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Silica nanotubes-doped alginate gel for yeast alcohol dehydrogenase immobilization

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Abstract

A novel alginate–silica nanotubes (ALG–SiNTs) composite was prepared through the incorporation of silica nanotubes (SiNTs) into the alginate (ALG) gel followed by Ca^{2+} cross-linking for encapsulating yeast alcohol dehydrogenase (YADH, EC 1.1.1.1) from *Saccharomyces cerevisiae*. Pre-adsorption of YADH onto the surface of SiNTs before encapsulating in alginate gel was adopted to circumvent the enzyme leakage. AFM and SEM characterization confirmed that YADH molecules were substantially adsorbed on the SiNTs. SEM and EDX studies showed that the SiNTs homogenously distributed in alginate matrix. The enzyme leakage from ALG–SiNTs–YADH composite was remarkably reduced about 50% compared to that of ALG–YADH composite. Meanwhile, the optimum reaction condition, catalytic activity and kinetic parameters of immobilized YADH in ALG–SiNTs composite were studied. The results showed that stronger affinity between substrates and enzyme, higher activity retention, improved storage and operational stability were achieved when YADH was immobilized in ALG–SiNTs composite instead of ALG–YADH composite.

Keywords: Alginate; Alcohol dehydrogenase; Silica nanotubes; Encapsulation; Adsorption

1. Introduction

A promising bio-pathway to convert greenhouse gas to methanol through three sequential enzymatic reaction catalyzed by co-immobilized formate dehydrogenase ($F_{ate}DH$), formaldehyde dehydrogenase ($F_{ald}DH$) and alcohol dehydrogenase (ADH) in silica gel has been presented in our previous study [1]. To deeply understand the whole process and increase the yield of methanol, it is necessary to study each separate enzymatic reaction. In this study, we will focus on the immobilization of yeast alcohol dehydrogenase for converting formaldehyde to methanol.

Polymer–silica hybrid composites have emerged as a novel class of materials, which may find promising applications for enzyme encapsulation with the potential use in biomedical, biocatalysis, bioseparation and biosensing areas [1–5]. Among those silica materials, nanosized silica materials such as nanosized silica xerogels and silica nanotube have been investigated and proved to be an ideal enzyme carrier due to high chemical and thermal stability, large surface area, and good compatibility with the environment [6]. Ding et al. have employed fine-structured silica nanotubes (SiNTs) as enzyme immobilization carrier to adsorb lysozyme. It was found that both immobilization efficiency and the bioactivity of immobilized lysozyme molecules were increased whereas desorption of enzyme would happen and thus cause the enzyme leakage in the long term run [7].

Therefore, incorporating SiNTs into polymer would be an effective approach to efficiently encapsulate enzymes, which could combine the adsorption of enzyme cooperating with the cage effect of the polymer and potentially increase the activity and stability of the immobilized enzyme.

The polymer used in present study is alginate (ALG) gel, which is one of the most widely used polysaccharides for enzyme immobilization owing to their superior biocompatibility, abundance in natural source from seaweed, low cost and easy preparation [8–12].

In this study, the alginate–silica nanotubes (ALG–SiNTs) hybrid composite prepared by the incorporation of silica nanotube into the alginate gel followed by Ca^{2+} cross-linking was

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used for encapsulating yeast alcohol dehydrogenase (YADH, EC 1.1.1.1) from *Saccharomyces cerevisiae*.

The enzyme leakage, enzyme activity retention, optimum temperature and pH, storage and recycling stability of YADH immobilized both in alginate gel (ALG) and ALG–SiNTs composite were investigated. The enzymatic activity of immobilized YADH for reduction of formaldehyde to alcohol with using nicotinamide adenine dinuncleotide (NADH) as coenzyme was evaluated by kinetic parameters.

2. Materials and methods

2.1. Materials

Yeast alcohol dehydrogenase (YADH, EC 1.1.1.1) and reduced nicotinamide adenine dinuncleotide (NADH, grade I, 98%) were purchased from Sigma, USA. Sodium alginate (average molecular weight 6.27×10^5) was purchased from Shanghai Tianlian, China. Silica nanotubes were kindly donated from Jilin University, China. Tris (hydroxymethyl amnomethane, 99.5%) was used to prepare the 0.05 M, pH 7.0 Tris–HCl buffer. All other chemicals were of analytical grade.

