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Short communication

Organic–inorganic hybrid material for the cells immobilization: Long-term viability mechanism and application in BOD sensors

Ling Liu, Li Shang, Shaojun Guo, Dan Li, Changyu Liu, Li Qi, Shaojun Dong*

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Renming Street 5625, Changchun 130022, Jilin, PR China

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ABSTRACT

In this paper, organic–inorganic hybrid material, which is composed of silica and the grafting copolymer of poly (vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)), was employed to immobilize *Trichosporon cutaneum* strain 2.570 cells. Cells entrapped into the hybrid material were found to keep a long-term viability. The mechanism of such a long-term viability was investigated by using confocal laser scanning microscopy (CLSM). Our studies revealed that arthroconidia produced in the extracellular material might play an important role in keeping the long-term viability of the immobilized microorganism. After the arthroconidia were activated, an electrochemical biochemical oxygen demand (BOD) sensor based on cell/hybrid material-modified supporting membrane was constructed for verifying the proposed mechanism. The results and insight gained from the present experiments can be widely used to various biosensor designs.

1. Introduction

Recently, the biosensor based on the immobilized whole cells has received considerable attention due to its application in industrial bioprocess (Baca et al., 2006). Immobilizing living cells in extracellular materials provides some advantages over free cells such as increased metabolic activity, protection from environmental stresses and toxicity, and enhanced stability (Alvarez et al., 2009). Based on the multiple intracellular enzyme systems, cells entrapped in the extracellular materials may provide inherent benefits in multitask (e.g. degrading the mixture composed of multiple organic compounds) for catalysis applications (Ferrer et al., 2003). However, the entrapped procedure usually exposed cells to an unnatural environment, and brought a constraint to entrapped cells, which would affect the viability of immobilized cells (Perullini et al., 2008). On the other hand, the long-term viability of the immobilized cells was a limiting factor for the use, storage and transportation of prepared biosensor in practical applications (Amoura et al., 2007; Avnir et al., 2006). Thus, investigation on the long-term viability of entrapped cells was of great importance (Di Vona et al., 2006).

The organic–inorganic hybrid materials have provided a good opportunity for cell encapsulation (Ferrer et al., 2006; Lin et al., 2006). So far, a large number of organic–inorganic hybrid materials have been developed for cell immobilization. Some prominent examples include PVA-cryogels hybrid material for the immobilization of *Bacillus subtilis* cells (Szczęsna-Antczak and Galas, 2001), silica–glycerol matrix for the investigation of viability of *Escherichia coli* (Nassif et al., 2002), alkoxysilanes organically modified silica for living cell immobilization (Böttcher et al., 2004). Those studies were successful for demonstrating the organic–inorganic hybrid materials used to immobilize cells and maintaining viability of such cells in measuring process. However, investigation on the long-term viability for storage of immobilized cells and its mechanism has rarely been reported, especially in the study based on the morphology of cells (Matys et al., 2004).

In this communication, the morphology of immobilized yeast cells in the organic–inorganic hybrid biomatrix membrane were investigated through confocal laser scanning microscopy (CLSM) technique for studying the mechanism of their long-term viability. Our results revealed that the arthroconidia produced in the extracellular material plays an important role in keeping the long-term viability of the immobilized cells. *Trichosporon cutaneum* 2.570 cells existed as an arthroconidia state when they were stored. These dormant form cells could resist the environmental stresses and could be made active in appropriate conditions. Based on this, an electrochemical biochemical oxygen demand (BOD) sensor was constructed for verifying the proposal mechanism.

* Corresponding author. Tel.: +86 431 85262101; fax: +86 431 85689711.
E-mail address: dongsj@ciac.jl.cn (S. Dong).

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2. Experimental

2.1. Staining and CLSM

The biomatrix was prepared by mixing the *T. cutaneum* cells, grafting copolymer PVA-g-P(4-VP) and silica sol–gel (supporting information). The morphology and integrity of cells were characterized by CLSM system Leica TCS SP2 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Fluorescein isothiocyanate (FITC, Sigma) was chosen to label all cells and propidium iodide (PI, Sigma) was used to label the damaged cells. FITC and PI were dissolved together in PBS (0.08 M KH_2PO_4 and 0.12 M Na_2HPO_4, pH 7) with a final concentration of 1 mg ml^{-1} and 0.05 mg ml^{-1}, respectively. The biomatrix with entrapped cell (supporting information) was incubated in the above solution containing fluorescence dyes for 6 h at 28 °C. Before imaging with CLSM, the biomatrix was washed thoroughly by PBS. The excitation wavelength was 488 and 543 nm for FITC and PI, respectively. A 50× objective was used for all imaging.

