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THE FABRICATION AND THE USE OF IMMOBILIZED CELLS AS TEST ORGANISMS IN A FERRICYANIDE-BASED TOXICITY BIOSENSOR

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Abstract:

Cell immobilization is an effective method to prolong the life time of microorganism and has been proved their feasibility in some other biosensors. Thus, we study on the use of *Escherichia coli* (*E. coli*) immobilized by agar, gelatin, agar-gelatin mixture (agar/gelatin), chitosan and polyvinyl alcohol (PVA) to screen toxicity electrochemically. The *E. coli* immobilized by PAV gel showed the highest apparent bioactivity and the longest storage time in pH 7.0 PBS solution. Furthermore, the *E. coli* immobilized by different gels was applied in the toxicity determination via a reported ferricyanide-mediated electrochemical method, where 3,5-dichlorophenol (DCP) was used as a model toxin. *E. coli* immobilized by PVA showed the highest sensitivity to DCP and the corresponding value of 50% inhibition concentration (IC_{50}) was 9.62 mg L^{-1}. The values of IC were in the range of 6.32 % and 13.75% when the *E. coli* immobilized by PAV was challenged by wastewater, which were comparable with those obtained with the standard luminescent bacterial method (value of EC was in the range of 7.96%–25.42% for the same samples). Given the apparent bioactivity, storage ability and sensitivity to toxin, PVA was the best polymer to confine cells among the polymers used in this work.

Keywords: Cell immobilization; Environmental toxicology; Toxic effects; Analytical toxicology

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INTRODUCTION

Rapid，cost-effective determination of toxicity has become more and more important, due to the increased pollution caused by chemicals. Microorganism with the advantages of sharp response to toxicity, susceptible modifications and so on, has proved their feasibility as test organism in designing novel toxicity biosensors for environmental risk assessment[1,2,3,4,5，6]. Recently, a ferricyanide-based toxicity biosensor was proposed，where respiratory inhibition was recognized as the response of microorganism to toxicity and could be determined electrochemically[7,8,9,10,11,12]. This toxicity biosensor showed comparable sensitivity to typical heavy metals and some organic materials to the commercial equipment[7,9,13,14]. However, the use of free cells as the test organism is not suitable for practical application of this toxicity biosensor because free cells can lose their bioactivity rapidly and have to be cultured before use. The immobilized cells are well known for its capability to maintain the biological activity of target cells in a long-term storage[15,16,17,18,19,20], and therefore the use of the immobilized cells instead of free cells can be expected to dramatically promote the field application of the toxicity biosensors[21,22,23].

The sensitivity of immobilized cells to toxicants is believed to be related with the porosity and the type of polymer matrix, which can dramatically affect the mass transfer of toxicants and further hinder the interaction between immobilized cells with toxicants[24,25,26,27]. The entrapment of cells in polymer matrix such as agar, gelatin and alginate, is an efficient and simple method to obtain a sensitive biological probe with good controls of the structure, shape and resultant component, which is thus a favorable method to prepare the target organisms in the toxicity determination[20,21,27,28].

In this work, agar, gelatin, chitosan, polyvinyl alcohol (PVA) and the mixture of agar-gelatin (agar/gelatin), were employed as different polymer matrices to immobilize *Escherichia coli* (*E. coli*) ATCC 25922 as the model microorganism. The contents of agar, gelatin, chitosan, PVA and agar/gelatin of the corresponding matrices of immobilized cells were optimized to be 30 g L^{-1}, 150 g L^{-1}, 5 g L^{-1}, 10 g L^{-1} and 30 g L^{-1}/150 g L^{-1}, respectively, in term of apparent biological activity. The concentration of the *E. coli* used for immobilization and the thickness of the resultant film of immobilized cells are also considered to affect the apparent biological activity, which were optimized to be 10.0 in optical density at 600 nm (OD_{600}=10.0) and 1mm, respectively. The resultant film of immobilized cells with PVA matrix exhibited the highest biological activity at first, and could maintain suitable its activity after 40-day storage in pH 7.0 PBS solution. Then all films of immobilized cells were further used as test organisms in the ferricyanide-based toxicity biosensor, and the highest sensitivity to 3, 5-dichlorophenol (DCP) was obtained with PVA-based film. Given the long-term storage stability and the high sensitivity, PVA is believed to be a suitable polymer matrix for immobilization of target cells used in toxicity sensors. The combination of toxicity biosensor and PVA-based film with immobilized *E. coli* was further challenged by a series of wastewater, exhibiting the similar toxicity response to that obtained by the standard luminescent bacterial test. All these results can prove the good promise of the ferricyanide-based toxicity biosensors using immobilized *E. coli* with PVA matrix as test organism in field application.