2.2. Immobilization of YADH in ALG gel beads

Sodium alginate was dissolved in deionized water at a final concentration of 2.5% (w/v). Four milliliters of the alginate solution was mixed with the enzyme stock solution prepared by dissolving 0.24 mg of YADH in 1.0 ml of, 0.05 M, pH 7.0 Tris–HCl buffer. This mixture formed was then added dropwise into the 20 ml, 0.2 M $CaCl_2$ solution through a 0.7 mm injection needle under constant stirring. The beads, thus formed rapidly, were cured in the gelation media for 30 min and then taken out, collected by filtration, rinsed with deionized water several times and stored in the deionized water. All procedures were carried out at room temperature.

2.3. Immobilization of YADH in ALG–SiNTs composite

Four milligrams of silica nanotubes, which were well dispersed by ultrasonic prior to use, was mixed with an enzyme stock solution prepared by dissolving 0.24 mg of YADH in 1.0 ml of 0.05 M, pH 7.0 Tris–HCl buffer and kept for 12 h to ensure that the adsorption equilibrium of YADH on SiNTs was achieved. Then this mixture solution was mixed with a 4 ml of 2.5% (w/v) alginate solution and the rest of procedure was as same as the procedure of immobilizing YADH in alginate gel beads.

2.4. Characterization

The adsorption of YADH on SiNTs was confirmed by both atomic force microscopy (AFM) and transmission electron microscope (TEM) (JEM-100 CXII, JEOL). The cross-sectional morphology of ALG gel and ALG–SiNTs composite were studied by using SEM. The existence of SiNTs in the alginate matrix was confirmed by means of energy dispersive X-ray spectrometer (EDX) (XL-30 ESEM, Philip).

2.5. Leakage of immobilized YADH

ALG–SiNTs composites and ALG gel beads containing YADH were firstly incubated in 20 ml, 0.2 M $CaCl_2$ aqueous solution for initial 4.5 h gelling period and then were transferred into a 20 ml of 0.05 M, pH 7.0 Tris–HCl buffer which was used as releasing medium for 20 h at 25 °C. The enzyme leakage during this procedure was determined by continuously measuring its absorbance at 280 nm using UV–vis spectrophotometer (U-2800, Hitachi). Meanwhile, the water loss during the bead formation was determined by the weight change of the beads.

2.6. Activity assays of immobilized YADH

The kinetic of YADH-catalyzed reduction of formaldehyde to methanol coupling with the oxidation of NADH to NAD^+ was studied (Eq. (1)).

$$CH_2O + NADH + H^+ \overset{YADH}{\rightleftharpoons} CH_3OH + NAD^+ \leq \quad (1)$$

The enzyme activity was determined spectrophotometrically by directly measuring the decrease in absorbance of NADH at 340 nm. The standard assay was carried out in 0.05 M, pH 7.0 Tris–HCl buffer containing different concentrations of HCHO and NADH at optimal pH and temperature. The kinetic parameters, maximum velocity of the reaction (V_{max}) and the Michaelis–Menten constant (K_m) were determined according to Dalziel's equation [13] as well.

To determine the optimum pH and temperature of immobilized YADH, the activity assays were carried out over temperature range of 20–50 °C in 0.05 M, pH 7.0 Tris–HCl buffer and pH range of 6.0–8.0 in 0.05 M Tris–HCl buffer at 25 °C. The highest activity of free YADH under its optimum condition was assigned to be 100% and the relative activities of immobilized YADH were defined as the ratio of its activity to the highest activity of free YADH.

The recycling and storage stability of ALG–SiNTs–YADH and ALG–YADH biocomposites were investigated, respectively. The enzyme activity retention of the immobilized YADH was determined as described in activity assays.

To evaluate the recycling stability of immobilized YADH, the biocomposite beads were collected after each reaction run and washed with 0.05 M, pH 7.0 Tris–HCl buffer to remove residual substrate and product within the beads. The beads were then reintroduced into fresh reaction media and enzyme activities were determined in a batch operation at optimum operating conditions.

The storage stability of immobilized YADH stored in the 0.05 M, pH 7.0 Tris–HCl buffer at 4 °C was measured in a batch operation mode at optimum conditions with the experimental conditions given above. Taking the initial activity of ALG–SiNTs–YADH biocomposite to be 100%, the relative activities of immobilized YADH was defined as the ratio of the activity to the initially activity.

