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Encapsulation of lactate dehydrogenase in carbon nanotube doped alginate–chitosan capsules

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ABSTRACT

Alginate–chitosan shell–core (AC) capsules doped with carbon nanotubes (CNTs) were prepared for lactate dehydrogenase (LDH, EC 1.1.1.27) encapsulation to convert pyruvic acid to lactic acid coupling with the oxidation of NADH to NAD^+. LDH was entrapped within the liquid core of the capsules and the CNTs were incorporated in the alginate or chitosan matrices or both. The physical properties of the capsules and the immobilized LDH activity were investigated. The AC capsules doped with CNTs showed better mechanical strength than that without CNTs. The LDH loading efficiency of the AC capsules with CNTs (10 mg/mL) doped in both the shell and the core was 30.7% higher than that without CNTs. The optimal pH value for the bioconversion catalyzed by immobilized LDH was 7.0, lower than that by free LDH (7.5). The optimal temperature was 35 °C for both immobilized and free LDH. Operational stability of the immobilized LDH was greatly improved by doping CNTs in AC capsules. The results showed that this method was efficient for enzyme encapsulation in the biotechnology applications.

1. Introduction

Enzyme immobilization has attracted great interest in biotechnology processes for its high operational stability and durability, easy separation from products and low cost of industrial applications. Among the various techniques of enzyme immobilization, microencapsulation has received increasing attention both in fundamental academic research and industrial applications [1]. Through microencapsulation (a mild process), the advanced structure of the enzyme entrapped in a semi-permeable support material will nearly not change and maintain a higher activity recovery. The support material prevents the enzyme from leaving while allowing substrates, products and co-factors to pass through [2]. Therefore, the selection of the support materials and the designation of the capsule are very important in enzyme immobilization.

Macromolecular materials such as alginate and chitosan, naturally occurring biopolymers, have been largely employed to make capsules. Alginate, composed of α-L-guluronic acid and β-D-mannuronic acid residues, is a typical anionic polymer with carboxyl groups in the molecular chain and can cross-link with cations to form gels [3,4]. Chitosan is a cationic polymer obtained by *N*-deacetylation of chitin, which is the second most abundant naturally occurring polymer [5,6]. Investigation on alginate–chitosan (AC) capsules has been reported in enzyme and microorganism immobilization and they can efficiently prevent the encapsulated enzyme from leaking out compared with only alginate or chitosan beads [7–12]. For example, Taqieddin and Amiji prepared AC core–shell capsules for β-galactosidase immobilization with solid and liquid core, respectively [13]. Won et al. entrapped lipase in Ca-alginate gel beads coated with chitosan and studied its activity and stability [14]. In addition, the alginate–chitosan polyelectrolyte complex can improve the structure of the alginate or chitosan beads [15]. However, enzyme leakage is still a critical problem and the poor mechanical strength of AC capsules also limit their applications in enzyme immobilization.

Carbon nanotubes (CNTs) doped in alginate or chitosan matrices have been reported to be able to reduce the enzyme leakage and improve the mechanical strength of the support for their unique large surface area, and mechanical and thermal properties [16–22]. Jiang et al. incorporated multiwalled CNTs (MWCNTs) into alginate gel to prepare hybrid composites for bovine serum albumin immobilization [23]. Protein leaking from the alginate–MWCNT composites was found much lower than that from the Ca-alginate beads. Moreover, enzyme–polymer–single walled CNTs (SWCNTs) composites were reported to be superior to enzyme–polymer–graphite composites [24]. However, CNTs used as the dopants in preparing AC capsules for enzyme immobilization has not been reported.

In this study, the AC capsules with chitosan as the core and alginate matrices cross-linked with Ca^{2+} as the shell were prepared as the basic and comparative support. Moreover, capsules with CNTs doped in the core, shell or both were prepared and compared in this

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work. Lactate dehydrogenase (LDH, EC 1.1.1.27) from rabbit muscle was employed as the model enzyme. The enzyme activity of immobilized LDH was measured using sodium pyruvate as substrate and nicotinamide adenine dinucleotide (NADH) as coenzyme. The influences of the CNTs addition on the characteristic of the capsules, enzyme loading efficiency, enzyme activity and the operational and storage stabilities of the immobilized LDH were investigated in this study. In addition, the optimal pH, temperature and enzyme kinetic parameters of immobilized LDH were also detected.

