Effects of modification of silica gel and ADH on enzyme activity for enzymatic conversion of CO_2 to methanol

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Abstract

CO_2 can be converted to MeOH through an enzymatic approach in three steps: (1) converting CO_2 to HCOOH by formate dehydrogenase (FateDH), (2) converting HCOOH to HCHO by formaldehyde dehydrogenase (FaldDH) and (3) converting HCHO to MeOH by alcohol dehydrogenase (ADH). Polyethylene glycol (PEG) was used to adjust the pore size of immobilization carrier silica gel and SC-PEG was used to improve the catalytic property of ADH. The catalytic properties of pegylated ADH were investigated using the conversion of HCHO to MeOH as model reaction. The results show that PEG modification of the silica matrix and/or the ADH significantly increases the enzymatic activities.
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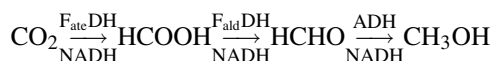*Keywords:* Carbon dioxide; Methanol; Dehydrogenase; PEG; Enzymatic conversion; ADH

1. Introduction

1.1. Bio-conversion of CO_2

In recent years, how to convert CO_2, the major man-made greenhouse gas, into useful chemicals and fuels, such as methanol, formate and formaldehyde, etc., has aroused global concern. Heterogeneous catalysis [1], electrocatalysis [2] and photocatalysis [3] are presently the three pre-dominant chemical methods for CO_2 utilization. These methods, however, suffer from the following inherent drawbacks: they need either high temperature and high pressure or additional electric or luminous energy, and the selectivity and yields are still very low. In comparison, biochemical conversion or bio-fixation of CO_2 employing whole cell or enzymes as catalysts has attracted a lot of attentions, due to its advantages such as high yield and selectivity at milder reaction conditions with no pollution [4–6]. For example, cyanobacteria have been used to produce H_2 by taking up CO_2 as carbon source and solar energy as energy source [7,8]. The enzymes used to fulfill

the conversion include carbonic anhydrase in converting CO_2 into carbonic acid [9], carbon monoxide dehydrogenase in reducing CO_2 into CO [10], pyrrole-2-carboxylate decarboxylase in catalyzing CO_2 and pyrrole into pyrrole-2-carboxylate [11], carboxylase in synthesizing benzoic acid from phenol and CO_2 [5], formate dehydrogenase in photosynthesis of malic, aspartic acid and formic acid from CO_2 and corresponding substrates [12], methanol dehydrogenase in the photochemical reduction of CO_2 into methanol [4], formate dehydrogenase and methanol dehydrogenase in electrochemical conversion of CO_2 to methanol [13] and a combination of formate dehydrogenase, formaldehyde dehydrogenase and alcohol dehydrogenase in consecutive reduction of CO_2 into methanol [6].

The present work focused on the consecutive reduction approach, considering that enzymes are cheap and easily available with clear reaction mechanism. The scheme of the approach in this work can be simply described as

$$CO_2 \underset{NADH}{\overset{F_{ate}DH}{\longrightarrow}} HCOOH \underset{NADH}{\overset{F_{ald}DH}{\longrightarrow}} HCHO \underset{NADH}{\overset{ADH}{\longrightarrow}} CH_3OH$$

where $F_{ate}DH$, $F_{ald}DH$ and ADH designate formate dehydrogenase, formaldehyde dehydrogenase and alcohol dehydrogenase, respectively, while reduced nicotinamide

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adenine dinucleotide (NADH) is used as a terminal electron donor for each reduction step. The feasibility and overall yields have been investigated in our previous work [14]. To understand the whole process more clearly and get the optimized operating conditions, the above three reactions need to be studied separately. In this paper, we concentrate on the third step.