2.2. Application of the biomatrix on BOD biosensor

The biomatrix membrane (SEM images were shown in Fig. S1, supporting information) was prepared by dropping the mixture (cells, grafting copolymer and sol–gel) on a supporting membrane. The biomatrix membrane was stored at 4 °C in the refrigeratory in the sterilized glass plate. The ambient humidity and the nutritious did not need to be controlled. This biomatrix membrane was combined with an oxygen probe to build an electrochemical sensor for BOD monitoring. The potential of gold electrode versus Ag/AgCl reference electrode was set at −700 mV, and Pt was used as the counter electrode. The BOD^{198} standard solution was prepared according to APHA method (American Public Health Association, 1997). This standard solution contained 150 mg l^{-1} glucose and 150 mg l^{-1} glutamic acid (GGA) with a BOD value of 198 ± 31 mg BOD_5 l^{-1}. Solution with other concentrations was prepared by appropriate dilution of the BOD^{198} standard solution. A 25.0 mg BOD_5 l^{-1} GGA solution was used to estimate performance of the biosensor (for activating of the biomatrix membrane, and the biosensor response within 2 months). Temperature of the detection chamber was maintained at 28 °C. The flow rate of solution was 3 ml min^{-1}, and all the results were obtained based on the average of measurement for three times.

3. Results and discussion

3.1. Arthroconidia formation in the biomatrix

It is known that *T. cutaneum* strain 2.570 itself has the ability to produce arthroconidia, which is a characteristic of *Trichosporon* spp. (Li et al., 2005). However, the report that arthroconidia could be produced in the biomatrix was relatively scarce. It is interesting that the arthroconidia were observed to be formed in the present biomatrix after being stored at 4 °C for over 10 days. As shown in Fig. 1a and b, a great number of the asexual conidia were produced by the segmentation of hyphae and several clear constrictions were indicated by arrows. All cells were labeled by FITC (green, Fig. 1a), whereas only very few damaged cells were labeled by PI (red, Fig. 1b), which indicated that almost all cells kept good integrity (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.). Fig. 1c and d displays the CLSM images of *T. cutaneum* strain 2.570 cells in the xero-matrix stored at 4 °C over 13 months without being cultured in