2. Materials and methods

2.1. Materials

LDH (EC 1.1.1.27) from rabbit muscle, NADH (reagent grade, >97%) and sodium pyruvate were purchased from Sigma, USA. MWCNTs (main range diameter <10 nm, length 1–2 μm, purity of MWCNs >95%, specific surface area 40–300 m^2/g) were purchased from Shenzhen Nanotech Port, China. Chitosan (86.5% degree of deacetylation, average molecular weight 30,000) was purchased from Weifang Kehai Chitin Corporation (Weifang, China). Sodium alginate (average molecular weight 2.4×10^5) and Tris(hydroxymethyl)aminomethane (99.9%) were bought from Shanghai Shenggong, China. All other chemicals were of analytical grade.

2.2. Preparation of LDH–AC capsules

LDH–AC capsules were prepared in two steps. Chitosan was dissolved in 0.5% (v/v) acetic acid to get a 2% (w/v) solution. LDH stock solution was prepared by diluting 5 μL original enzyme with 1 mL 0.05 mol/L pH 7.0 Tris–HCl buffer and then it was mixed homogenously with 1 mL 2% chitosan solution. Then the mixture was added dropwise into 30 mL 1% sodium alginate solution through a 0.7 mm injection needle under constant stirring. The AC capsules formed rapidly followed by being incubated in alginate solution for 10 min, and then filtrated and rinsed with deionized water. The capsules were transferred into 20 mL 2% (w/v) $CaCl_2$ solution and hardened for 15 min and then filtrated and rinsed with deionized water until neutral. The capsules were stored at 4 °C and all the procedures were carried out at room temperature.

2.3. Preparation of LDH–CNT–AC capsules

For the preparation of LDH–CNT–AC capsules with CNTs doped in the alginate shell (CNT–AC I capsules), 250 mg CNTs were well dispersed in 50 mL 0.05 mol/L Tris–HCl buffer (pH 7.0) by ultrasonic before use. Fifty microliters LDH stock solution was mixed uniformly with 1 mL 2% chitosan solution. The mixture was then added dropwise into 15 mL 2% sodium alginate solution doped with 15 mL 2 mg/mL CNT dispersion through a 0.7 mm injection needle under constant stirring. The remaining procedures were the same as that of AC capsules.

For the preparation of LDH–CNT–AC capsules with CNTs doped in the chitosan core (CNT–AC II capsules), 0.5 mL 5 mg/mL CNT dispersion were mixed homogeneously with 50 μL LDH stock solution. After 12 h adsorption of LDH onto CNTs at 25 °C on a shaking incubator at 100 rpm, the mixture was incorporated into 0.5 mL 2% chitosan solution. Finally, they were added dropwise into 30 mL 1% sodium alginate solution through a 0.7 mm injection needle under constant stirring. The remaining procedures were the same as that of AC capsules.

For the preparation of LDH–CNT–AC capsules with CNTs doped both in the core and shell (CNT–AC III capsules), the shell was produced following the process of CNT–AC I capsules and the core following that of CNT–AC II capsules. The other preparation procedures were similar as those described above.

All the capsules prepared above were used to determine the kinetic parameters, optimal reaction conditions, operational and storage stabilities of the immobilized LDH.

2.4. Characterization

2.4.1. Scanning electron microscopy (SEM)

The surface and cross-sectional morphology of the freeze-dried capsules were analyzed by SEM (XL-30TEP ESEM, Phlilps, Holand). The freeze-dried samples were mounted on a slide with and sputter coated with gold to minimize surface charging. They were observed at a 20 kV accelerating voltage.

2.4.2. Mechanical strength determination

An Electronic Universal Testing Machine (CSS-44001, Changchun Research Institute for Testing Machines, China) was used to examine the mechanical strength of the capsules at room temperature. The compress rate was selected as 5 mm/min until the capsules broke and the compression intensity of the capsules was measured. Each sample was detected in five replications.