1.2. Enzyme immobilization and modification

Enzymes are usually expensive and sensitive. In order to reduce the enzyme cost, increase the enzyme stability and facilitate the late purification, enzymes have to exist in an immobilized form instead of free form [15,16]. There is a long history for enzyme (protein) immobilization using solid supports via physical adsorption, covalent attachment and entrapment. One of the most extensively used methods for immobilizing enzymes is encapsulation inside sol–gel silica [17,18]. The crucial advances in sol–gel bio-encapsulation came from the pioneering efforts of Avnir and coworkers [19]. In 1990, this group disclosed that proteins could be encapsulated using facile sol–gel protocols based upon TMOS and TEOS precursors. Catalytically active and stable and transparent silica xerogels doped with enzymes like alkaline phosphatase, chitinase, aspartase and β-glucosidase were prepared and tested [19]. This was followed in 1992 with the work of Ellerby et al. [20], who used this methodology to entrap the metalloproteins copper–zinc superoxide dismutase, cytochrome *c* and myoglobin in silica sol–gels. Confirming the findings of Avnir and coworkers [19], they showed the protein–silica nanocomposites for typical catalytic, metal-exchange, oxidation–reduction and ligand-binding reactions of the soluble proteins. Numerous works have since then shown that the technique is generic and can accommodate a wide variety of labile biological materials including enzymes. Considering some pertinent features of enzymes, several critical aspects should be borne in mind when choosing the sol–gel encapsulation of enzymes: (a) the encapsulation of enzymes should proceed via their aqueous solutions or suspensions; (b) encapsulation and subsequent processing must employ media at ambient/sub-ambient temperatures, in the biological pH range, at low ionic strength, and with the minimal use of solvents or other organic species; (c) the formed polymer structure must allow for sufficient access to the enzyme as well as its desired conformational mobility, while preventing its leakage from the matrix.

Unfortunately, most studies concerning sol–gel bio-encapsulation showed lower specific activity than that of free enzymes in solution [21] due to the following reasons: (a) small pore size and non-open-pore structure; (b) the three-dimensional structures were significantly damaged by the alcohols produced during the hydrolysis and condensation steps involved in sol–gel process. Herein, a modified sol–gel method using polyethylene glycol (PEG) as a structure-directing reagent to enlarge the pore size of silica was applied [22]. In addition, the pegylation procedure using methoxypolyethylene glycol (mPEG) as a modifier was introduced to enhance enzyme's resistance towards the attack from organic solvents (alcohols).

In this work, properties of unmodified and modified gels were characterized by BET, IR, CD, etc. The catalytic activities of free and immobilized ADH were investigated using the conversion of HCHO to CH_3OH as model reaction.

2. Experimental

2.1. Chemicals

ADH (393 U/mg solid), β-NADH (98% purity) and trinitrobenzene sulphonic acid (TNBS, 5%, w/v, aqueous solution) were purchased from Sigma. Tetraethylorthosilicate (TEOS, 28% Si content), hydrochloric acid (37%, HCl content), sodium hydroxide (99% purity), sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$, 99% purity), disodium hydrogen phosphate dodecahydrate ($Na_2H-PO_4 \cdot 12H_2O$, 99% purity), formaldehyde (36–40% HCHO content), PEG200, PEG400, PEG600, PEG5000 and other reagents were all commercially obtained.

2.2. Preparation of SC-PEG

Succinimidyl carbonate derivative of methoxypolyethylene glycol (SC-PEG) was prepared according to Zalipsky's method [23]. Sixty grams of mPEG of molecular weight 5000 (Union Carbide) was dissolved in 200 mL toluene/dichloromethane (volume ratio 3:1) and treated with 30 mL toluene solution of phosgene (57 mmol) overnight. The solution was evaporated to dryness and the remainder of phosgene was removed under vacuum. The residue was redissolved in 150 mL toluene/dichloromethane (volume ratio 2:1) and treated with solid *N*-hydroxysuccinimide (2.1 g) followed by triethylamine (1.7 mL). After 3 h, the solution was filtered and evaporated to dryness. The residue was dissolved in 600 mL warm (50 °C) ethyl acetate, filtrated again and cooled to facilitate precipitation of the polymer. The product was collected by filtration and then recrystallized once more from ethylacetate. The yield of SC-PEG was 52.5 g.

To determine the contact of active carbonate of the product, samples of SC-PEG were reacted with benzylamine in dichloromethane. The unreacted amine was titrated with perchloric acid in dioxane. These titrations indicated that 1 g of the product contained 1.97×10^{-4} mole of active carbonate.

