–1000 mg O/L by (gPVP/*E. coli*/NR)p/GCE.

time and insufficient biodegradation. In other words, some compounds are not easily assimilable by microbe in such a short time. Therefore, we further adopted a method based on TNTs pretreatment for enhancing electrochemical signal. Fig. 5a shows the SEM images of the cross-sectional and the top view of TNTs. The nanotubes have an average diameter of 100 nm and a length of approximately 900 nm. Fig. 5b shows that the N-TNTs consist of both anatase and rutile. The mechanism of TiO_2 photocatalysis toward organic compounds was not quite clarified yet, but it was likely photoinduced electrons and holes are illuminated by UV light, yielding OH• radicals in water solution, the most reactive hydroxy radicals formed competitively attack the aromatic ring and/or the ethoxy group to form the hydroxyl intermediates. Finally, the organic compounds decompose into CO_2 via various oxidation steps [15,16]. This indicated that the degradation of organic compounds by TNTs was gradually. After TNTs pretreatment, those lower molecules could be easily assimilated and degraded by microbes. Thus the TNTs pretreatment is expected to enhance the electrochemical signal. As shown in Fig. 6, the calculation of result was that by using the difference of ten figures at above 180 s and 350 s. The electrochemical signals obtained at (gPVP/*E. coli*/NR)p/GCE for evaluating GGA solution were enhanced by 6% and 39% upon using TNTs for 15 min and 60 min, respectively, and the OECD were 88% and 91%, respectively. Meanwhile, the signal of urea was found to be decreased by 66% and 21%, respectively. The results revealed that the macromolecule organic compounds could be degraded to the lower molecular weight compounds by TNTs, and lower molecular compounds would be faster assimilated to microorganism cells. So this mechanism could be employed for degradation of organic compounds at a short time and used in BOD_{Med} method. But it was obvious that an unsatisfactory result was obtained for the measurement of urea. The reason was speculated that the urea was a lower molecular compound, and it was over degraded. So the present co-immobilized microbe and mediator BOD_{Med} method would be developed in the further research. But it's all the more important that the present method would provide a feasible alternative for applying BOD^{Med} method in practice.

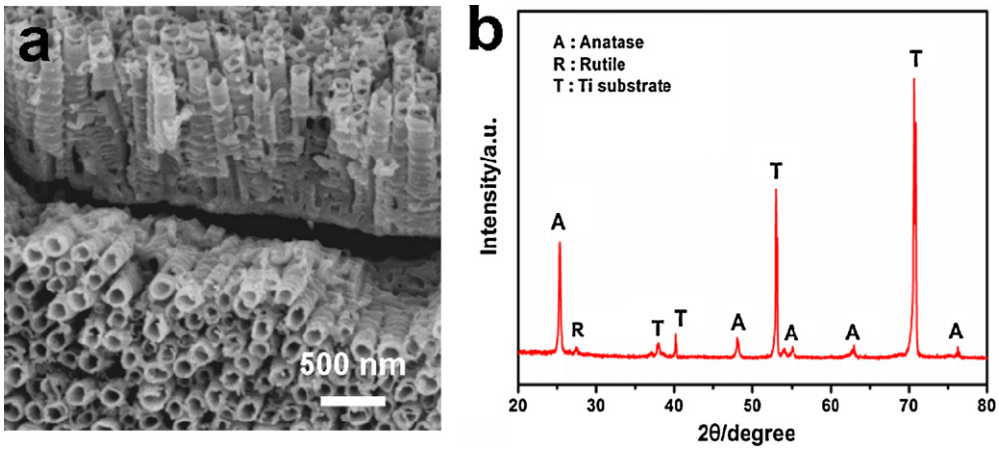

Fig. 5. SEM images of the cross-sectional and the top view of TNTs (a) and XRD results (b).