2.4.3. Loading efficiency of LDH in the capsules

A known amount of capsules were grinded and transferred into 0.05 mol/L pH 7.0 Tris–HCl buffer to release the protein thoroughly. The mixture was filtrated and the protein content in supernatant was determined by the Bradford method [25,26]. The loading efficiency of LDH was calculated by the following formula:

$$\text{Loading efficiency } (\%) = \frac{C_e V_e}{C_i V_i} \times 100 \quad (1)$$

where C_i and V_i are the initial protein concentration and the initial volume of LDH solution during the capsules preparation; C_e and V_e are the protein concentration and the volume of LDH solution entrapped in the capsules.

2.5. Determination of kinetic parameters of immobilized LDH

The activity of LDH was determined using sodium pyruvate (SP) as substrate and NADH as coenzyme. LDH catalyzed the reversible change of pyruvic acid into lactic acid coupling with the oxidation of NADH to NAD^+:

$$CH_3COCOOH + NADH + H^+ \overset{LDH}{\rightleftharpoons} CH_3CHOHCOOH + NAD^+ \quad (2)$$

The enzyme activity was determined spectrophotometrically by a decrease in absorbance at 340 nm resulting from the consumption of NADH. This reaction follows ordered sequential reaction mechanism and Dalziel's equation [27], which describes the kinetic behavior of LDH catalytic reaction:

$$v = \frac{V_{max}[A][B]}{K_m^A \cdot K_m^B + K_m^B[A] + K_m^A[B] + [A][B]} \quad (3)$$

where v is the apparent initial catalytic rate, V_{max} is the maximum reaction velocity, K_m^A and K_m^B are Michaelis–Menten parameters of substrates A (NADH) and B (SP). By keeping the concentration of one substrate constant and changing that of the other, two groups of data were obtained and plotted to gain Lineweaver–Burk double reciprocal profiles, respectively. The kinetic parameters of K_m^A, K_m^B and V_{max} were calculated from Eq. (3) and the graphs.

2.6. Optimization of the reaction conditions of immobilized LDH

To determine the optimum pH and temperature of the immobilized LDH, the activity of LDH was measured at the temperature

range of 25–50 °C in 0.05 mol/L Tris–HCl buffer (pH 7.0) and pH range of 6.0–8.5 at 35 °C, respectively. The highest activities under the optimum conditions were considered as 100% for both free and immobilized LDH, and the relative activities under other reaction conditions were defined as the proportion of the highest activity.

2.7. Determination of the operational and storage stabilities

Operational stability of the immobilized LDH was investigated and the relative enzyme activity of immobilized LDH was measured. In brief, the capsules were filtered after each reaction run and washed with 0.05 mol/L Tris–HCl buffer (pH 7.0) to remove substrate and product. The capsules were then reintroduced into fresh reaction media and the enzyme activity was measured as described above in a batch operation. The initial activities of the four immobilized LDH were considered as 100%, and the relative activity of each immobilized LDH was defined as the ratio of the activity to their initial activities, respectively.

Storage stability of the immobilized LDH was studied at 4 °C in a batch operation mode and the enzyme activity was determined.

3. Results and discussion

3.1. Characterization

The typical pictures of the capsules prepared in this study were shown in Fig. 1. Spherical capsules with an external diameter of about 3.5 mm were found for either doped with CNTs or not. There was no obvious difference between these four capsules in size. The introduction of CNTs into AC capsules was easily confirmed from the color of the capsules.