2.3. Synthesis of mPEG-modified ADH

3.5 mg ADH and 10.5 mg SC-PEG were dissolved in 5 mL of 0.1 M phosphate buffer (pH 7.0) and the solution was kept gently stirring for 2 h at room temperature. The degree of modification was determined by TNBS method

that employed trinitrobenzene sulphonic acid (TNBS) to react with the unpegylated amino groups left on the enzyme. The amount of the product that has a characteristic adsorption at 420 nm was measured by an UV–vis spectrophotometer (U-2800, Hitachi, Japan). The degree of modification was then calculated as 90%. Circular Dichroism Spectrometer (J810, Jasco, Japan) was employed to analyze the secondary structure change of the native ADH and pegylated ADH.

2.4. Preparation of gel and immobilization of ADH

The improved sol–gel immobilization processes are illustrated in Fig. 1. Two approaches were involved: (1) immobilization of enzyme in modified gels and (2) immobilization of modified enzymes in typical gels. In approach (1), the precursor (tetraethylorthosilicate, TEOS), the catalyst (HCl) and the gel-modifier (PEG) were mixed together. Then, the pH of the solution was adjusted to 7.0 by adding NaOH aqueous solution to form the modified sol. Afterwards, a certain amount of ADH or pegylated ADH phosphate buffer solution (pH 7.0, 3.5 mg/mL) was added to the sol. Transparent enzyme-containing gels were formed shortly. In approach (2), TEOS was only mixed with HCl and the subsequent steps were the same with approach (1). Transparent gels were formed in a short period. The addition amount of PEG was defined as its mol concentration based on the amount of TEOS. All the gels prepared by the above two ways were aged at 4 °C for 7 days. The aged gels containing ADH were equilibrated with 0.1 M phosphate buffer solution (pH 7.0) at 4 °C by repeated buffer exchange until there is no alcohol detected by Gas Chromatographer (Agilent 6890). Finally, we got the following four types of gels: (A) typical gel with ADH; (B) typical gel with mPEG-modified ADH; (C) PEG-modified gel with ADH; (D) PEG-modified gel with mPEG-modified ADH. Additionally, typical gel and

modified gel without enzyme were also prepared for analysis and comparison.

The pore size distribution and the most probable pore diameter of the typical gel and PEG-modified gel were determined by the BET nitrogen adsorption–desorption method (CHEMBET3000, Quanta Chrome). Their chemical structures were characterized by infrared spectroscopy analyses (Nicolet-560, MAGNA-IR).

2.5. Enzymatic reactions

Enzyme activities of ADH were evaluated based on the reaction rates and kinetic Michaelis constants (K_m) of the reduction of HCHO to CH_3OH. Since, during the reaction, the cofactor (NADH), which has a characteristic adsorption at 340 nm, will be converted to NAD^+ that has no adsorption at 340 nm, the reaction progress can be recorded by the detection of the amount of NADH. UV–vis spectrophotometer (U-2800, Hitachi) was used for determination of the concentration of the NADH and the reaction rates were calculated accordingly. At 25 °C and pH 7.0, the concentrations of NADH and HCHO were varied in the range 50–250 μM and 3–30 mM, respectively, while the concentrations of free ADH and immobilized ADH were fixed at 0.0035 and 0.087 mg/mL. Then, the kinetic parameters of ADH-catalyzed conversion of HCHO to CH_3OH were obtained by the following equation, which is based on the ordered mechanism proposed by Dalziel [24]:

