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# Viable but nonculturable cells used in biosensor fabrication for long-term storage stability

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ABSTRACT

In this paper, we first reported the viable but nonculturable (VBNC) cells used for fabricating biosensor. The organic–inorganic hybrid material composed of silica and the grafting copolymer of poly(vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)) was used to immobilize microbial cells for biosensor fabrication. The VBNC cells were formed after the hybrid material dried, showing the cell walls were sacrificed. With the intracellular enzymes as core and the "sacrificed" cell walls as shell, the present VBNC cells maybe considered as a core/shell structure. The extracellular material worked as the scaffold for core/shell structure. The core/shell structure and the scaffold structure were demonstrated by single-cell level image analysis using confocal laser scanning microscopy (CLSM). The electrochemical method was adopted for further examining the enzyme activity of VBNC cells. The VBNC cells did not need nutrient treatment and other physicochemical factors for cell growth, which is a significant contribution for storing biosensor. A glucose–glutamic acid biosensor fabricated by the VBNC cells exhibited long-term storage stability for 100 days.



## 1. Introduction

The living cells and the purified enzymes have been extensively used to design of biosensors in a long time. However, they were limited in industrial applications with some disadvantages. For example, the substrate and product were required to control for the cell-type biosensor, and the response of the biosensor was not easy to keep constant due to the cell growth [1,2]. For enzyme-type biosensor, the purification process was complicated, and the purified enzymes are expensive. Furthermore, the purified enzymes are more susceptible and denatured by their unnatural exterior environment [3,4]. As is known, the stability was an important index of the biosensor, but the stability of the cell-type and the enzyme-type biosensors would be difficultly controlled due to above disadvantages. Here, the viable but nonculturable (VBNC) cells were introduced for designing biosensor, which could avoid the above interference commonly coexisted in the cell-type and the enzyme-type biosensors.

The term VBNC means that "cells cannot grow on or in selective and nonselective media in which they would normally develop into colonies but retain metabolic activity and remain in this state for long periods of time" reported by Dinu et al.. [5]. Accordingly, the VBNC cells possess some excellent properties such as resistance to environmental stresses and good permeability. More importantly, the growth control of VBNC cells did not needed [6,7]. To our knowledge, previous reports about the VBNC cells were generally focused on some aspects such as induction, resuscitation or determination of the VBNC cells, but using VBNC cells for biosensor fabrication has been scarcely reported [8–12]. It is noted that the VBNC cells applied here is distinguishable from the conventional concept, as the cells were able to form colonies after resuscitation in present work. The VBNC cells were grown under the standard aerobic conditions in a rotating shaker at 37 °C in the 3% (w/v) commercial CASO broth medium for about 3–7 d.

In the present work, the state of microbial cells was estimated by valuate the cell integrity based on the different permeability of the LIVE/DEAD fluorescing reagent [13]. The microbial cells were encapsulated into an organic–inorganic hybrid material. After dried, the cells could be stained by propidium iodide (PI), which indicated that those cells were sacrificed. However, they kept metabolic viability and could be further applied to biosensor fabrication. Here, we develop a glucose–glutamic acid biosensor based on the VBNC cells. Because the cells had lost their cultivability, the growth of VBNC cells would not be controlled, where the intracellular enzyme activity could be favorably remained due to their homologous biological environment and a long-term storage stability of the biosensor was obtained [14–16].

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## 2. Experimental

### 2.1. Materials

CASO broth medium was bought from Fluka (Fluka Chemie GmbH CH-9471 Buchs). RoTrac® capillary pore membranes were bought from Orbisphere. The CASO Broth contained casein peptone soybean flour peptone broth. BOD seed (E-05466-00, Cole-Parmer) cells were composed by *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Serratia liquefaciens*, *Yersinia enterocolitica*, *Citrobacter amalonaticus*, *Enterbacter sakazakii* and *Enterobacter cloace* [28]. Tetraethyl orthosilicate (TEOS) was purchased from Tianjin reagent factory (Tianjin, China). Cetyltrimethyl ammonium bromide (CTAB) was purchased from Dingguo Ltd. (Beijing, China). Grafting copolymer of poly(vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)) was prepared as described previously [17]. Chemicals used in this study were of analytical reagent grade and all solutions were prepared with deionized water being sterilized.