SEM micromorphs of the surface, sectional and inner structures of the capsules were shown in Fig. 2. Fig. 2a showed cutaway image of a freeze-dried capsule and the hollow capsule structure could be seen after the liquid core being dried. The surface micrograph of the freeze-dried capsule and a smooth and uniform shell with a network structure was seen in Fig. 2b. The inner image of the capsule was shown in Fig. 2c, and a denser and smoother layer inside with a network structure could be found comparing to the external surface. This was attributed to the diffusion of Ca^{2+} into the alginate matrices and the cross linkage of them. Therefore, with the indiffusion and consumption of Ca^{2+}, an inhomogeneous structure formed with the loose and porous outside. This porous structure could help the substrates and products to pass through. The difference of SEM micromorphs of the surface, sectional and inner structures between AC and CNT–AC capsules was not obvious under the lower powered microscope. However, some claviform materials with one end exposed and the other embedded in the matrices could be observed in the sectional micrograph of the CNT–AC capsules under the high powered microscope (Fig. 2d).

The results of mechanical strength measurements showed that the compression intensity of the CNT–AC capsules was much higher than that of AC capsules (Table 1). In addition, the compression intensity increased about 80.91% with the addition of CNTs increasing from 1 to 10 mg/mL for CNT–AC III capsules. This was mainly because of the enhancement of the interaction between CNTs and alginate matrices. It was obviously indicated that the mechanical strength of AC capsules was greatly improved after being doped

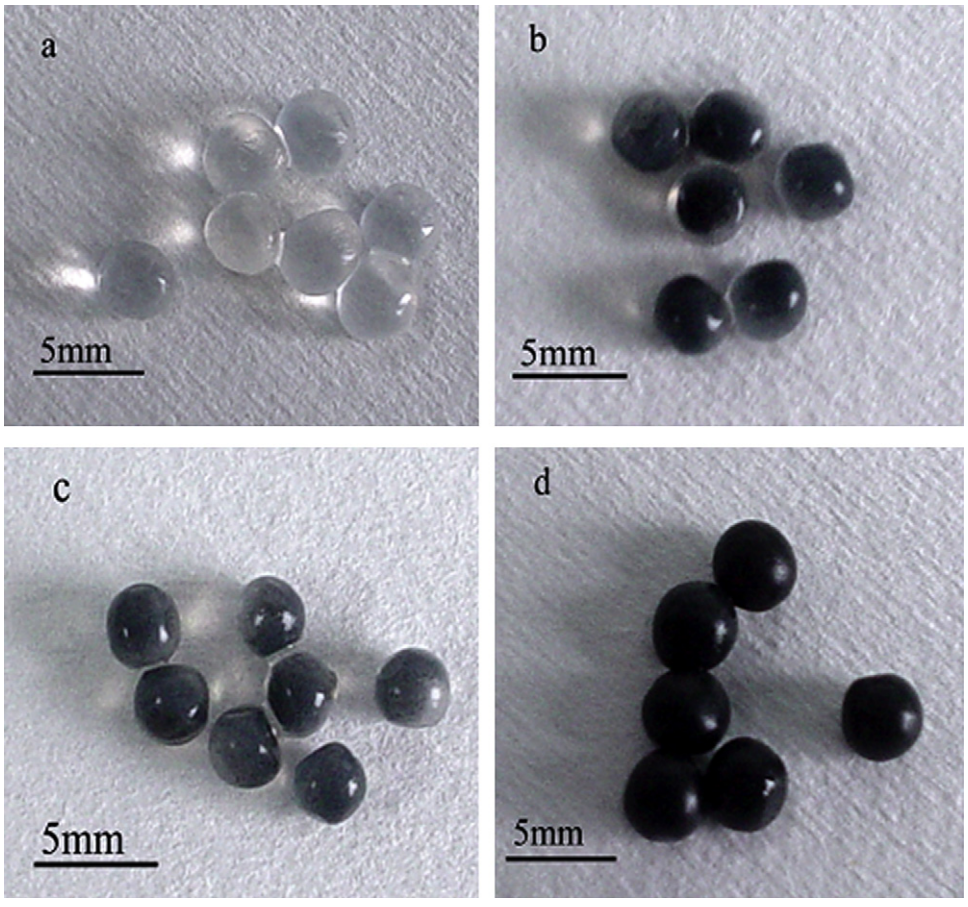

Fig. 1. Photographs of the actual capsules: (a) AC capsules; (b) CNT–AC II capsules; (c) CNT–AC I capsules; (d) CNT–AC III capsules (photos taken by camera of Nikon coolpix 5200).