MATERIALS AND METHODS

Materials

DCP, peptone and PVA were obtained from Beijing AoBoXing Bio-tech co., Ltd (China). Yeast extract, chitosan, glucose and glutamic acid were from OXOID Ltd (England). NaCl, Na_2HPO_4, KH_2PO_4, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, agar and gelatin were purchased from Tianjin Chemical Reagent Factory (China). All the reagents used in this study were of analytical grade except peptone and yeast extract. Agar, gelatin, agar/gelatin and PVA solutions with desired concentrations were prepared with sterilized phosphate buffer solution (PBS), and the chitosan solution was prepared with 0.05 g L^{-1} acetic acid. The fresh mixture of 150 mg L^{-1} glucose and 150 mg L^{-1}glutamic acid with the BOD_5 value of 220 mg L^{-1} were prepared as a nutrient and assigned as GGA^{220}.

E. coli ATCC 25922 was the quality-control strain and gifted from The First Affiliated Hospital of Jinzhou Medical University. A Luria Bertani (LB, 10 g L^{-1} peptone and NaCl, 5 g L^{-1} yeast extract) broth was adjusted to pH 7.0 and sterilized at 120 ºC for 20 min. The sterilized LB broth was vaccinated with a colony of *E. coli*, and then a bacterial culture was grown at 37 ºC for 12 h (late exponential phase of growth) in a shaking incubator at 150 rpm. At room temperature, the cells of *E. coli* were harvested by centrifugation at 4500 rpm for 10 min. The collected cells were washed twice with PBS and re-suspended in PBS at desired concentrations valued by OD_{600}[29]. The experimental bacteria were required on the day of training.

Preparation of immobilized cells

All polymer solutions were sterilized at 120 ºC for 20 min and then kept in the inoculating worktable at room temperature. The concentrations of agar, gelatin, agar/gelatin and PVA solution were adjusted by PBS solution. But the chitosan solution was adjusted by acetic

solution. All polymer solutions were mixed with different OD values of *E. coli* cells to prepare polymer-cell suspensions. Then each suspension was casted to a homemade device of height-adjustable scrapers for producing an intact film with desired thickness. It was noted that all suspensions were directly sealed in the scrapers at 4 ºC for 2 h except PVA. The saturated boric acid had to be used to handle the crosslink process of PVA, and then the handled PVA was sealed in the scrapers at 4 ºC for 24 h. All solidified films with immobilized *E. coli* were peeled carefully and washed twice with a large amount of sterilized PBS.

Determining biological activities of immobilized cells

A total volume of 10.0 mL liquid substrate that contained 45mM ferricyanide and standard GGA^{220} solution was used for each test. To screen the optimum immobilization conditions, biological activities of immobilized *E. coli* with different ploymers were determined by electrochemical methods after incubating in 1 h at 37 ºC in the liquid substrate mentioned above. During the incubation time, ferricyanide ions as a common mediator could be reduced into ferrocyanide ions and shuttle electrons produced by the respirations of immobilized cells to the electrode surface. Obviously, the apparent biological activity of immobilized cells was then detected in term of amperometric measurements related to ferrocyanide ions yields. PBS was chosen to supplement deficient volume of liquid substrate.