$$V = \frac{V_{max}[A][B]}{K_{mB}[A] + K_{mA}[B] + [A][B] + K_{SA}K_{mB}}$$

where A and B indicate NADH and HCHO, respectively, V and V_{max} are the reaction and maximum reaction rates, respectively, K_{mA} and K_{mB} are Michaelis constants for NADH and HCHO, respectively, K_{SA} is the dissociation constant of the ADH–NADH pairs.
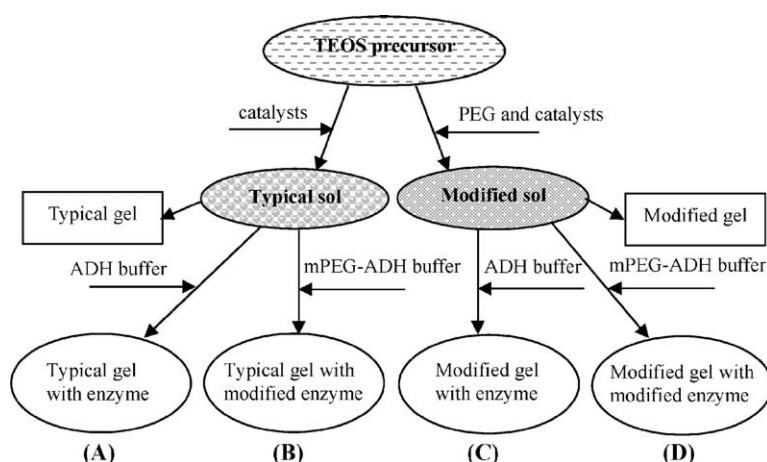

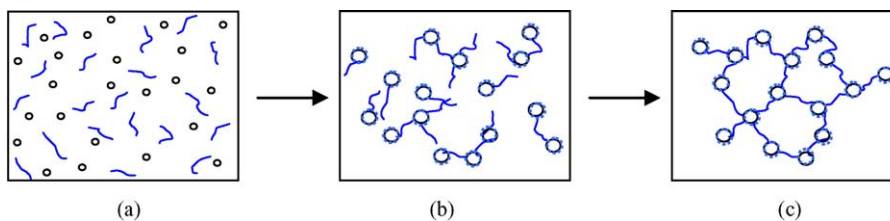Fig. 1. Experimental sol–gel immobilization processes.

Fig. 2. Schematic illustration of PEG-modified sol–gel process: (a) initial formation of sol particles; (b) sol particle growth and linkage between PEG and sol cluster; (c) gelation.

3. Results and discussion

3.1. PEG-modified sol–gel process and structure characterization of gels

The final gel is formed through three stages: formation of sol particles due to the hydrolysis of precursor, gradual aggregation of sol particles into small and then larger clusters and finally gelation through condensation. The PEG was added into the initial precursor solution. During the initial sol-forming process, the PEG molecules and the sol particles (about several nanometers in size) were separated and dispersed in the solution. As the sol particles grew up gradually, the PEG molecules began to wrap the surface of sol particles to form a layer that prevents the sol particles from further growing up. At the same time, the active hydroxyl groups of the PEG molecule ($HO-(CH_2CH_2O)_n-OH$) on the particle surface will facilitate the aggregation of sol particles by crosslinking, resulting in larger PEG–sol clusters. Moreover, PEG molecules with linear structure may act as ''floating bridges'' between the PEG–sol clusters, leading to a more ordered and flexible network matrix with larger pore sizes compared with conventional gel without PEG [25]. Fig. 2 demonstrates the PEG-modified sol–gel process.

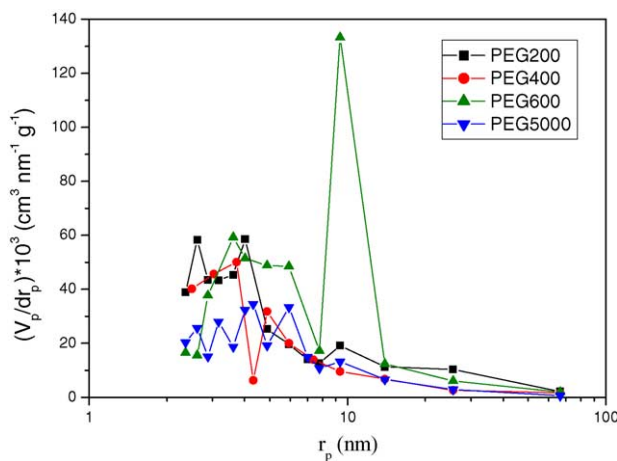Four PEGs with different molecular weight ranging from 200 to 5000 were used as gel modifiers and the pore size distribution of the resulting four gels is shown in Fig. 3. The pore size corresponding with the curve peak was taken as the most probable pore size. It appeared that there exited a most suitable molecular weight of PEG for enlargement of gel pore size. Too small and too large molecular weights were both unfavorable (the most probable pore size were 4–6 nm), and PEG600 had the best effect according to the resulting largest (and) most probable pore size (9.3 nm) among the four kinds of PEGs tested. It can be figured out that, when the molecular weight of PEG is very low, it is difficult for PEG to cover the entire surface of sol particle. The particles will aggregate as it does in the conventional sol–gel process. And the ''bridge'' is relatively too short to affect the pore size. On the contrary, when the molecular weight is too large, the PEG molecules tend to aggregate themselves, reducing or losing their enveloping and bridging functions. PEG600 was chosen for further experiments.

