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NANOSTRUCTURED ZINC OXIDE AS A TRANSDUCER FOR BIOSENSORING OF SULPHATE-REDUCING BACTERIA

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ABSTRACT. Sulphate-reducing bacteria is widely applied in the environmental biotechnology for treatment of acid mine drainage water in passive and active systems. The use of biosensoring methods to describe its microbial populations in natural communities has attracted considerable interest. In particular, we are applying nanostructured ZnO thin films, prepared by magnetron sputter deposition, as a transducing material for bacterial detection aiming to optimization and monitoring of dissimilatory sulphate-reduction. Our innovation opens the opportunity to increase the sensitivity of the reported nanobiosensor for detection of sulphate-reducing bacteria. We are improving the transducer efficiency toward longer life-time utilization by engineering a novel biosensor design.

РАЗРАБОТВАНЕ НА БИОСЕНЗОР НА БАЗАТА НА НАНОСТРУКТУРИРАН ЦИНКОВ ОКСИД ЗА МОНИТОРИНГ НА СУЛФАТ-РЕДУЦИРАЩИ БАКТЕРИИ

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РЕЗЮМЕ. Сулфат-редуциращите бактерии са широко използвани за третиране на киселинни руднични дренажни води при пасивни и активни системи. Използването на биосензорни методи за мониторинг на техните микробни популации в естествена среда придобива все по-значителен интерес. В тази насока е конструиран датчик, получен от тънък филм –1 μm наноструктуриран ZnO (чрез отлагане при магнетронно напръскване) за откриване на сулфат-редуциращи бактерии, като по този начин е възможно да се извършва мониторинг на процеса. Предложен е нов дизайн на биосензора, който дава възможност да се повиши чувствителността на детекция на ниски концентрации на клетки. Така не само се подобрява ефективността на работа на датчика, но и се удължава времето му на експлоатация.

1. Introduction

Sulphate-reducing bacteria (SRB) are anaerobic microorganisms that use sulfate as a terminal electron acceptor, resulting in the production of hydrogen sulphide (Beijerinck et al., 1985). Hydrogen sulfide is highly corrosive and toxic, thus it can be a serious problem for the ecological systems. On other hand SBR can be used as an alternative biotechnological approach for treatment of acid mine drainage water. Their metabolic products include bicarbonate, which can neutralize water acidity, and hydrogen sulfide, which forms highly insoluble precipitates with many toxic metals (Bless et al., 2008). Although using SRB to treat acid mine drainage water is now fairly wide-spread, there are not rapid and sensitive approaches for their detection and environmental monitoring. In general, conventional methods for monitoring microbial SBR populations, such as the most probable number method, involve a pre-enrichment step or a selective enrichment step followed by a biochemical test, and the sophisticated series of assays required can take up 15 days to complete (Starkey 1948, ABD-EL-Malek et al., 1958). A variety of protocols have been presented for monitoring microbial SRB populations, including enzyme-linked immunosorbent assay (ELISA) (Gaylarde et al., 1990), and pertinent molecular techniques such as polymerase chain reaction (PCR) (Cook et al., 2008) or fluorescence in situ hybridization (FISH) (Lücker et al., 2007). Although promising, these techniques are very time-consuming and still in development phase. However, several problems occur when they are used in situ and in real time, mainly due to the longer exponential growth phase of SRB compared to other microorganisms.

Recently, a very promising protocol that uses enzyme electrochemical biosensor based on nanostructured ZnO was reported (Zhao et al., 2010). This method involves immobilizing antibodies that are specific to the target microorganisms on the surface of electrodes to detect minute bacteria. The nanostructure of this material provides high surface to volume ratios and high surface activity, and thus possess unique advantages over other conventional materials in terms of enzymatic immobilization and signal transduction. The nanostructured ZnO not only possesses high surface area, good biocompatibility and chemical stability and is non-toxic, but it also shows biomimetic and high electron communication

features (Tian et al., 2002, Sberveglieri et al., 1995, Rodriguez et al., 2000), making it great for biosensoring application. Nevertheless, the ZnO material possess solubility in water (0.16 mg/100 ml at 30 °C), which getting worse its practical utilization, especially when the pH of analyte is acid as in the case of SRB. In this paper, we present the first study of degradability of nanostructured ZnO in bacterial biosensor, when SRB is applied. We have conducted a systematic study on the dissolving behavior of ZnO in various solutions with moderate pH values and SRB suspension taken from bioreactor for treatment of acid mine drainage water. We created a design of the biosensor, which enables long-time utilization of nanostractured ZnO transducer for SRB detection.

2. Experimental procedures

2.1. Preparation of ZnO nanostructured thin film.

The nanostructured thin film was prepared by magnetron sputter deposition of above substance on glass at substrate temperatures (T_s) 250 and 500 °C, respectively. The obtained polycrystalline films are about 1000 nm thick. Their structural properties were characterizes by X-ray diffraction, scanning electron microscope and atomic force microscope.