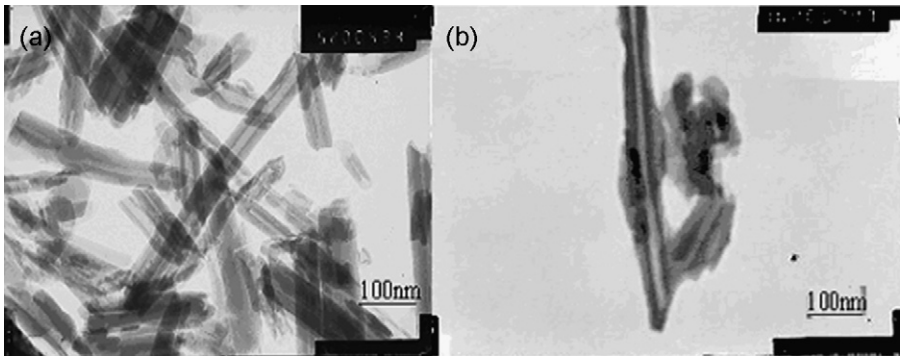

Fig. 1. TEM image of SiNTs before (a) and after (b) adsorption of YADH.

3. Results and discussion

3.1. Characterization

The immobilization of YADH on SiNTs was investigated by TEM and AFM. TEM images of SiNTs before and after incubating in enzyme stock solution for 12 h are shown in Fig. 1a and b. It can be clearly observed that some YADH molecules are indeed adsorbed on the SiNTs. Fig. 2a and b show the AFM images of SiNTs without and with adsorbed YADH molecules, respectively. Before the enzyme immobilization, the outer diameters of SiNTs are quite close throughout the whole length. After the enzyme immobilization, it is observed in Fig. 2b that the tubes were covered with certain amount of enzymes and the outer diameters became uneven. The TEM and AFM images confirmed that the YADH molecules have been substantially immobilized in SiNTs by adsorption.

The structure of ALG–SiNTs composite and the distribution of the SiNTs in the composite are observed by SEM equipped with EDX (Figs. 3 and 4). As shown in Fig. 3, many tublar structure materials are found to be implanted into the alginate matrix. EDX spectrum confirms the existence of element Si which arises from SiNTs doped in the alginate. In addition, the SEM image of the composite indicates the SiNTs homogeneously distribute in the alginate matrix and has not aggregated considerably.

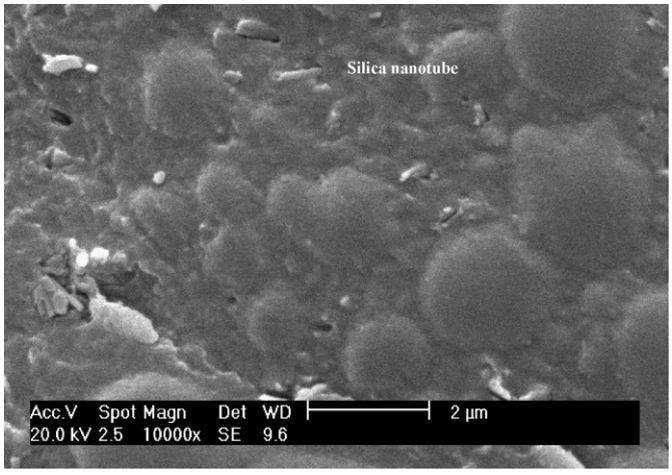

Fig. 3. SEM image of SiNTs–ALG composite.

3.2. Leakage of immobilized YADH

Enzyme leakage is always a big concern while using alginate gel as matrix for enzyme immobilization [8,14,15]. Herein, the leakage of YADH immobilized in ALG–SiNTs composites was measured during the initial gel formation period and subsequent storage period. The results of water loss and enzyme leakage of ALG–YADH and ALG–SiNTs–YADH biocomposite are listed in Table 1.