The effect of addition amount of PEG600 was also explored, as shown in Fig. 4. It can be seen that 1% of PEG seemed to be a suitable amount. The water in the TEOS hydrolysis exists in three forms: bound water (water bound to the PEG molecule by hydrogen bonds), trapped water (water trapped in the loosen structure of bound-water-solvated PEG) and free water. When the addition amount of PEG is small, water exist mainly in free form, and the bound water and trapped water take only small percent. In this case the PEG thereby functions only as a dispersant and has no

Fig. 3. The pore size distribution of gels modified by PEG of different molecular weight (PEG content = 1%).
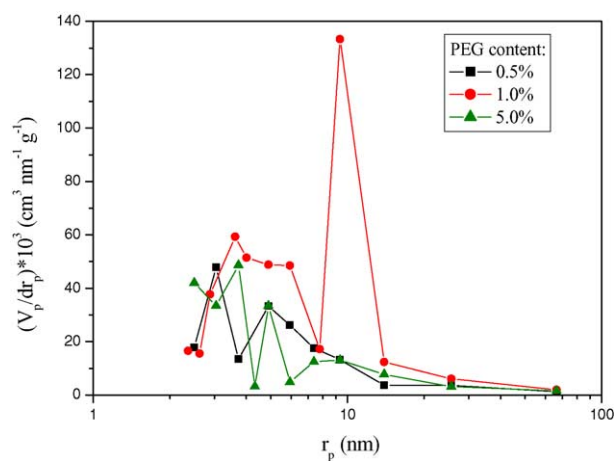

Fig. 4. The pore size distribution of gels modified by PEG600 of different content.
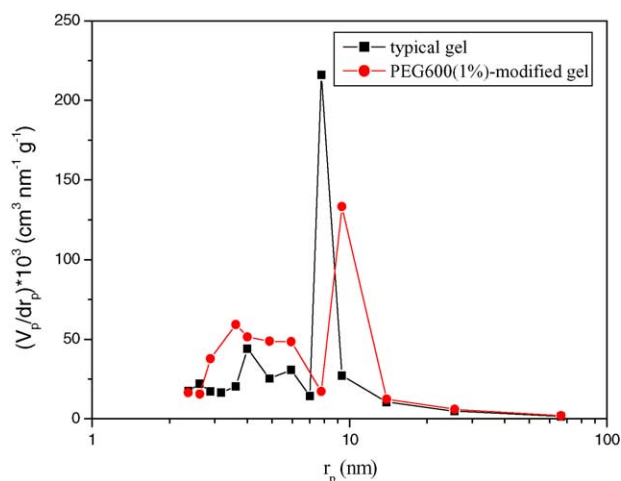
Fig. 5. The pore size distribution of typical and PEG600-modified gels.

visible effect on the pore size enlargement. As the increase of the PEG addition, more water become bound and trapped. The hydrolysis will be restrained to some extent. Its enveloping and crosslinking functions begin to work. However, if too much PEG is added to the solution, too much bound and trapped water will increase the thickness of the solvated layer surrounding the PEG, which will inhibit the formation of ordered network. Furthermore, too abundant PEG molecules may crosslink among themselves and maybe aggregate together to block the gel pores. Addition of 1% PEG600 to the precursor solution was found to be the best amount within the range of this study.