2.2. Sulfate-reducing bacteria cultivation.

Mixed seeds of SRB used in this study were separated from effluent samples collected from the mus. SBR cultures were inoculated in a medium contained 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 2.0 g $MgSO_4$, 0.5 g Na_2SO_4, 0.1 g $CaCl_2$, 1.0 g yeast, 4 ml sodium lactate in 1 L seawater. The pH value was adjusted to 7.2 after the deaeration using nitrogen. Then the medium was autoclaved at 120 °C for 15 min. The SRB seeds were incubated at 30 °C. The bacteria growth was monitored by 0.5 g $(NH_4)_2Fe(SO_4)_2.6H_2O$ and 0.1 g L-ascorbic acid. The $(NH_4)_2Fe(SO_4)_2.6H_2O$ and L-ascorbic acid were sterilized by ultraviolet radiation for half an hour and were put into the media together with the incubation of the SRB seeds. This bacterial culture was introduced into a bioreactor and tested at real conditions with acid mine drainage water.

2.3. Bacterial detection and measurement of the transducer element thickness.

Aliquots of 10 ml moderate solutions with various pH (3.5, 5.0 and 9.0) and bacterial suspension from passive system were applied on 1 μm thick nanostructured ZnO film as a transducer element (2 cm^2 surface). The film thickness changing was measured optically by transmission and absorbance analysis at various fixed wavelengths (360, 420 and 520 nm) in a high-sensitivity Shimadzu spectrophotometer. ZnO sections were also observed in a scanning electron microscope (SEM). The data were collected and proceeded to calculate accurate the thickness change of ZnO. The diameters of generated in the air flux biosensor aqueous micro droplets were measured by fluorescence microscope Leica DM5500B. The adsorbed on the ZnO film SRB were proved and analyzed in 80 kV SEM machine.

3. Result and discussion

We investigated the growth structure of ZnO on the transducer element by using of x-ray diffraction (XRD). XRD spectra proved the polycrystalline structure of the nanostructured films with preferential crystallographic orientation (002) and *c*-axis perpendicular to the substrate surface (Fig. 1a). Scanning electron micrograph (Fig. 1b) and atomic force micrograph (Fig. 1c) reveal the columnar structure of the films with rough top surface. The combination of these three techniques for analysis concludes that the transducer films are of high crystalline quality and the ZnO material is absolutely homogeneous and stoichiometric.

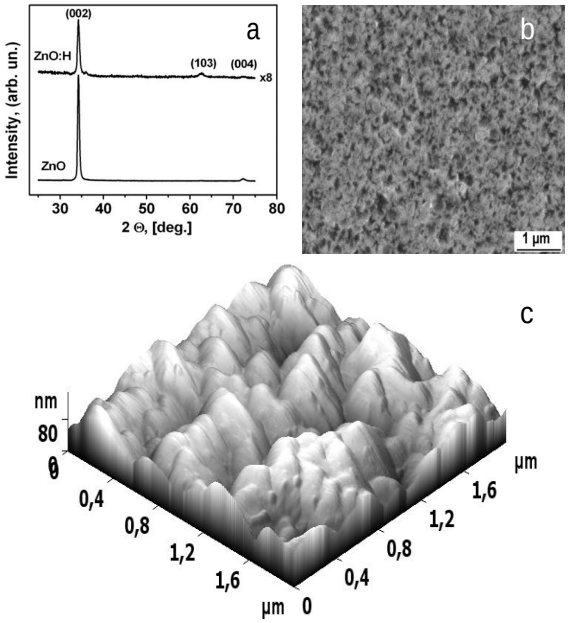

Fig. 1. Analysis of nanostructured thin film ZnO as a transducer element by: (a) x-ray diffraction, (b) scanning electron microscope (SEM) and (c) atomic force microscope.

We studied the dissolving behavior of ZnO transducer element in contact with bacterial suspension and various aqueous solutions with moderate pH values. To our knowledge ZnO is poorly soluble in water. The deionized water used in our experiment had a pH 5.0. Nevertheless, ZnO dissociate slowly in water according to the chemical equation (*process 1*):

$$ZnO + 2H_3O^+ \longrightarrow Zn^{2+} + 3H_2O \quad (1)$$

The efficiency of *process 1* was investigated optically by measurement of the transmitted light trough a Si glass covered with 1 μm nanostructured ZnO. The result shows that its contact with deionized water for about 30 min results in intensively decreasing of the thickness from 1000 nm to 300 nm (70 % dissolving). The second liquid used in our study was ammonia (with moderate pH 8.7-9.0). The dissolving of ZnO by ammonia can be attributed to the following chemical reaction (*process 2*):

$$ZnO + 4NH_3.H_2O \longrightarrow Zn(NH_3)_4^{2+} + 2OH^- + 3H_2O \quad (2)$$

The obtained product $Zn(NH_3)_4^{2+}$ is soluble in water and the dissolving intensity of *process 2* is even higher in comparison with deionized water. The optical measurement shows that for 30 min the film thickness dissolved from 1000 nm to 150 nm (85 % dissolving).