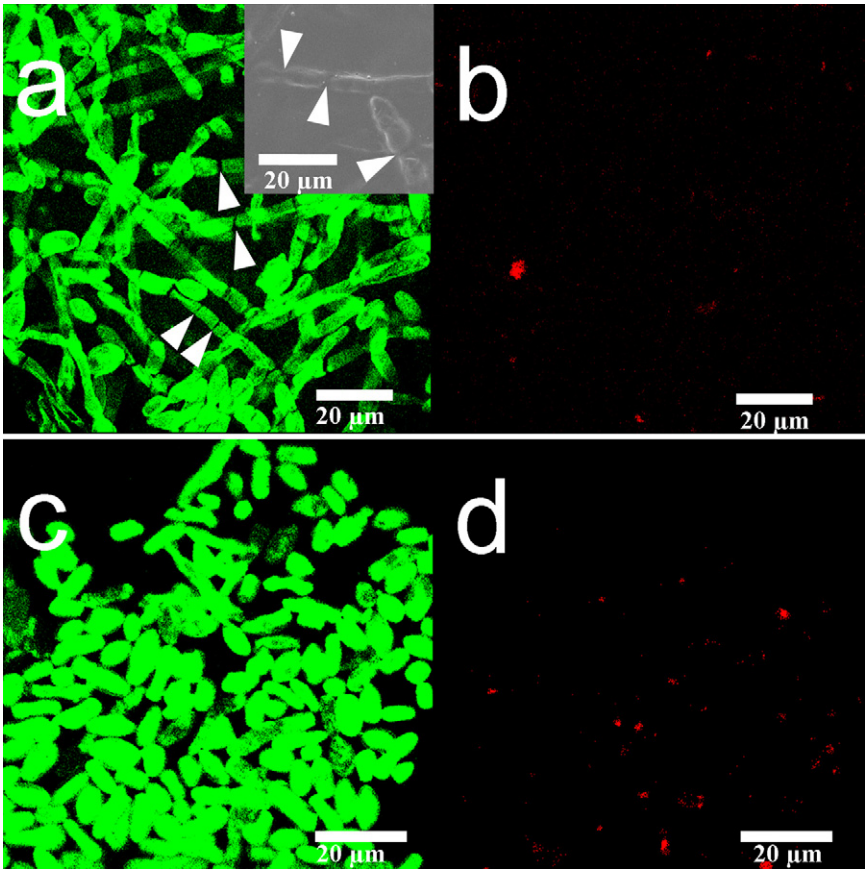

Fig. 1. CLSM images of the arthroconidia in the biomatrix. (a and b) The formation of arthroconidia; (c and d) the arthroconidia in biomatrix after stored at 4 °C for 13 months. Inset: magnified scanning electron microscopy (SEM) images of typical constrictions for arthroconidia production.

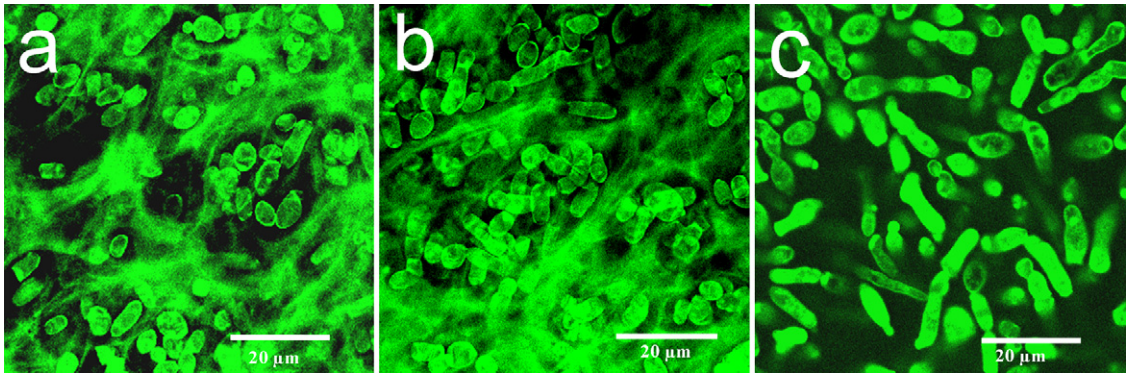

Fig. 2. CLSM images of *T. cutaneum* cells in the biomatrix after activated for 2 (a), 4 (b) and 7 (c) days.

any specific substrates. Fig. 1c (stained by FITC) shows that the cells are almost ellipsoidal arthroconidia. According to the corresponding Fig. 1d (stained by PI), very few damaged cells were observed, although they have been stored in the biomatrix for over 1 year. The present results indicated that all arthroconidia still kept their good integrity, which could be attributed to that the arthroconidia with the concentrated protoplasm has a stronger tolerance than vegetative cells toward stress of the environment (pH, ionic strength and thermal condition, etc.). Therefore, cells located at the arthroconidia phase are suitable for their long-term storage. Furthermore, the arthroconidia can be activated to vegetative cells in a suitable condition (nutrition substances exist), as shown in the following. It is noteworthy that previous studies showed that the cells with the energetic metabolism in the vegetative phase have great potential for application in biocatalysis and biosensors (Nassif et al., 2002; Ferrer et al., 2003).

3.2. Arthroconidia germination in the biomatrix

Fig. 2 displays CLSM images of the arthroconidia (immobilized in the biomatrix and stored for 13 months) after being activated under suitable nutrition conditions at 28 °C for different times. It is clearly observed that with the activation time extended, the amount of stretched-out vegetative cells increased gradually. The process that the immobilized cells changed from vegetative form to arthroconidia form and recovered to vegetative cell form was called a cell life cycle. After 1-week activation, as shown in Fig. 2c, the community of *T. cutaneum* cells in the biomatrix consisted of hyphae, arthrocondia, disarticulating hyphae and budding cells, which indicated that the stage of cell life cycle had entered a stable period. When further extending the activation time (more than 10 days, data not shown), the shape of the cells kept unchanged. It is noteworthy that these cells did not evolve into the primal filamentous hyphae, which was probably due to the restricted effect of extracellular material, but the metabolism level of the vegetative cells was enough for application in the biosensor.