Fig. 5 shows the pore size distribution of typical gel and PEG600-modified gel. The most probable pore size of PEG600-modified gel (9.3 nm) was larger than that of the typical gel (7.8 nm). The pore size was enlarged by the linking function of PEG between sol particles that resulted in

a branched structure with larger mesopores. Experimental results also showed that there was no ADH leakage from both the typical gel and the PEG-modified gel, indicating that the enlarged pore size was still small enough for confining the ADH.

The infrared spectra of the typical gel, PEG-modified gel and SC-PEG-containing gel are shown in Fig. 6. The band at $1100 \, \text{cm}^{-1}$ is attributed to the Si–O bond absorption. The absorbance bands at $2912 \, \text{cm}^{-1}$ for PEG-modified gel and $2917 \, \text{cm}^{-1}$ for mPEG-containing gel are attributed to the C–H stretching vibration. The absorbance bands at 1456 and $1352 \, \text{cm}^{-1}$ for PEG-modified gel and 1474 and $1353 \, \text{cm}^{-1}$ for mPEG-containing gel are attributed to the C–H bending vibration. It is clear that there are no ester bonds or other new bonds formed in the modified gel. Thus the linkage between PEG and silica sol particles was most probably held through molecular interactions such as van der Waal's forces or hydrogen bonds rather than stronger chemical reactions. This is in accordance with the observation in Ref. [26].

3.2. mPEG-modification for ADH

Besides gel modification, mPEG, which has a similar linear structure as PEG but the ending hydroxyl groups are substituted by methoxyl groups, was used to modify the ADH enzyme. Enzyme modification is effectively applied to improve the enzyme properties, especially its activity, or to protect enzyme from harmful environment by introducing or removing some specific groups. Its stable and amphiphilic property makes mPEG a most frequently used modifier for many enzymes and proteins by reacting with the amino groups on the enzyme [27]. Herein, it is supposed that the enzyme ADH with mPEG linked on its surface by reaction between PEG and the lysine residues of enzyme may be more flexible when confined into the gel pore due to the
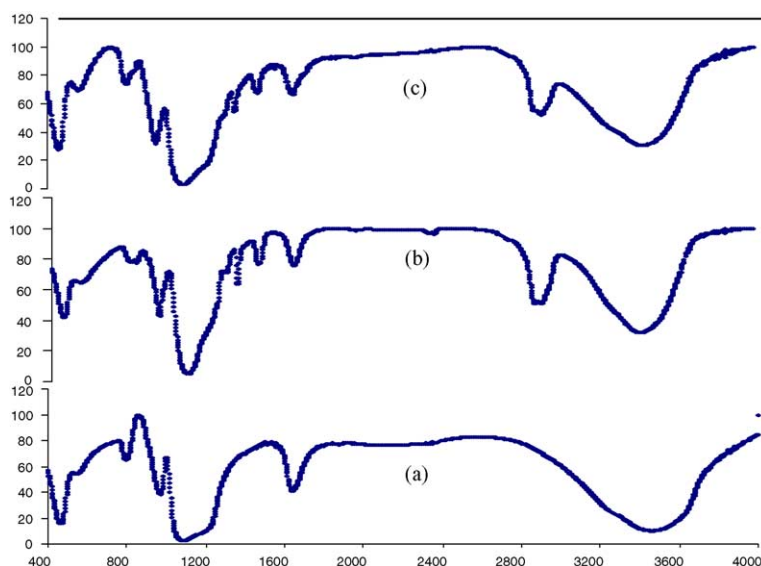

Fig. 6. IR spectra of (a) typical gel, (b) PEG-modified gel and (c) mPEG-containing gel.
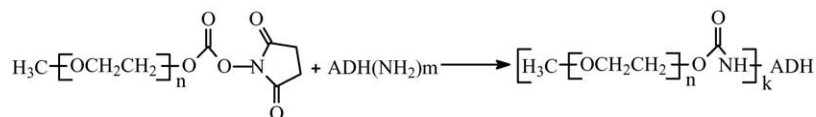
Fig. 7. Principles of ADH modification by SC-PEG.