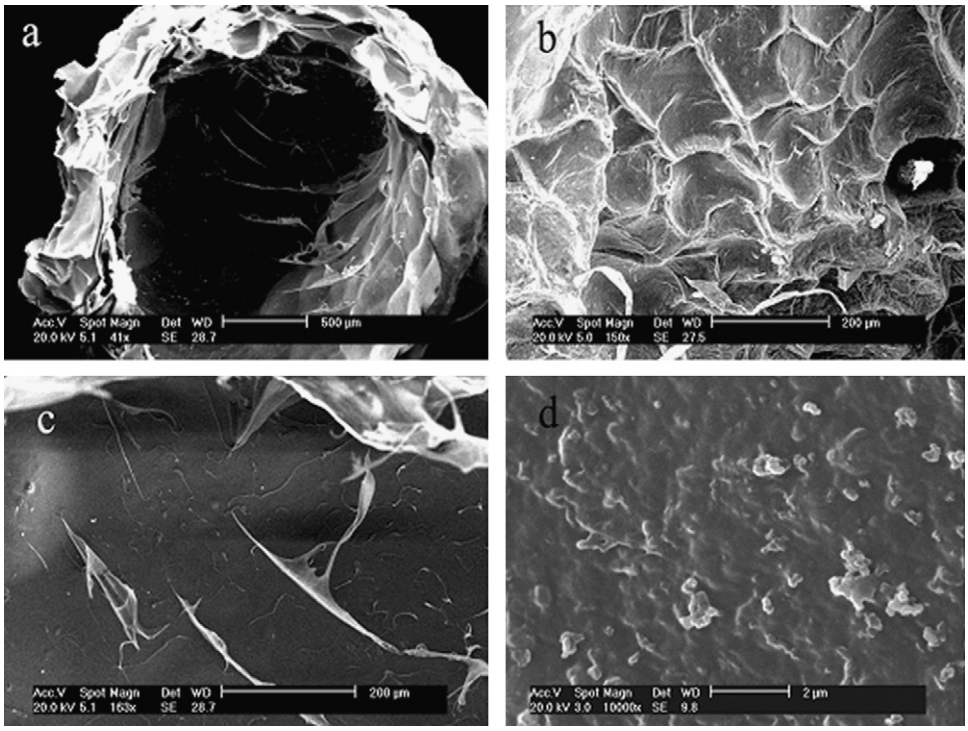

Fig. 2. SEM image of (a) a cutaway freeze-dried AC capsule; (b) the surface structure of the AC capsule; (c) the inner structure of the AC capsule; (d) the sectional structure of the CNT–AC III capsule.

Table 1
Compression intensity of the capsules under the same alginate, chitosan and $CaCl_2$ concentration and gelation time

CNT addition (mg/mL)	Compression intensity (*N*)			
	0	1	5	10
AC capsules	53.8	–	–	–
CNT–AC I capsules	–	68.6	85.3	117.2
CNT–AC II capsules	–	57.4	66.5	79.8
CNT–AC III capsules[a]	–	72.8	102.1	131.7

[a] For CNT–AC III capsules, CNT addition of 1, 5 and 10 mg/mL meant that CNTs were added in both shell and core of 1, 5 and 10 mg/mL, respectively.

with CNTs, which was in favor of continuous and repetitious use of immobilized enzyme.