### 2.2. Evaluation of the property of hybrid material scaffold

*Saccharomyces cerevisiae* strain was maintained by our laboratory on yeast extract peptone dextrose (YPD) nutrient agar (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) plates at 4 °C in refrigerator. The culture of *S. cerevisiae* was prepared according to the previous report [18]. After 1 week incubation in the YPD substrate, the cells were arrested at $G_0$ phase of the cell cycle in uniformly round [19]. The microorganism cells were collected by centrifuging at 4000 rpm for 10 min at room temperature. Then the cell mass was washed twice with PBS and resuspended in PBS at the concentration of 1.5 g/ml and was used for preparing the biomatrix immediately. The preparation of silica sol–gel was referred to our previous paper [20]. 100 μl microorganisms were mixed adequately with 200 μl grafting copolymer PVA-g-P(4-VP) and 100 μl silica sol–gel. Then 200 μl of the mixture was dropped on the surface of a glass slide and was covered with cover glass. The obtained membrane was dried at 4 °C and stored before use. Formation of the biomatrix depended on a lower temperature and mild synthetic conditions. After dried, the biomatrix was treated by 2% (w/v) CTAB for lysing the cells to obtain a hybrid material scaffold.

### 2.3. Preparation of VBNC cells

Commercial BOD seed cells were grown under the standard aerobic conditions in a rotating shaker at 37 °C for 10 h in the 3% (w/v) commercial CASO broth medium. The microorganism cells were collected by centrifuging at 8000 rpm for 3 min at room temperature. The preparation of mixture of the cell and extracellular hybrid material was identical as in Section 2.2.

### 2.4. Construction of biosensor

Then 5 μl of the mixture was dropped on the surface of a capillary pore membrane. The membrane contained bacteria was dried at 4 °C and stored, and used for fabricating biosensor. Formation of the membrane depended on a lower temperature which was a mild condition for maintaining the viability of intracellular enzyme. After dried, the VBNC cells were formed. The membrane was used as a cap covered on surface of a three-electrode system for establishing biosensor. The amperometric mode has been adopted in the present work. A gold electrode was used as the working electrode, Pt as the auxiliary electrode, and a potential of −700 mV versus Ag/AgCl reference electrode was applied to the Au-working electrode in 0.1 M potassium chloride (KCl) as the supporting electrolytes.

### 2.5. Characterization and evaluation of the VBNC cell

The structure of the extracellular material was evaluated by employed *S. cerevisiae* cells. The protection of the intracellular enzyme was demonstrated by employed the commercial BOD seed. The characterization of the VBNC cells was carried out by confocal laser scanning microscopy (CLSM, Leica TCS SP2, Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Fluorescein isothiocyanate (FITC, Sigma) was chosen to stain all cells and propidium iodide (PI, Sigma) was used to label the damaged cells (the VBNC cells). FITC and PI were dissolved together in PBS with a final concentration of 1 and 0.05 mg/l, respectively. The cells were incubated in above solution for 0.5 h at 30 °C. Before detected by CLSM, samples were washed thoroughly with PBS. The fluorescence images for the sample were photographed with the excitation wavelengths of 488 and 543 nm for FITC and PI, respectively. A 100× oil-immersion objective was used for all imaging. After stained, the VBNC cell biomatrix membrane was washed three times by PBS thoroughly for avoiding the background interferences. The biomatrix membrane was processed by PBS (pH 7) at 80 °C to dissolve the hybrid material for the single-cell level analysis. The single-cell level analysis was employed by the LCS Leica confocal software. The projections were obtained from *xy*-confocal slices of an image stack display them in a two-dimensional image and to 3D view analysis.

### 2.6. Metabolic activity of the VBNC cells

Metabolic activity of the VBNC cells was evaluated by electrochemical method according to Catterall et al.. [12]. Briefly, 500 μl phosphate buffer solution (PBS, 0.12 M $Na_2HPO_4$/0.08 M $KH_2PO_4$) with 5 pieces of the biofilm (0.15 g microorganism cells per pieces), 160 μl of 330 mM potassium ferricyanide, and 340 μl of glucose solution, were mixed with a final volume of 1 ml for incubation. The controlled experiment was conducted using 500 μl 1.5 g/ml BOD seed instead of the biofilm. The endogenous controlled experiment was prepared using PBS instead of glucose solution. The final concentration of the microorganism solution was 0.375 g/ml. The samples were incubated at 37 °C for 4 h in a water bath, and then centrifuged with 8000 rpm at room temperature for 3 min. The supernatant solution was then used for detection of ferrocyanide produced by microbe. The amperometric mode has been adopted in the present work. A platinum microelectrode array (2 × 2 microdiscs with 25 μm diameter each) was used as the working electrode, Pt foil as the auxiliary electrode, and a potential of −450 mV versus Ag/AgCl reference electrode was applied to the Pt-working electrode in 0.1 M potassium chloride (KCl) as the supporting electrolytes.