The electrochemical analysis

The electrochemical measurements involved in this study were performed by a CHI832C electrochemical workstation (Chen Hua, Shanghai). An ultramicroelectrode array (UMEA) composed of 15 pieces of isolated Pt ultramicroelectrode with the diameter of 25μm, was used as the working electrode. A platinum wire and an Ag/AgCl (saturated KCl solution) electrode were used as the counter and reference electrode, respectively. All potentials given

here were versus the KCl-saturated Ag/AgCl electrode. Amperometric measurements were carried out by holding the working potential at 450 mV and the anodic limiting current of ferrocyanide-oxidation at UMEA could be obtained within 15 s.

The biological activities of the immobilized cells were determined with equation 1:

$$Ratio = \frac{i_m}{i_0} \qquad (Eq.1)$$

where i_m meant that the limiting current was from respiration of immobilized cells; the value of i_0 was blank control that meant limiting current was gotten from the empty polymer matrix.

The respiratory inhibition of microorganism was the biological response to toxicants in this work, which could be evaluated by the following equation (2):

$$Inhibition\% = \frac{i_b - i_t}{i_b} \times 100\% \qquad (Eq.2)$$

where the degree of the respiratory inhibition (inhibition%) was the percentage of inhibition after 1 h incubation with 45 mM ferricyanide, GGA^{220} and toxin; i_b meant that the limiting current was from response of unaffected cells to control substrate; i_t was the electrochemical response of injury cells to toxicants. The liquid substrate without toxicants was used as the control.

Determining toxicities of lab wastewater samples with electrochemical method and luminescent bacterial method.

A set of wastewater collected from chemical lab of Jinzhou Medical University from April 13, 2015 to May 26, 2015. Standard luminescent bacterial method (LUMIStox 300, Hach

Ltd.) and the ferricyanide-based toxicity sensor using the immobilized cells as the test organism were used to evaluate the toxicity of the samples. All samples were analyzed on duplicate by two methods mentioned above.

RESULTS AND DISCUSSION

The influences of polymer matrix on the biological activity of the immobilized cells

The immobilized cells with different polymer matrices were fabricated to investigate the influences of the polymer matrix on the apparent biological activity of the films. The films with immobilized *E. coli* exhibited a wafer shape, and the thickness and the diameter were 1 mm and 1 cm, respectively. The OD_{600} value of the *E. coli* used was 5.0. The immobilized cells were grouped based on the types of polymer matrix used and were subjected to the apparent biological activity test. The results were shown as Figure 1. The apparent biological activity of the corresponding immobilized cells in each group increased firstly and then decreased with the increase of the content of the polymer matrix. The highest apparent biological activities in each group were obtained when the contents of agar, gelatin, agar/gelatin, chitosan and PVA were 30 g L^{-1}, 150 g L^{-1}, 30 g L^{-1} -150 g L^{-1}, 5 g L^{-1} and 10 g L^{-1}, respectively. The nature of the polymer matrix is gel with a large amount of pores in it, and the higher content of the polymer solution used for gel formation but in the same thickness leads to the smaller pore size. At low content of polymer matrix, the pore size maybe so large that partial of cells embedded in the films can be washed away at the washing step, and thus present lower apparent biological activity even though the large pores can facilitate the mass transfer in the films. With increase in the content of polymer matrix, the *E. coli* could be well confined by the polymer matrix and thus the enhanced apparent biological activity to be observed. However, further increase in the content of the polymer matrix could, of course, result in smaller pores and retard the mass transfer of substrates

in the films, and thus the decrease in the apparent biological activity occurred. Moreover, the immobilized *E. coli* with 10 g L^{-1} PVA as matrix exhibited the highest apparent biological activity among all test immobilized methods.

The influences of the thickness of films with immobilized cells on the apparent biological activity

The homemade device of height-adjustable scraper related to our present skill could adjust the minimum height of scraper in 1 mm, and thereby the thicknesses of the films with the optimized polymer matrix contents to be controlled from 1 to 3 mm. Figure 2 showed the thickness-dependent apparent biological activity changes. It was obvious that all the immobilized cells exhibited the decrease in the apparent biological activity with the increase in the film thickness. It is reasonable since the thicker film leads to the higher barrier for immobilized cells to exchange the substrate with solution around it. Obviously, 1 mm was a suitable choice of "active thickness" for the further tests.