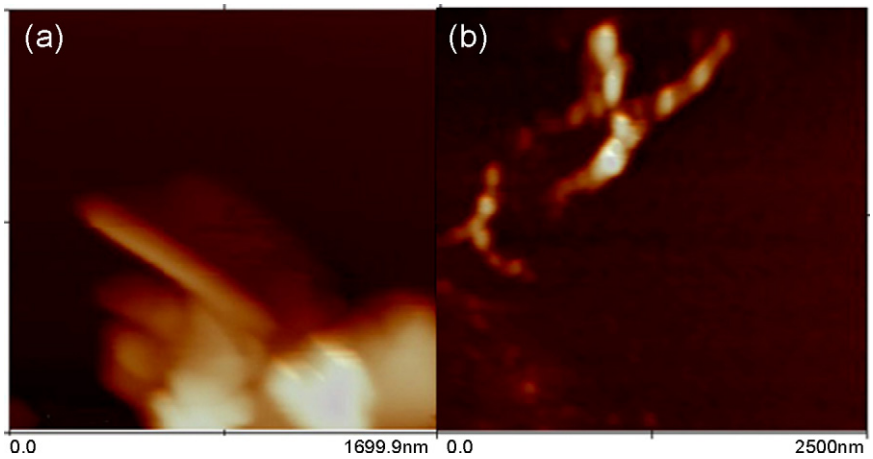

Fig. 2. AFM height images of the SiNTs before (a) and after (b) adsorption of YADH.

Table 1
Leakage of YADH in ALG gel and ALG–SiNTs composite

	Water loss (%)	YADH leakage during formation (%)	YADH leakage during storage (%)	Total YADH leakage (%)
ALG–YADH	59.3 ± 0.9	25.2 ± 1.8	6.4 ± 1.1	31.6 ± 3.9
ALG–SiNTs–YADH	52.6 ± 1.0	13.0 ± 1.6	3.2 ± 0.9	16.2 ± 2.5

As shown in Table 1, the YADH leakage in ALG gel mainly took place during the formation of alginate gel beads within initial 4.5 h. During this period, droplets were hardened to form gel beads due to the syneresis phenomenon: the carboxylate groups of guluronate monomers complexed with calcium cations and the water in the beads was expelled out. And the enzyme in the beads can be brought into the bulk solution and result in severe enzyme leakage [16].

The result also showed that after immobilized in ALG–SiNTs composites, the enzyme leakage was reduced from 25.2% to 13.0% during gel formation while the water loss decreased from 59.3% to 52.6%. After 20 h storage, 83.8% of the total immobilized YADH was retained in ALG–SiNTs composite while only 68.4% was retained in ALG gel. Total enzyme leakage in ALG–SiNTs–YADH biocomposite was remarkably reduced about 50% compared to that in ALG–YADH biocomposite. We assumed that SiNTs could effectively adsorb enzyme and hold water to prevent them from bringing enzyme out of the alginate matrix due to its hydrophilicity and high surface energy, which thus result in remarkably reduced enzyme leakage. In addition, this result was in agreement with the experimental result obtained in our previous study on BSA leakage in ALG–SiNTs composite [17].

3.3. Optimum enzymatic reaction condition of immobilized YADH

To determine the optimum condition of enzymatic reaction of immobilized YADH, the activity assays were carried out over pH range of 6.0–8.0 and temperature range of 20–50 °C. The profile of relative activity of immobilized YADH at different temperatures and pH values is shown in Figs. 5 and 6, respectively. Both the highest relative enzymatic activity of YADH immobilized in ALG–SiNTs gel and ALG gel was obtained at pH 7.0, 25 °C, which was in consistent with that of the free YADH [18]. Under these conditions, the relative activity of ALG–SiNTs–YADH composite was up to 61.3%, while the relative activity of ALG–YADH was only 51.6%.

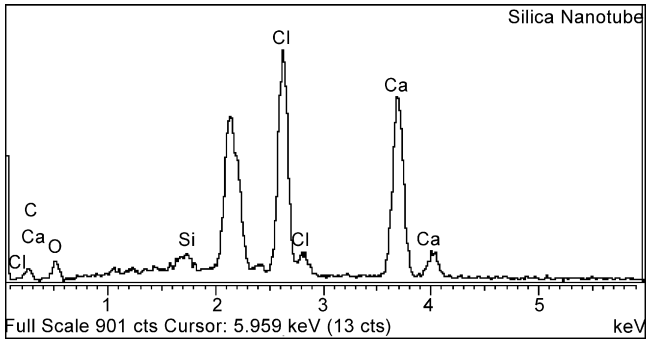

Fig. 4. EDX spectrum of SiNTs–ALG composite.