We present by SEM micrographs how intensive is decreasing of ZnO thickness if SRB suspension with pH 3.5 is applied (see on Fig. 2). As it is shown on Fig. 2a the ZnO

thickness in the beginning of the biosensoring is about 1 μm. After 30 min reaction the thickness is less than 500 nm and after 1 hour contact with the the suspension the film is almost completely dissolved. The reaction mechanism in that case mainly follows the chemical equation of *process 1* (the authors do not exclude the possibility for other reactions with the components of acid-mine drainage water).

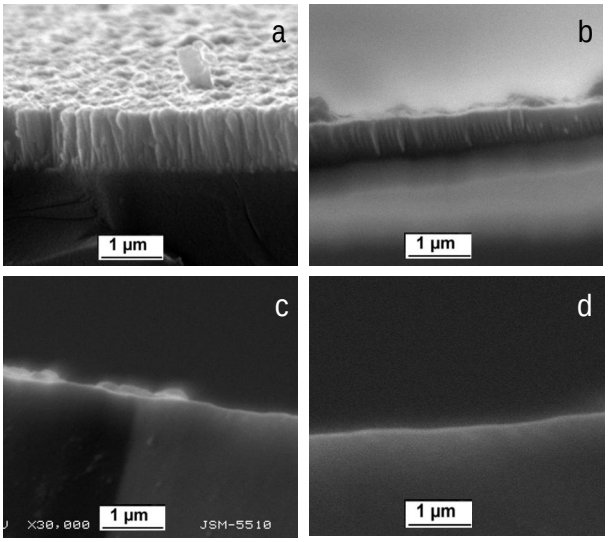

Fig. 2. SEM images of nanostructured ZnO thin film, that has interact with sulfate-reducing bacterial suspension taken from bioreactor for treatment of acid mine drainage water (pH 3.5). The dissolving time is (a) 1 min, (b) 30 min, (c) 60 min and (d) 90 min. Scale bar = 1 μm.

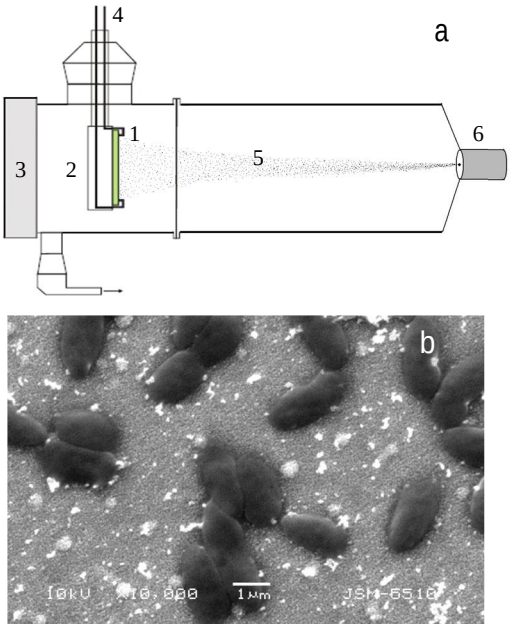

Fig. 3. Bacterial biosensor based on air flux detection principal. (a) Components of air flux biosensor (AFB): *1* ZnO transducer element, *2* sample holder, *3* heater, *4* electronic device, *5* air flux, *6* pulverizer. (b) SEM image of SRB on ZnO transducer film after 8 hours exploitation. Scale bar = 1 μm.

The results of this study are of great significance. First, biosensor made of ZnO nonmaterial has a certain time to perform a device function. Secondly, once completing the corresponding service, the ZnO wires can eventually dissolve into ions that can be completely absorbed by the cells and become part of the nutrition. To overcome these problems we engineered a new design of the biosensor in order to achieve longer life-time utilization of the transducer element (see on Fig. 3a). The basic difference in our AFB is that bacteria are reaching the transducer element by air flux, which is produced by pulverizer. Our additional experiment shows that the distance between the pulverizer and the transducer is of significant importance for the droplet size and amount of water which is deposited on the film. By using of dye colored liquid and fluorescent microscope, we found the best conditions to obtain mixture of SRB and microemulsion droplets with diameter around 1 μm. This mixture obtains a mist in the AFB interior. The heavy droplets easy gravitate in the camera and never reach to the transducer. However, as it is shown on Fig. 3b, SRB can carry through the air flux and adsorb easily on the ZnO transducer. Our experiment proved that in this case even after 8 hours exploitation of AFB the ZnO thickness change is difficult to be registered.

4. Conclusion

From the obtained results we can conclude that ZnO possess high degradability in aqueous solutions with different pH values, especially when SRB in acid-mine water are applied. Due to this reason it has potentially low possibilities for applications in *in situ* biosensoring or biodetection in aqueous environment. ZnO has potential to be used as transducer element in the biosensor if the analyte is applied in the state of air flux. In that case the "survival lifetime" is highly increased.

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