The effects of E. coli concentration on the apparent biological activity

The immobilized *E. coli* with different concentrations were fabricated at the optimized contents of polymer matrices and grouped according to the types of the matrix used. The thicknesses of the films all were in 1 mm. Figure 3 exhibited the plots of the apparent biological activity versus the concentrations of *E. coli* immobilized. With the increase in the concentration of *E. coli*, the apparent biological activity of the immobilized cells in the same group increased firstly due to the increased amount of viable *E. coli*, and then decreased probably due to the negative effect of high concentrated *E. coli* on the mass transfer within the films. The highest apparent biological activities were estimated to be 9.93, 12.86, 14.24, 7.56 and 16.86 at the OD_{600} value of 10.0 in the case of agar, gelatin, agar/gelatin, chitosan and PVA used as polymer matrix, respectively. Therefore, the *E. coli* with the OD_{600} value of 10.0 was embedded in the

films for the following experiments.

The long-term storage stability of the immobilized cells

The films fabricated with the optimized conditions, were stored in the glass-surface vessel at 4 ºC to investigate the long-term storage ability of the immobilized cells, which is very important to fulfill the practical application of the microbial sensor. Figure4 showed the apparent biological activity decay of the immobilized cells upon storage. The immobilized cells with the agar/gelatin as polymer matrix showed the longest storage time and the apparent biological activity could maintain 10% of its initial value after 70-day storage. Conversely, the immobilized cells based chitosan as matrix only sustained 20 days, and the residual activity was 2.23. The films with PVA and gelatin as polymer matrix showed 1.47 and 1.61 residual activities of the immobilized cells in 60 days, respectively.

In order to study the potentialities of immobilized cells with different matrices for online monitoring, we attempted to examine their stabilities in pH 7.0 PBS solution. Comparing with other immobilized cells, the film fabricated with PVA showed the longest storage time and the suitable residual activity after 40-day storage when the 80% rupture occurred in the PBS solution (shown in table 1). In fact, PVA has been studied quite extensively as a biological material for cell immobilization. It is thought that the hydrophilic hydroxyl groups of boric acid partly react with PVA, and then formation of some large pores and stable structure increases internal mass transfer and material stability. Therefore, PVA-based films could sustain the suitable residual activity of immobilized cells after long time storage in the solution. For the immobilized cells fabricated by agar, gelatin and agar/gelatin mixture, the lifetimes were 15 days, 30 days and 35 days when the 80% rupture occurred in the PBS solution. The chitosan-based film showed the

shortest lifetime just in 10 days comparing with other polymer-based film.

Screening the most sensitive matrix of immobilized cells to the toxic tests

The immobilized cells with the optimized conditions were employed in the ferricyanide-based toxicity sensors to evaluate the feasibility of their use as test organisms for sensing toxicity. The toxicity responses of the immobilized cells to the commonly used model toxicant, DCP, were recorded and shown in Figure 5. The immobilized cells based on all polymer matrices exhibited obvious toxicity responses to DCP, and the ratio of respiratory inhibition calculated with Eq.2 was increase with the increase in DCP concentration, which was identical with that obtained with free microorganism[7,8,9,29]. The values of 50% inhibition concentration (IC_{50}) obtained with immobilized cells based on matrices of agar, gelatin, agar/gelatin, chitosan and PVA were 19.21 mg L^{-1}, 19.86 mg L^{-1}, 17.76 mg L^{-1}, 15.63 mg L^{-1} and 9.62 mg L^{-1}, respectively, indicating that PVA as polymer matrix for *E. coli* immobilization was more sensitive to DCP.

Table 2 showed the comparison of the IC_{50} values and EC_{50} values for DCP obtained with different toxicity sensors. The IC_{50} value obtained with immobilized cells using PVA as polymer matrix was comparable with those obtained with free *E. coli* and lux maked *E. coli*, but was an order of magnitude smaller than those obtained with *Preucomonas putida* and *Vibrio fischeri*, confirming the feasibility of the immobilized cells as test organism in toxicity biosensors.