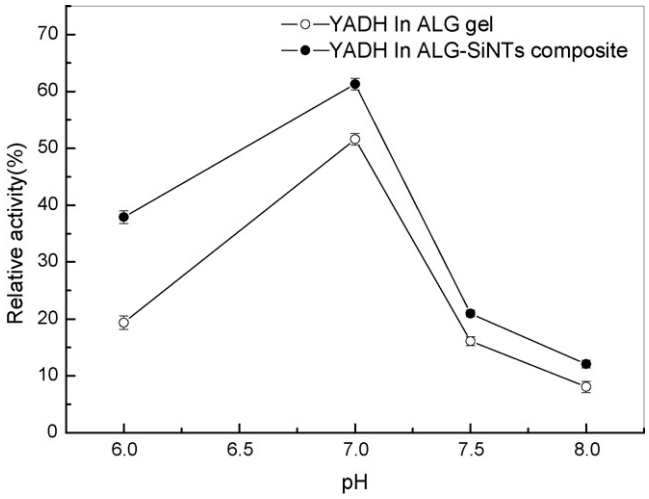

Fig. 5. Effect of Temperature on the activity of immobilized YADH.

3.4. Kinetic studies of enzymatic reaction for immobilized YADH

Kinetic studies of reduction of formaldehyde to methanol of free and immobilized YADH were investigated at 25 °C, pH 7.0 while varying the initial concentration of formaldehyde and NADH. Kinetic parameters, K_m and V_{max}, for free and immobilized YADH were calculated according to Dalziel's equation [13] and values are presented in Table 2.

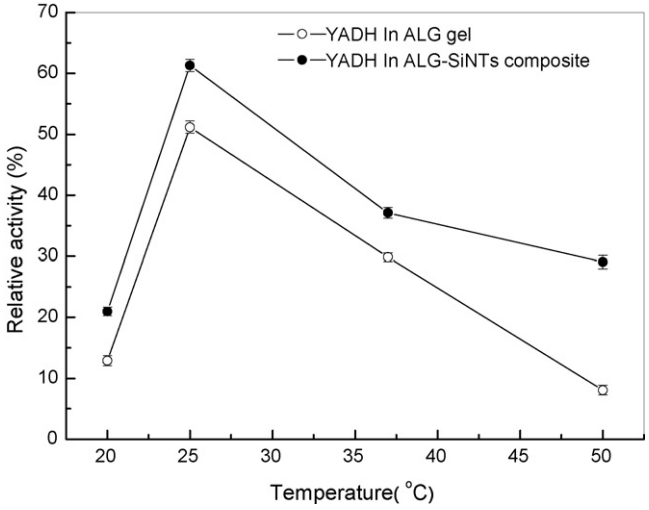

Fig. 6. Effect of pH on the activity of immobilized YADH.

Table 2
Kinetic parameters of free and immobilized YADH

	V_m ($\mu M\,S^{-1}$)	K_{NADH} (μM)	K_{HCHO} (mM)
Free YADH	0.71 ± 0.03	28.70 ± 0.15	10.15 ± 0.23
ALG–SiNTs–YADH	0.53 ± 0.03	57.54 ± 0.12	11.13 ± 0.18
ALG–YADH	0.47 ± 0.04	66.70 ± 0.18	16.69 ± 0.17

As was expected, the reaction with free YADH had the highest V_{max} and lowest K_m. Furthermore, the result showed that the kinetic parameter for YADH immobilized in ALG–SiNTs composite was much close to that for free enzyme compared to ALG gel. As shown in Table 2, the V_{max} value for YADH immobilized in ALG–SiNTs composite was higher that for ALG gel. From the result of previous leakage study, it can be concluded that immobilization of YADH in ALG–SiNTs composite could provide good preservation of enzyme with high loading efficiency which would directly result in the increase of immobilized enzyme activity.

The K_{NADH} and K_{HCHO} value for YADH immobilized in ALG–SiNTs composite was found to be much lower than that for ALG gel, which suggests YADH immobilized in composite had higher affinity towards the substrate.

Usually V_{max} and K_m of the enzyme immobilized might be affected by some factors including the steric hindrance of the active site by the matrix, the enzyme flexibility necessary for substrate binding, and importantly, diffusion limitation of substrates and products.

In our previous study [19], diffusion characteristic of NADH in ALG gel and ALG–SiNTs composite have been investigated. The results showed that the maximum value of effective diffusion coefficient (D_e) of NADH in ALG–SiNTs composite was up to $2.54 \pm 0.2 \times 10^{-10}\,m^2\,s^{-1}$, close to that in bulk solution ($3.30 \pm 0.2 \times 10^{-10}\,m^2\,s^{-1}$) [20] and remarkably larger than that in ALG gel ($1.89 \pm 0.2 \times 10^{-10}\,m^2\,s^{-1}$). It suggests that ALG–SiNTs composite facilitates the rapid diffusion of NADH and its facile access to immobilized YADH during the enzymatic reaction. Moreover, Wratten's product inhibition studies showed that the kinetic mechanism of ADH was ordered with coenzyme binding first [21]. Therefore, the increase of D_e of NADH might make contribution to the increase of V_{max} as well.