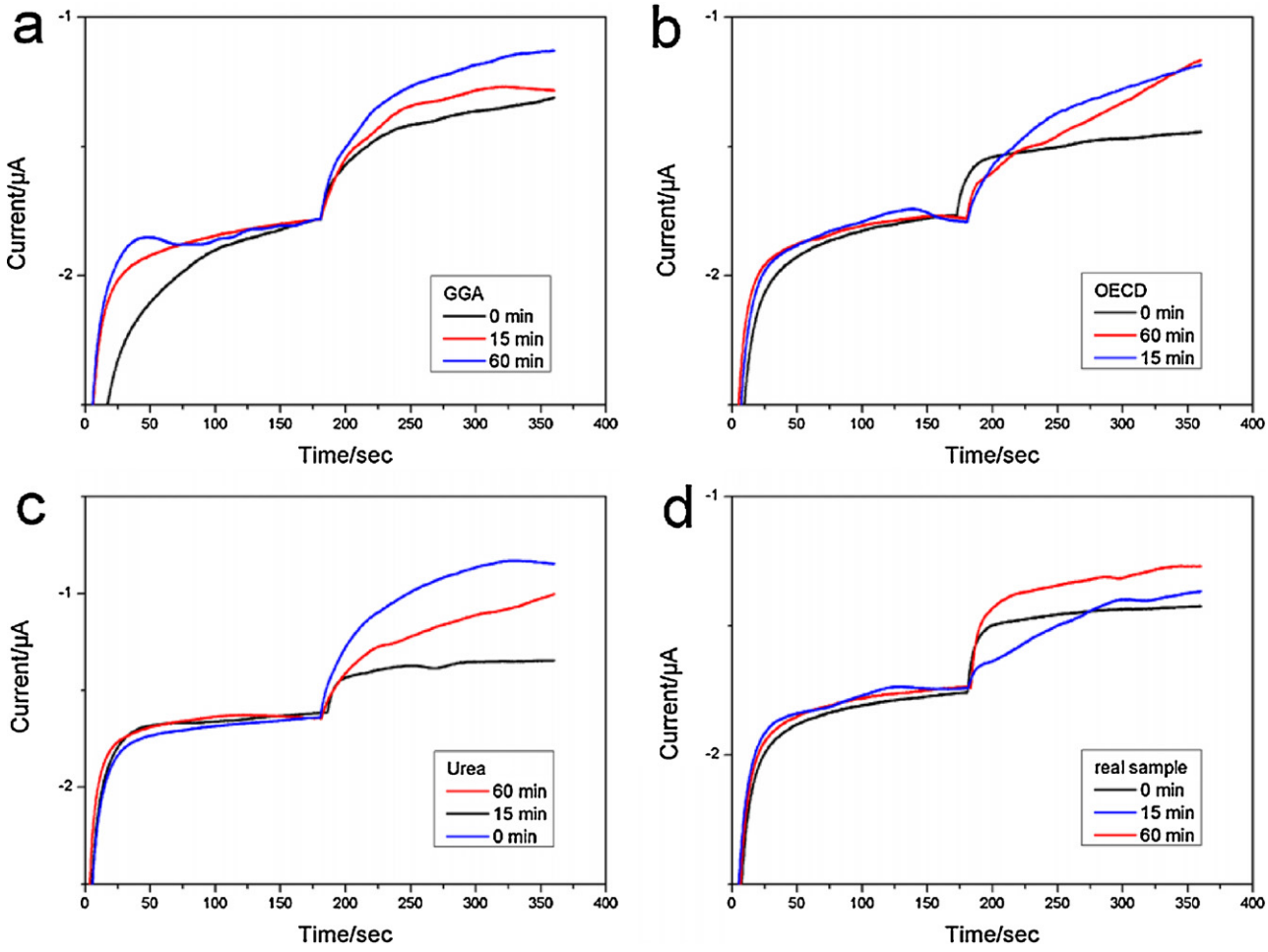

Fig. 6. Effect of TNTs pretreatment on evaluating GGA (a), OECD (b), urea (c) and real wastewater (d).

3.5. Analysis of environmental samples

The TNTs pretreatment was used for evaluating BOD of the real wastewater treatment. As shown in Fig. 6d, the results obtained with and without TNTs pretreatments were compared. The electrochemical signal was enhanced by about 12% and 40% within 15 min and 60 min after pretreatment, respectively. Gab-Joo et al. evaluated each compound of artificial wastewater (AWW) by using TNTs [21], and obtained different degrees of photodegradation. The environmental sample was much more complex than AWW, so the TNTs pretreatment approach just one of methods for improving the electrochemical signal. Furthermore, other approaches such as mixed microbe species, based on its broad spectrum of substrate range, were expected used for real wastewater measurement. But the results of electrochemical signal were slightly in disorder by using mixed microorganisms and not benefit for comparing experiment. If the stability of electrochemical signal obtained by mixed microorganisms would be studied, the BOD_{Med} method could be further developed.

4. Conclusion remarks

In conclusion, we have developed a method by using the commonly immobilized microbes and mediator for BOD measurement. Two approaches for fabricating the modified GCE were carried out and compared. The results showed that the performance at (gPVP/*E. coli*/NR)p/GCE was better than (gPVP/*E. coli*)/PNR/GCE. To develop the results obtained at (gPVP/*E. coli*/NR)p/GCE further, TNTs pretreatments were utilized for enhancing the electrochemical signal. A large enhancement was obtained for OECD, and a middle for real wastewater, and a small for GGA, and a reduction for urea.

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