The enzyme loading efficiencies of these four capsules were shown in Table 2. Under the same alginate, chitosan and $CaCl_2$ concentrations and capsule formation and hardening time, the loading efficiency had an obvious difference between these four capsules. The LDH loading efficiency of the CNT–AC III capsules with 10 mg/L CNTs addition was the highest and 30.7% higher than that of the AC capsules. Fewer enzyme molecules leaked out during the capsule formation stage because of the viscous properties of the droplet and the fast gelation reaction which was helpful for confining the enzyme molecules within the capsules [28]. The enzyme leakage mainly occurred during the capsule hardening stage. The combination of the carboxylate groups of guluronate monomers with Ca^{2+} reduced the space occupied by alginate and led to the shrinkage of the membrane as well as the inner size of the capsule. Therefore, a certain amount of water extruded out of the capsules carrying more or less enzyme molecules out. Due to the high hydration energy released on the surface of CNTs and their large specific surface area, more water molecules were adsorbed on their surface and thus more enzyme molecules were confined within the LDH–CNT–AC capsules [29]. In addition, the loading efficiencies of CNT–AC I and CNT–AC II capsules were between the values of AC capsules and CNT–AC III capsules, which also proved that CNTs played an important role in preventing enzyme molecules from leaking out. Obviously, more LDH leakage from the capsules would result in the slowing down of the catalytic reaction when the same amount enzyme was immobilized in different capsules. Moreover, it could be found that the loading efficiency increased with the addition of CNTs increasing.

Table 2
LDH loading efficiency of the capsules under the same alginate, chitosan and $CaCl_2$ concentration and gelation time

CNT addition (mg/mL)	LDH loading efficiency (%)			
	0	1	5	10
AC capsules	71.7	–	–	–
CNT–AC I capsules	–	80.3	84.4	88.2
CNT–AC II capsules	–	77.4	82.1	87.5
CNT–AC III capsules[a]	–	84.8	89.3	92.7

[a] For CNT–AC III capsules, CNT addition of 1, 5 and 10 mg/mL meant that CNTs were added in both shell and core of 1, 5 and 10 mg/mL respectively.

3.2. Optimization of the reaction conditions of immobilized LDH

The influences of pH and temperature of the reaction system on free and immobilized LDH were shown in Figs. 3 and 4, respectively. The relative activity was the highest at pH 7.5, 35 °C for free LDH and pH 7.0, 35 °C for immobilized LDH. The highest relative activities for both free and immobilized LDH were defined as 100%. The optimal pH and temperature for free LDH were consistent with the former reports [30]. In Fig. 3, the decrease of the optimal pH for immobilized LDH could be explained by the electrostatic attraction of OH^- by the polycationic polymer, chitosan [31], which led to the little increase of the pH in the enzyme microenvironment. Therefore, the pH value in the bulk solution for immobilized enzyme should be lower than that for free enzyme in order to obtain the optimal reac-

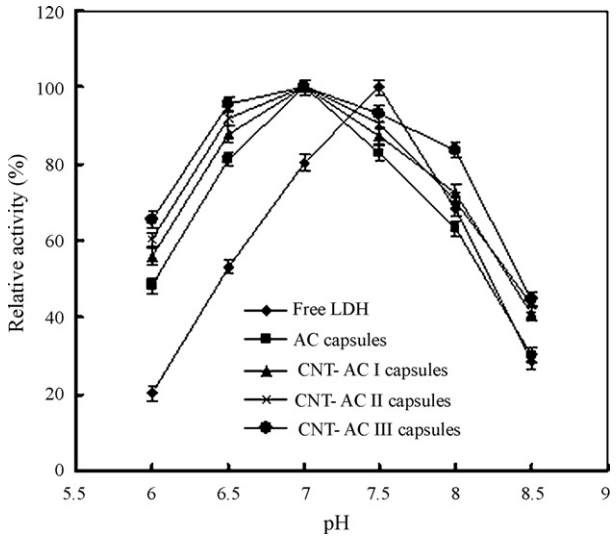

Fig. 3. Effect of pH on the activity of immobilized LDH (for CNT–AC III capsules, CNT addition of 5 mg/mL was used).

tion conditions for the immobilized LDH. It was reported that the shift of the optimum pH value depended on the enzyme reaction as well as the structure and the charge of the matrices. For the immobilized LDH in this study, shift toward the acid directions was found. In addition, as shown in Figs. 3 and 4, the immobilized LDH had a higher stability over a broad pH and temperature range than the free LDH, which could be explained by the protection and buffer effect of the microenvironment for the immobilized LDH. The unique thermal properties of CNTs might be the reason that resulted in the highest stability on pH and temperature of the reaction system for CNT–AC capsules.