"spring-like" action of PEG, compared with unmodified enzyme in the "stiff" gel network. A succinimidyl carbonate derivative of mPEG (SC-PEG) was chosen to be the activated form of mPEG due to its appropriate reactivity and stability [28]. The principle of the modification was illustrated in Fig. 7.

Fig. 8 shows the circular dichroism (CD) spectra of ADH before and after pegylation. The native ADH has a strong negative bond between 225 and 250 nm, which still appeared but was greatly reduced after pegylation. This may suggest that the ADH molecule chains were partly wrapped and linked by the highly hydrated SC-PEG molecule chains. The weak bond of ADH at 280 nm was broadened and shifted to lower wavelength. This indicates that the secondary structure of ADH after pegylation was somewhat changed.

3.3. Activity of free ADH and mPEG-modified ADH

The Michaelis constants of the reaction catalyzed by free ADH and mPEG-modified ADH, respectively, are listed in Table 1. K_{mA} and K_{mB} indicate the affinity of the enzyme to the substrates, NADH and HCHO, respectively. K_{SA} means the dissociation ability of the ADH–NADH pairs. In addition to the structure change after pegylation as shown by CD spectra, the steric hindrance owing to the long linear structure of SC-PEG molecules connected to the enzyme surface may also have two effects on the reaction rate. First, it will impede the access of the substrates to active sites of the enzyme, showing higher K_{mA} and K_{mB}. Second, it will make the NADH–ADH pair uneasy to be dissociated, resulting in lower K_{SA}. Overall, the activity of SC-PEG-

modified ADH was lowered. However, it cannot be predicted that the activity of modified enzyme after immobilization will be surely reduced.

3.4. Stability of free ADH and mPEG-modified ADH in alcohol

During hydrolysis and condensation of sol–gel process using TEOS as a precursor, ethanol, which is harmful for enzyme, will be produced. Alcohol resistance of the enzyme is very important to maintain their activity after immobilization. In order to compare the resistance to alcohol of the enzyme before and after mPEG modification, both native and mPEG-modified ADH were studied by measuring their activities of free form. After being immersed in 1% ethanol solution for 2 min, the mPEG-modified ADH maintained 50% of its original activity, while the native ADH retained 36% of its original one. This may be explained by the following two reasons: (1) less flexibility of the enzyme with a long-chain PEG molecule linked to its surface, leading to steric hindrance of protein unfolding and (2) hydrophilicity of PEG, creating a more hydrophilic microenvironment that is more favored for enzyme to stay and function. Combining the results in the previous section with the above test, we can see that, although the modification has no good to the free-form activity, it is really helpful for strengthening the stability of enzyme in harmful alcohol environment. Therefore, it is probable that the mPEG-modified ADH might have a better or at least not worse activity after immobilization comparing with the unmodified ADH.

3.5. Activity of immobilized enzymes

The experiments confirmed that all the enzymatic reaction mechanism followed the Michaelis–Menten kinetics and the kinetic parameters were calculated by Dalziel's double-substrate model [24]. Michaelis constants of the reaction catalyzed by ADH in various forms (Fig. 1(A)–(D)) are shown and compared in Fig. 9. The lower the K_m is, the higher activity the immobilized ADH owns. One
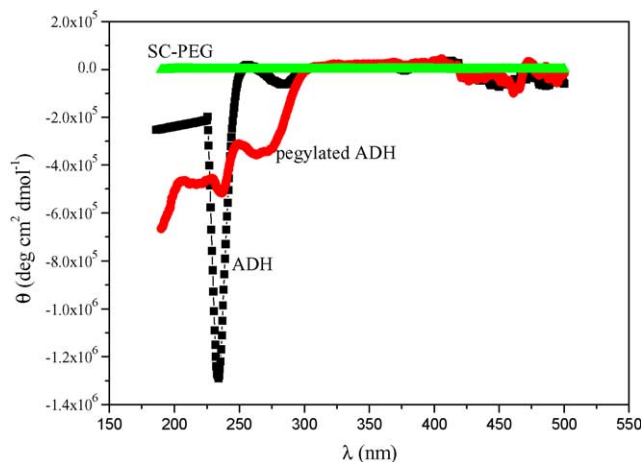

Fig. 8. CD spectra of ADH, SC-PEG and pegylated ADH.