## 3. Results and discussion

### 3.1. Strategy and analysis of the VBNC cells

As a biocompatible functional polymer, PVA-g-P(4-VP) contains a great amount of hydroxyl group and polar nitrogen atom in the pyridine structure. 4-Vinylpyridine (4-VP) monomer is known to be strongly hydrophilic and biocompatible [21]. PVA can act as a protective agent for microorganisms, and there is also evidence that PVA can weakly adsorb to the cell membrane through hydrogen bonds of the hydroxyl group [22]. So the present organic–inorganic hybrid material is perfectly compatible and miscible with the PVA-g-P(4-VP) via strong hydrogen bond interactions between the nitrogen atom in the pyridine and the hydroxyl groups within the PVA-g-P(4-VP), and between both PVA-g-P(4-VP) and silica sol–gel [23]. The cells were capable of being encapsulated into the hybrid material. The cell membrane could be destroyed by drying and

shrinkage of the matrix, which then possibly resulted in increasing the permeability of the cell walls and membranes. Previous work showed that VBNC enterococcal cells were endowed with a wall more resistant to mechanical disruption than dividing cells [24]. When the cells sacrificed, the intracellular enzyme of the VBNC cells were protected by the matrix composed of extracellular material and permeable cell wall. The construction is like a core/shell structure where the enzymes are maintained as the core, and the substrates and products are allowed to permeate the permeable cell wall [25]. Therefore, the strategy of sacrificing cellular cultivability combined with the hybrid materials matrix for fabricating intracellular enzyme biosensor is of importance in practical applications.

In order to demonstrate the scaffold structure of an extracellular material, we choose *S. cerevisiae* cells as a model. The *S. cerevisiae* was entrapped into the organic–inorganic material. After dried, the cells in the biomatrix were fragmented by cetyltrimethyl ammonium bromide (CTAB) to obtain a hybrid material scaffold. Fig. 1 shows the CLSM image of the scaffold stained by FITC. The mesh position by the cells occupied originally was seen in Fig. 1. The diameter of the meshes was almost the same as that of the cells (4.5–6.1 μm) which clearly indicated the scaffold construction of the hybrid material. This result showed that the original morphology of the matrix was kept due to the good physical rigidity of the hybrid material when the cells were leaked out.The formation of VBNC cell was valuated using confocal laser scanning microscopy (CLSM). The commercial BOD seed microbe was employed in the present work. Fig. 2 shows the images of the cells in three experimental conditions: for control (Fig. 2A), after entrapped in the hybrid material for 6 h (Fig. 2B), and after dried (Fig. 2C). The percentages of cells stained by PI were 25, 34 and 98% as shown by A to C. This result suggested that the cell viability was almost remained for the non-dried material, and the VBNC cells were formed in the process of drying. The core/shell structure of the present VBNC cell was demonstrated by single-cell level analysis. As shown in Fig. 2D, some green closed oval loops were composed by the voxels indicating the isolines form of the fluorescence intensity by 3D view in the CLSM image. The cell has been filled by red flocculent voxels, which indicated that the cell membrane was permeable (Fig. 2E). This result may be caused by the total cross-linking of the VBNC