Obviously, the sensitivity of the toxicity sensors is affected by determining method and test organism. Otherwise, it is entirely possible to enforce the influences on the sensitivity by changing storage time. The relevant studies of the bacterial response to the toxicity throughout the lifetime of the immobilized cells will be investigated in our further work. Based on our

present investigations, the polymer of PVA should be chosen as an optimum matrix to immobilize *E. coli* cells. Then a technique of scanning electron microscope (SEM) was used to study the changes of cell morphology after immobilized cells suffering different storage times at 4 ºC refrigerator (as shown in Figure 6). The results of SEM revealed that immobilized cells kept relatively intact morphologies in the first ten days storage at 4 ºC. But the cells were shriveling even to be found walls scattering when the storage times were increasing from 30 days to 50 days. These images well explained the apparent bioactive losses of immobilized cells depending on the increase of storage time.

Sensing toxicities of the wastewater samples

The ferricyanide-based toxicity biosensor was further challenged by a set of wastewater collected from the chemical laboratory of Jinzhou Medical University, where the cells immobilized with PVA as polymer matrix was used as the test organism. As a comparison, all of the wastewater samples were tested by standard toxicity sensor based on luminescent bacterial (shown in EC values). As shown in Figure 7a, both toxicity sensors could exhibit toxicity response to the wastewater samples, and the obtained EC values were in the range of 7.96 and 25.42% and the IC values in the range of 6.32–13.75%. The evident linear correlation between the IC values and the EC values was shown in Figure 8b, indicating the promise of the ferricyanide-based toxicity biosensors using the immobilized cells as test organism in practical applications.

CONCLUSIONS

In this work, the immobilized cells have been successfully and simply fabricated with the polymer matrix and the *E. coli*. The polymer matrices used here were gar, gelatin, agar/gelatin, chitosan and PVA, and the optimized contents for all films of immobilized cells were 30 g L^{-1},

150 g L^{-1}, 30/150 g L^{-1}, 5 g L^{-1} and 10 g L^{-1} respectively. To get high apparent biological activity, the OD_{600} value of the *E. coli* immobilized was optimized to be 10.0 and the thickness of the films to be 1 mm. The immobilized cells with PVA matrix could be stored over 40 days in pH 7.0 PBS solution and exhibit the highest sensitivity to DCP, indicating the PVA was the best polymer matrix among all test polymers. The IC_{50} value of the immobilized cells with PVA matrix to DCP was 9.62 mg L^{-1}, which was comparable with that obtained with free *E. coli* and the lux marked *E. coli*, suggesting the feasibility of the use of immobilized cells instead of free microorganisms as test organism. Then the film fabricated with *E. coli* and PVA matrix was used for sensing the toxicity of the wastewater of the chemical laboratory, and the test result was linear correlation with that from the standard bioluminescent method, further confirming the promise of the ferricyanide-based toxicity biosensors using immobilized cells as test organism in practical applications.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (liuchang@jzmu.edu.cn).

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Figure 1 The influences of polymer matrix on the apparent biological activity of the immobilized cells grouped based on the type of polymer matrix. Each result shown in the figure was the average of three samples.

Figure 2. The thickness influence of each film on the apparent biological activity of immobilized cells. Each result shown in the figure was the average of three samples.

Figure 3. The relationship between the apparent biological activity and the concentration of *E. coli* embedded. Each result shown in the figure was the average of three samples.

Figure 4. The bioactive changes of immobilized cells depending on the changes of storage time:

(a) 30 g L^{-1} of agar; (b) 150 g L^{-1} of gelatin; (c) 30/150 g L^{-1} of agar/gelatin mixture; (d) 5 g L^{-1} of chitosan; (e) 10 g L^{-1} of PVA. The different films with the same biomasses of *E. coli* cells in 10.0 at OD_{600} were divided into the wafer with size of 1.0 mm thickness and 1.0 cm diameter. Each result was the average of three samples.