3.3. Determination of kinetic parameters of immobilized LDH

Kinetic studies of free and immobilized LDH were carried out at pH 7.5, 35 °C and pH 7.0, 35 °C by varying the initial concentration of SP and NADH. Kinetic parameters, K_m^A, K_m^B and V_{max}, were calculated according to Dalziel's equation and the results were listed in Table 3. From the table, V_{max} value of the enzymatic reaction catalyzed by free LDH was 37.83 nM min^{-1}, which was higher than that

Table 3
Kinetic parameters of free and immobilized LDH

	V_m (nM min^{-1})	K_m^A (mM)	K_m^B (mM)
Free LDH	37.83	0.032	0.043
AC capsules	18.56	0.185	0.216
CNT–AC I capsules	22.67	0.106	0.154
CNT–AC II capsules	26.02	0.092	0.138
CNT–AC III capsules[a]	30.83	0.071	0.097

[a] For CNT–AC III capsules, CNT addition of 5 mg/mL was used.

of immobilized LDH; K_m^A and K_m^B values of free LDH were 0.032 and 0.043 mM, which were lower than that of immobilized LDH. This was the rational phenomenon because of the decrease of enzyme activity after enzyme immobilization for reasons such as diffusion limitation of substrate and product, steric hindrance of the active site by the carrier and so on. Therefore, the affinity of the immobilized enzyme towards the substrates decreased. However, for immobilized LDH, V_{max} value of LDH immobilized in CNT–AC III capsules was 30.83 nM min^{-1}, which was higher than that in AC capsules without CNTs doped, 18.56 nM min^{-1}; K_m^A and K_m^B values of LDH immobilized in CNT–AC III capsules were 0.071 and 0.097 mM, which were lower than that in AC capsules without CNTs doped, 0.185 and 0.216 mM. The results indicated that LDH had a higher affinity towards substrate after immobilizing in AC capsules after doped with CNTs both in the shell and core. This was consistent with the results of loading efficiency studies. Therefore, the relative activities of LDH immobilized in CNT–AC I and CNT–AC II capsules were somewhat lower than that in CNT–AC III capsules and higher than that in AC capsules without CNTs doped.

3.4. Storage and operational stabilities of the immobilized LDH

The storage and operational stabilities of immobilized enzyme are critical and basically concerned to evaluate the immobilization method or carrier selected. After investigating the storage and operational stabilities of immobilized LDH, the results were shown in Figs. 5 and 6, respectively. After 20 days storage, there was a very small change in the relative activity for the immobilized LDH, while that for free LDH was only 20% (Fig. 5). After 1 month storage, the relative activity of the immobilized LDH was about 80%, which indicated the slow decrease of the activity of LDH and the good storage stability after immobilization. In addition, the relative activity of LDH immobilized in AC capsules was a little lower than that in CNT–AC III capsules. This was attributed to the lower enzyme load-

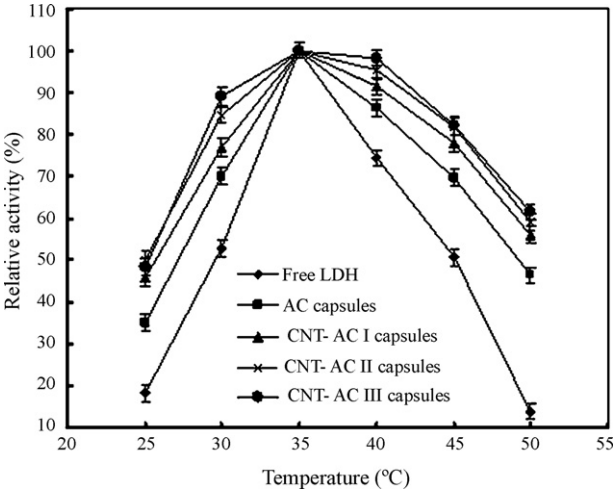

Fig. 4. Effect of temperature on the activity of immobilized LDH (for CNT–AC III capsules, CNT addition of 5 mg/mL was used).