3.5. Storage and recycling stability of the immobilized YADH

The ALG–SiNTs–YADH biocomposites and ALG–YADH biocomposites were stored at 4 °C to investigate the effect of storage on the enzyme activity and the result is shown in Fig. 7, from which it could be found that YADH immobilized in ALG–SiNTs composite maintained significant activity during 1-month storage. During the first 7-day storage, there was only a 14.2% drop in activity. Thereafter, the activity declined more slowly and a total of 31.6% loss of in 1 month, equivalent to an approximate loss of 1% per day. Under the same storage period, the relative activity loss of YADH immobilized in control ALG gel amounted to about 84.2% after 1-month storage.

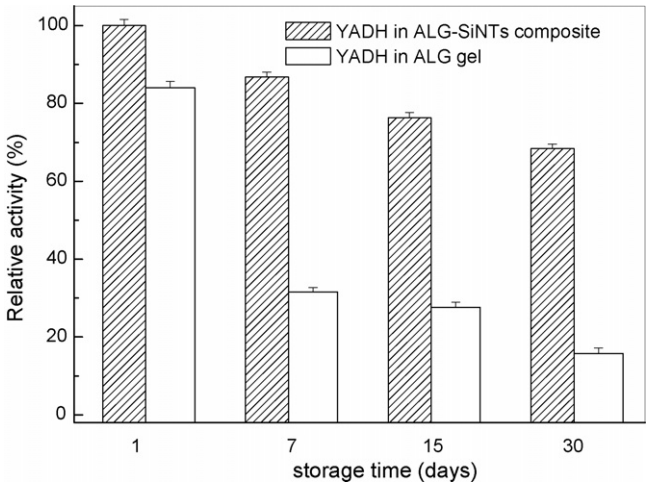

Fig. 7. Storage stability of immobilized YADH in ALG–SiNTs composite and ALG gel.

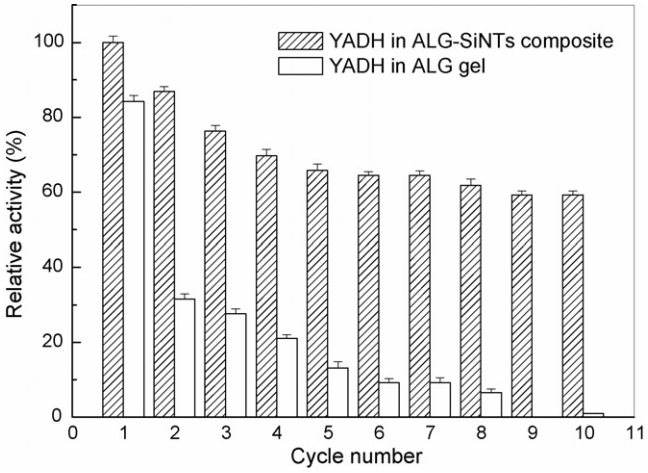

Fig. 8. Recycling stability of immobilized YADH in ALG–SiNTs composite and ALG gel.

The effect of recycling on the enzymatic activity of immobilized YADH was investigated as well. Fig. 8 shows the relative activity as a function of recycling times. As shown in Fig. 8, after 10 cycles, relative activity of YADH immobilized in ALG gel decreased almost to zero, while 60% of relative activity of YADH immobilized in ALG–SiNTs composite.

Compared to ALG–YADH biocomposite, ALG–SiNTs–YADH biocomposite showed higher initial enzyme activity retention and significantly improved enzyme storage and recycling stability.

4. Conclusions

The immobilized of YADH in a novel ALG–SiNTs composite was realized by pre-adsorbing enzyme on the hydrophilic SiNTs and then encapsulating them in the alginate matrix, followed by Ca^{2+} cross-linking. The enzyme leakage from ALG–SiNTs–YADH biocomposite decreased remarkably compared with that from ALG–YADH bicomposite. Incorporation of silica nanotube into alginate matrix facilitated the diffusion

of substrates and increased the affinity between enzyme and substrates, which lead to higher initial enzyme activity retention and significantly improved storage and recycling stability. Due to these advantages, we can expect that this composite can be used as an effective carrier for enzyme or cell immobilization and will find wide utilization in biomedical and biocatalysis field.

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