Figure 5. The inhibitory curves of DCP on the *E. coli* cells immobilized by matrices of 30 g L^{-1} of agar (a); 150 g L^{-1} of gelatin (b); 30/150 g L^{-1} of agar/gelatin mixture (c); 5 g L^{-1} of chitosan (d) and 10 g L^{-1} of PVA (e). The different polymer matrices with the same biomasses of *E. coli* cells in 10.0 at OD_{600} were divided into the wafer shape with size of 1.0 mm thickness and 1.0 cm diameter. Each result was the average of three samples.

Figure 6. The images of SEM for PVA-base film at 4 ºC storage in original immobilized day (a); in 10 days (b); in 30 days (c) and in 50 days (d) (10 000×).

Figure 7 Comparing the toxicities of real wastewater samples in 10 groups determined by electrochemical method based on immobilized cells with PVA matrix and the standard luminescent bacterial method (a); Scatterplot of electrochemical method equivalent values versus luminescent bacterial method values (b). The samples were collected from chemical lab wastewater in the data from April 13, 2015 to May 26, 2015. Each result was the average of two samples.

Table 1. The lifetimes of 80% rupture occurred and the residual bioactivities for immobilized cells with different polymer matrices.

Polymer	Original activities of immobilized cells (Ratio)	Time (days)	Activities of immobilized cells after 80% rupture occurring (Ratio)	Declining activities of immobilized cells after 80% rupture occurring (Ratio%)
Agar	9.93	15	1.31	86.8
Gelatin	12.86	30	1.26	90.2
Agar/gelatin	14.24	35	1.41	90.1
Chitosan	7.56	10	1.03	86.4
PVA	16.86	40	1.32	92.2

Table 2. Comparing IC_{50} or EC_{50} values of DCP toxicity with different determining methods and detected organisms.

Biological test	Lux marked *E. coli*[30]	Lux marked sunfish[30]	*Pseudomonas putida*[31]	*Vibrio fischeri*[32]	Suspensed *E. coli*[29]	*E. coli* immobilized by matrix of PVA*
IC_{50}/EC_{50} (mg L^{-1})	6.5–22.1	52	60	37.5	7.6	9.62*

* was from Fig. 5.

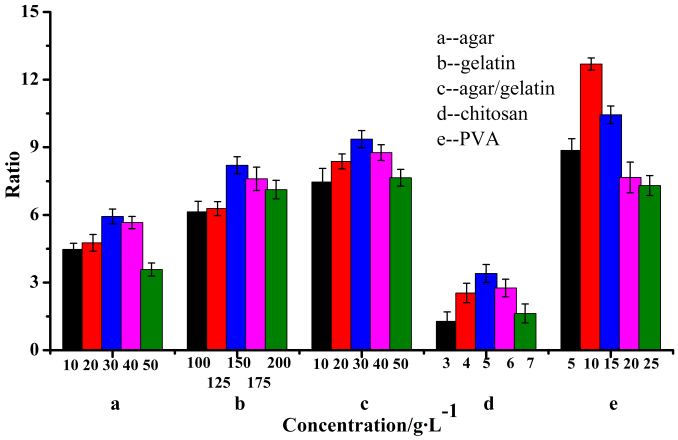


Fig. 1 The influences of polymer matrix on the apparent biological activity of the immobilized cells grouped based on the type of polymer matrix. Each result shown in the figure was the average of three samples.

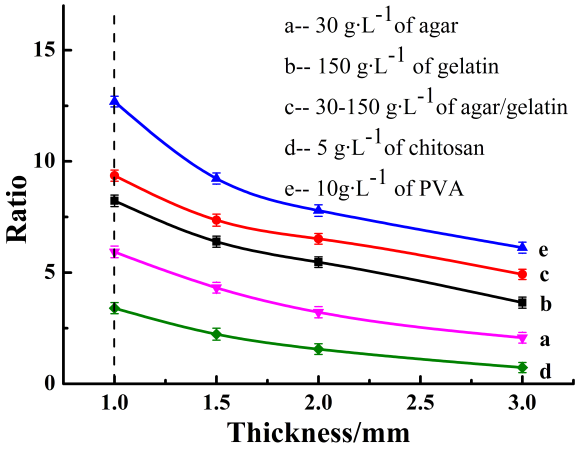


Fig. 2. The thickness influence of each film on the apparent biological activity of immobilized cells. Each result shown in the figure was the average of three samples.