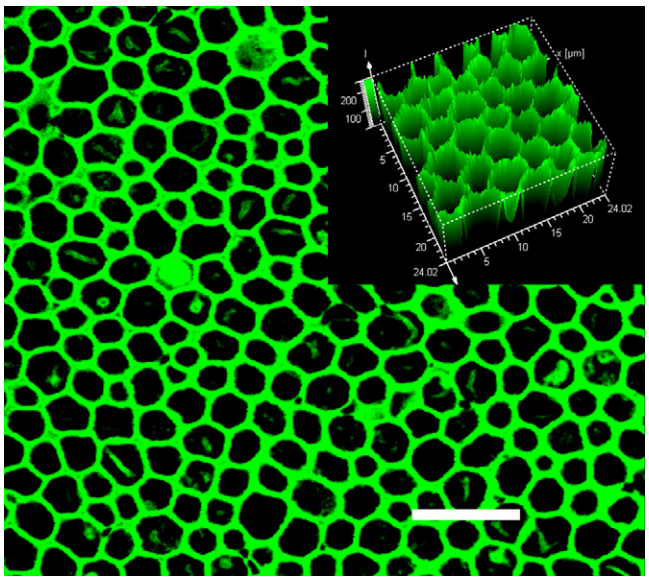

**Fig. 1.** CLSM images of the organic–inorganic hybrid material scaffold template obtained by segment *S. cerevisiae* cells and labeled by FITC. The bars represented 10 μm. Inset, the 3D view.

cell walls being changed, and the PI readily crossed the permeable membranes and intercalated into GC-rich segments of DNA [26]. Combined Figs. 1 and 2, the intracellular enzymes of VBNC cells could be protected by the matrix composed of the permeable cell membrane, cell wall and extracellular material. Furthermore, the homologous intracellular enzyme systems could be remained in the relevant specific biological and ecological conditions.

### 3.2. Metabolic activity of the VBNC cells

According to the conventional understanding, the cell was sacrificed when it was stained by PI [27], but the result of the glucose assays from the present work was on the contrary. As shown in

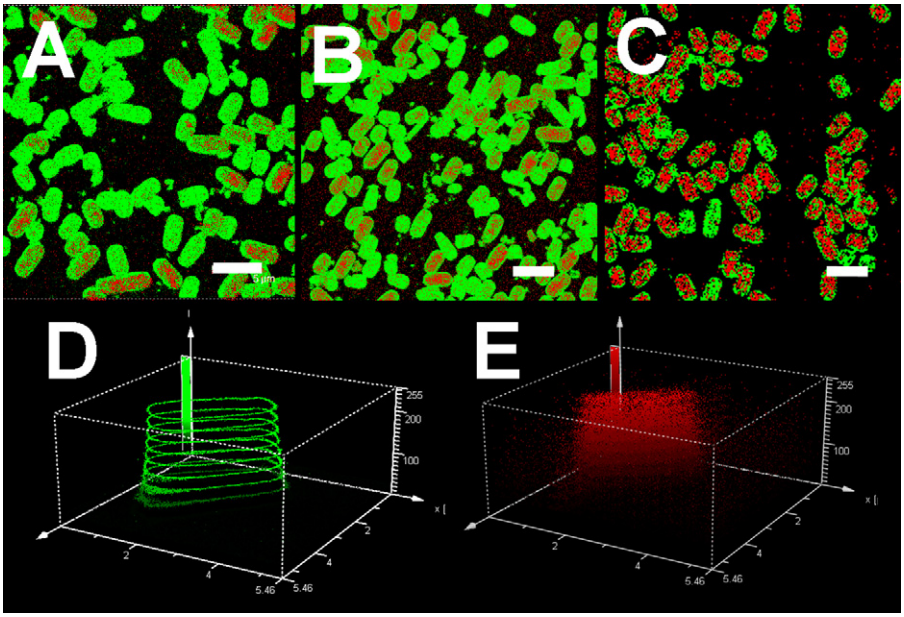


**Fig. 2.** CLSM images of the cells from (A) to (C): control, immobilized for 6 h, and after dried. The figures were obtained by superposing two images of FITC (green, for all cells) and PI (red, for sacrificial cells) stained for corresponding area of sample. The bars represented 2 μm. Images (D) and (E) showed the isolines form visualization of single VBNC cell for 3D view of the CLSM image. The unit of *X*- and *Y*-axis is μm. The *Z*-axis displays pixel intensity value. A 100× objective was used for imaging. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