3.3. Application of the biomatrix membrane on BOD biosensor

After being activated, the biomatrix membrane was used to construct a BOD biosensor. The time of measurement for the biosensor was 10 min and followed by a recovery time of less than 60 min. All measured signals were obtained after reducing the background current. Fig. 3a shows the net current response (ΔI) of the BOD electrochemical biosensor versus the activation time. The sample was 25.0 mg BOD_5 l^{-1} standard GGA solution. The current signal can reflect the activation degree of cells that entrapped in the biomatrix. After the cells were activated for 8 or 9 days, the net current

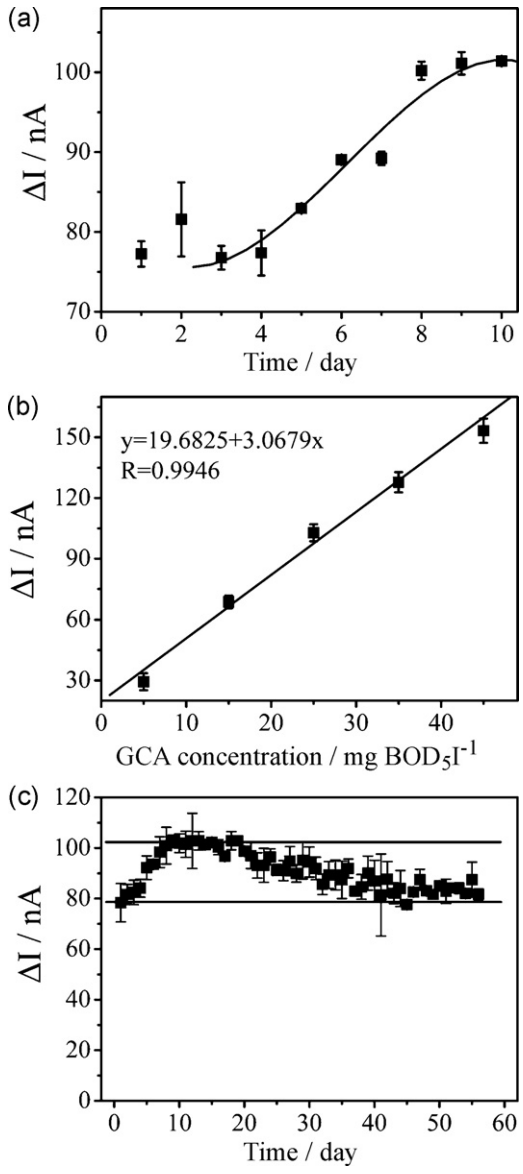

Fig. 3. Response of the BOD electrochemical biosensor was based on *T. cutaneum* yeast biomatrix membrane. (a) Process for activating the biomatrix membrane at different times; (b) calibration curve from GGA standard solution; (c) response of the biosensor within 2 months.

response of the biosensor for 25.0 mg BOD_5 l^{-1} standard solution reached a maximal signal (102 nA). The result demonstrated that the mechanism, long-term viability of arthroconidia for storing biosensor, became an effective means for sensor application. As shown in Fig. 3b, a calibration curve established from the standard solution was obtained for measuring the BOD of real polluted water sample. The calibration curve of GGA solutions at various concentrations from 5 to 45 mg BOD_5 l^{-1} was analyzed. A linear regression equation of $y = 19.6825 + 3.0679x$ ($R = 0.9946$) was obtained, where y was the current response (ΔI) and x was the concentration of the standard solution. The result revealed a satisfactory performance of the present biosensor according to previous work (American Public Health Association, 1997; Jia et al., 2003). Even after 2 months for continuous measurement, the biosensor could still keep 82.2% original response. Fig. 3c shows the operational stability of the present BOD biosensor within 2 months, where relative standard deviation (RSD) of the response was ±8.37% (for three reduplicate measurements). The result indicated that the biocompatibility of hybrid material was good and the activated cells could be applied for biosensor fabrication.

4. Conclusions

In summary, the PVA-g-P(4-VP)/sol–gel hybrid material has been successfully applied to immobilize *T. cutaneum* cells. It was found that the as-prepared biomatrix exhibited a biocompatible microenvironment which favored the keep of long-term viability of entrapped cells. The mechanism of long-term viability of immobilized cells was further explored through CLSM technique. The results revealed that arthroconidia produced in the extracellular material plays an important role for keeping the long-term viability of microorganism, which might because arthroconidia own the ability of resisting the environmental stresses. Furthermore, an electrochemical BOD sensor was constructed on cell/hybrid material-modified supporting membrane after activating the immobilized arthroconidia. The result indicated that the mechanism of long-term viability, the cell life cycle, would have a general meaning for biosensor strategy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.08.004.

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