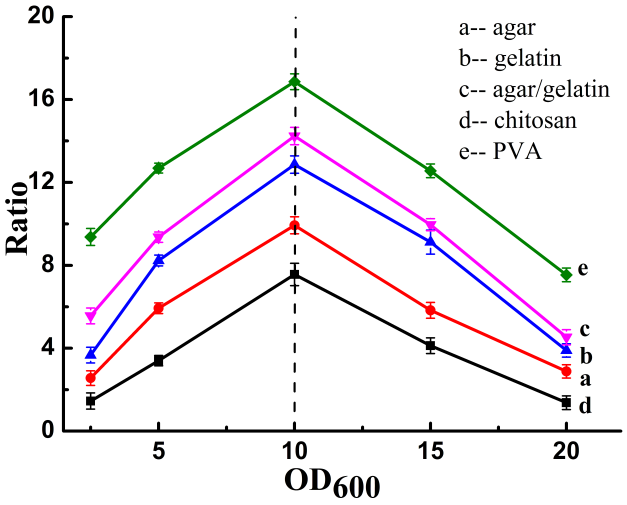


Fig. 3. The relationship between the apparent biological activity and the concentration of *E. coli* embedded. Each result shown in the figure was the average of three samples.

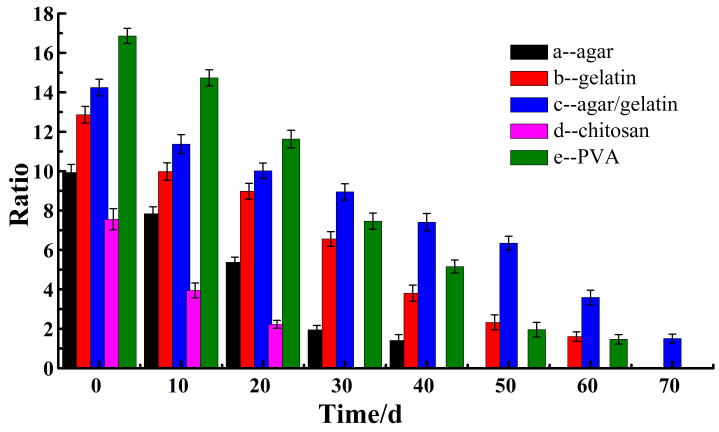


Fig. 4. The bioactive changes of immobilized cells depending on the changes of storage time: (a) 30 g L^{-1} of agar; (b) 150 g L^{-1} of gelatin; (c) 30/150 g L^{-1} of agar/gelatin mixture; (d) 5 g L^{-1} of chitosan; (e) 10 g L^{-1} of PVA. The different films with the same biomasses of *E. coli* cells in 10.0 at OD_{600} were divided into the wafer with size of 1.0 mm thickness and 1.0 cm diameter. Each result was the average of three samples.