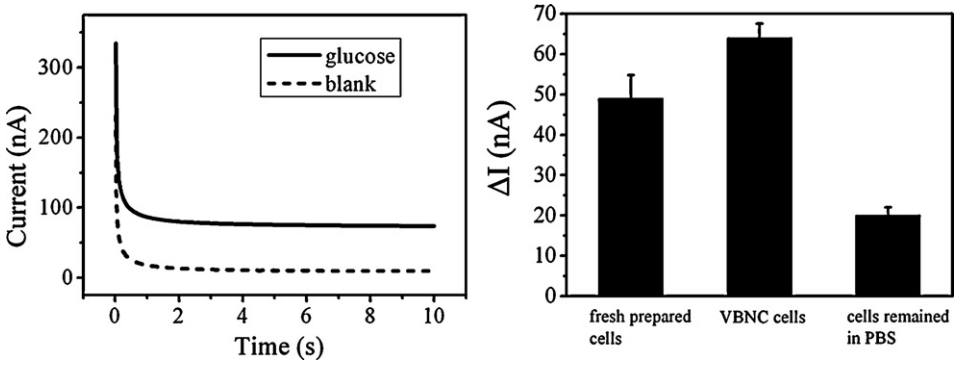


**Fig. 3.** (A) The response of VBNC cells biosensor for measuring 5.65 mg/l glucose solution. (B) The net current response ($\Delta I$) of the BOD seed cells in order were fresh prepared cells, VBNC cells and the cells remained in PBS.

Fig. 3, all measured current signals were obtained by background subtraction. The glucose solution with concentration of 5.65 mg/l was measured by the VBNC cell biosensor shown in Fig. 3A. In Fig. 3B, the net current response of the intracellular enzyme of the VBNC cells was 64.2 nA, and the net current responses of the cells prepared freshly and maintained in PBS were 48.8 and 19.9 nA, respectively. It must be point out that the result of former is not always higher than the latter, but in a short time when membrane dried. The result indicated that the metabolic activity of the cellular enzyme systems was still remained and the electron transport chain was completed in the permeable cells.

### 3.3. Fabrication of the glucose–glutamic acid biosensor

A glucose–glutamic acid biosensor was constructed using the VBNC cells. The time for measurement of the biosensor was 10 min and followed by ~60 min recovery. All the current signals were obtained by background subtraction. Fig. 4 shows the stability of the present biosensor with 10.91 mg/l glucose–glutamic acid within 100 days. The current signal can reflect the activation degree of the cells entrapped in the biomatrix. At the first day, the current response to the glucose–glutamic acid solution exceeded 85.4 nA which was a maximal signal. As shown in Fig. 4, the biosensor could still give 94% (80.0 nA), 82.4% (70.4 nA), 86.4% (73.8 nA), 53.9% (46.0 nA) of the original response after kept in ~4 °C for 18, 30, 58 and 100 days, respectively. This result indicated that the biosensor constructed based on the VBNC cells exhibited a long-term storage stability. The reasons of the excellent property were conjectured that the cultivability was sacrificed and did not need to control the condition for growing and proliferation. Furthermore, the condition for obtaining VBNC cells was much milder than conventional methods as physical photoelectric, thermal and chemical reagent process for purifying enzyme.

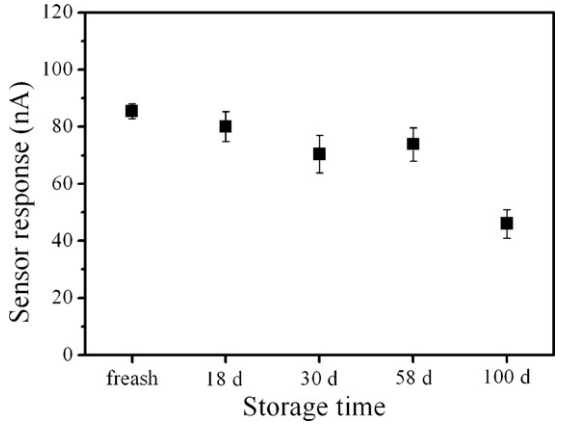


**Fig. 4.** Stability of the biosensor.

## 4. Conclusion remarks

The VBNC cell as a special state of microbial cells has been introduced in the present strategy for biosensor fabrication. A long-term storage stability of the biosensor was obtained. To compare with the conventional cell-type biosensor and enzyme-type biosensor, the present VBNC cell biosensor possessed some advantages as: the physical and chemical conditions for controlling cell growth were not needed, the conditions for protecting intracellular enzymes were native and mild, the operation of biosensor fabrication was simple and the cost was effective. The result obtained from the present work has a broad significance for biosensor fabrication.

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