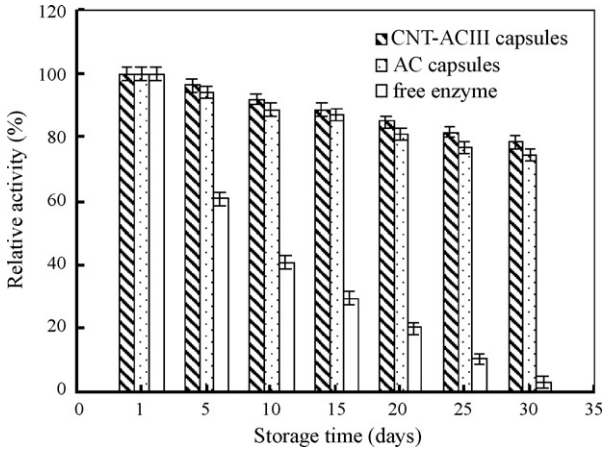

Fig. 5. Storage stabilities of free LDH and immobilized LDH in AC and CNT–AC III capsules (with CNT addition of 5 mg/mL).

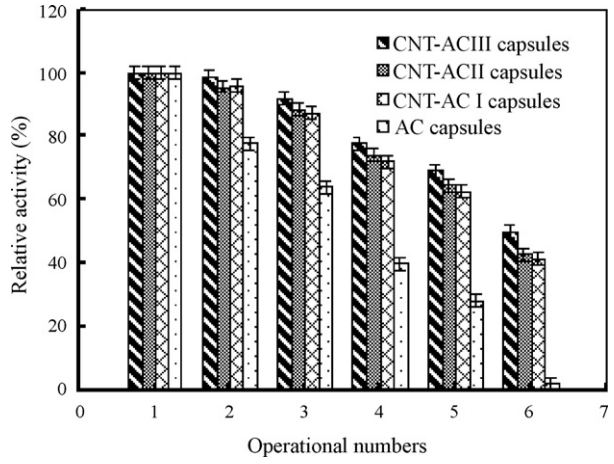

Fig. 6. Operational stabilities of immobilized LDH in AC and CNT–AC capsules (for CNT–AC III capsules, CNT addition of 5 mg/mL was used).

ing efficiency during the preparation process and higher enzyme leakage during the storage stage for AC capsules.

In Fig. 6, the enzyme relative activity as a function of reuse numbers of the immobilized LDH could be seen clearly and the difference between AC capsules and CNT–AC capsules was obvious. After three cycles, the enzyme activity was almost unchanged for the CNT–AC III capsules while AC capsules retained about 65% of the initial activity. About 70% and 30% of the initial activity lost after five cycles for AC capsules and CNT–AC III capsules, respectively. After six cycles, the relative activity of LDH immobilized in AC capsules was almost zero, while 50% of the initial activity still left for LDH immobilized in CNT–AC III capsules. This could be explained by the easy enzyme losing during the reuse process of the AC capsules compared with the CNT–AC III capsules. Moreover, it was identified that the mechanical strength of the capsules became weaker and some of them began to break during the filtration after four repeated cycles. This led to the enzyme leakage and thus the loss of the activity due to the loosened membrane. However, the dope of CNTs in the AC capsules could improve the mechanical strength somewhat from the former studies in this work and thus reduce the loss of the enzyme in a certain extent.

4. Conclusions

In summery, alginate–chitosan shell–core (AC) capsules doped with CNTs were proved to be an efficient biocompatible carrier for lactate dehydrogenase (LDH) encapsulation within the liquid core. By studying the physical properties of the capsules, better mechanical strength and higher enzyme loading efficiency were obtained by doping CNTs into the AC capsules. The optimal pH value for the bioconversion catalyzed by immobilized LDH was 7.0, lower than that by free LDH (7.5). The optimal temperature was 35 °C for both free and immobilized LDH. Storage stability of LDH was improved a lot after immobilization compared with the free LDH and the operational stabilities of the LDH immobilized in CNT–AC capsules were much higher than that in AC capsules without CNTs. This work illustrated an efficient method for LDH encapsulation and it was expected to be employed in other enzyme or microorganism immobilization.

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