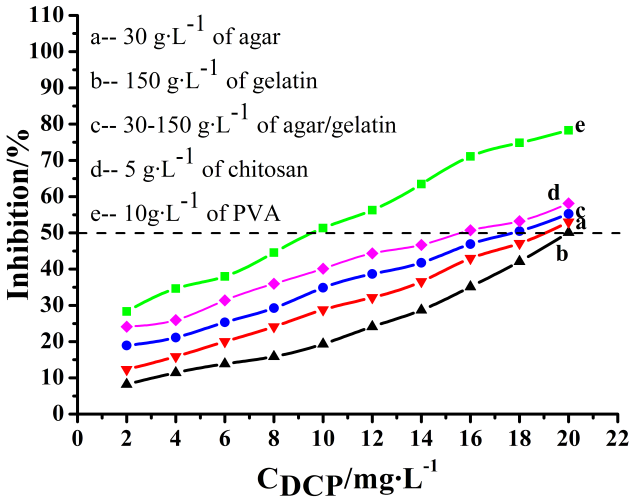


Fig. 5. The inhibitory curves of DCP on the *E. coli* cells immobilized by matrices of 30 g L^{-1} of agar (a); 150 g L^{-1} of gelatin (b); 30/150 g L^{-1} of agar/gelatin mixture (c); 5 g L^{-1} of chitosan (d) and 10 g L^{-1} of PVA (e). The different polymer matrices with the same biomasses of *E. coli* cells in 10.0 at OD_{600} were divided into the wafer shape with size of 1.0 mm thickness and 1.0 cm diameter. Each result was the average of three samples.

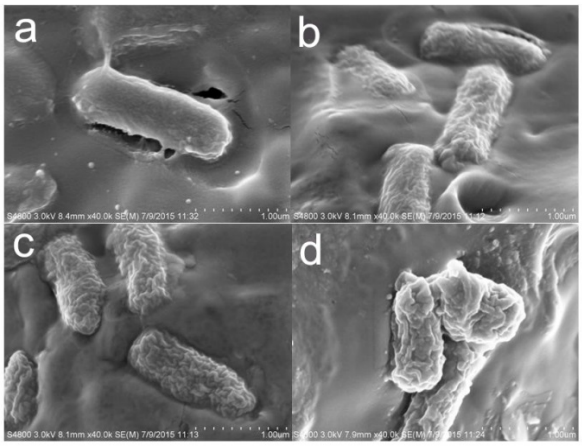
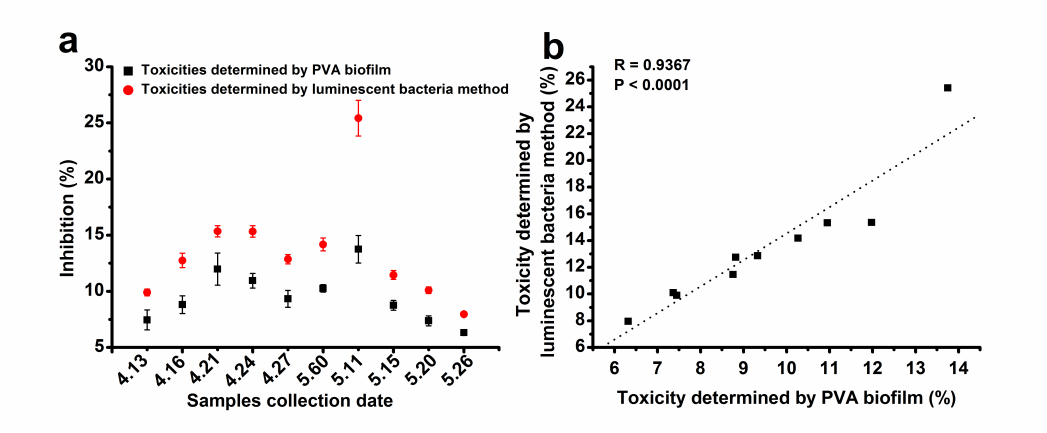


Fig. 6. The images of SEM for PVA-base film at 4 ºC storage in original immobilized day (a); in 10 days (b); in 30 days (c) and in 50 days (d) (10 000×).



把上面的图a的标注：Toxicities determined by PVA biolfilm改为Toxicities determined by immobilized *E. coli* with PVA matrix

Fig. 7 Comparing the toxicities of real wastewater samples in 10 groups determined by electrochemical method based on immobilized cells with PVA matrix and the standard luminescent bacterial method (a); Scatterplot of electrochemical method equivalent values versus luminescent bacterial method values (b). The samples were collected from chemical lab wastewater in the data from April 13, 2015 to May 26, 2015. Each result was the average of two samples.