Table 1
Michaelis constants of the reaction catalyzed by free ADH and mPEG-modified ADH

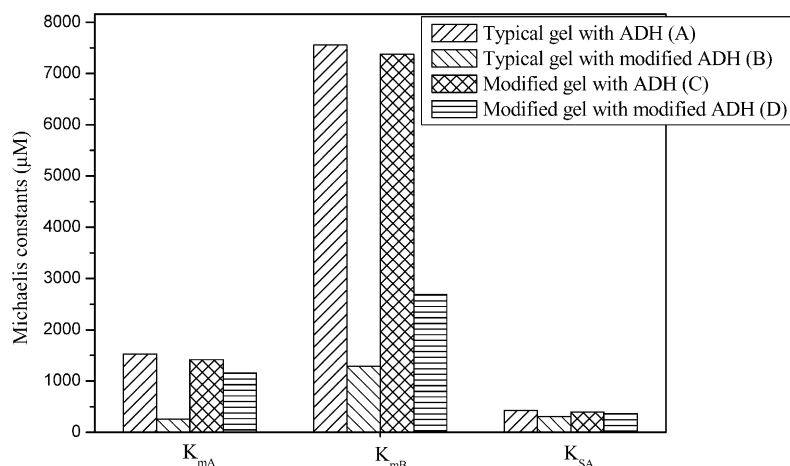| Michaelis constants	ADH in free form	mPEG-modified ADH in free form
K_{mA} (µM)	26.5	232.9
K_{mB} (µM)	1667.6	3640.1
K_{SA} (µM)	127.3	19.2w

Fig. 9. The kinetic Michaelis constants of reactions catalyzed by ADH.

thing should be pointed out first that the activity of all of the ADH-containing gels was lower than that of ADH in free form as shown in Table 1. For easier understanding and better comparison, the microstructure and microenvironment of the four types of immobilized enzymes in silicate matrix may be described as in Fig. 10. Based on the experimental results and referred to the structure sketch, the following analysis was made. Compared with the unmodified ADH in unmodified gel (A), both the gel and enzyme modifications worked (B–D). The fact that the catalytic activity of ADH immobilized in PEG-modified gel (C) was higher than that in typical gel (A) might mainly be attributed to the easier diffusion resulted from the enlarged pore size. These enlarged pores are not only more "comfortable" for enzymes to stay but also easier for substrate to diffuse through the pore channel and access to the enzymes. In addition, the improved gel properties such as hydrophilicity and mechanical strength may also provide higher biocompatibility for enzymes. The mPEG-modified ADH encapsulated in typical gel (B) showed the highest activity among the four cases. Compared the effects of the two modification methods on the final enzyme activity, it can be seen that the modification on ADH (B, D) seemed to have a greater favorable effect than the modification on gel (C). It might be concluded that the more hydrophilic microenvironment

maybe more important than the modified gel structure for the enzyme to stay and function, in addition to the enhanced stability by enzyme modification. However, it could be seen from the results of (B) and (D), too highly hydrophilic microenvironment actually was not an advantageous factor as it was originally imagined. The thick solvated water layer around the enzyme would hinder the substrate to access to the enzyme. The dissociation constants (K_{SA}) did not change so much as the combination ones (K_{mA}, K_{mB}), although the changing trend was similar.

3.6. Storage stability of immobilized ADH

As shown in Fig. 11, the activity of the native ADH encapsulated in typical silica gel (A) was reduced by 5% of its original activity after 21 days of storage and by 17% after 92 days. In contrast, the enzyme activities of the other three forms (B–D) almost showed no decrease after storage for the same periods. This encouraging result proved that both the PEG modification of gel and the mPEG modification of enzyme helped to maintain the activity most probably by confining or grasping the enzyme and preventing it from denaturation. Further study on the microstructure of the modified gel and modified enzyme needs to be conducted.
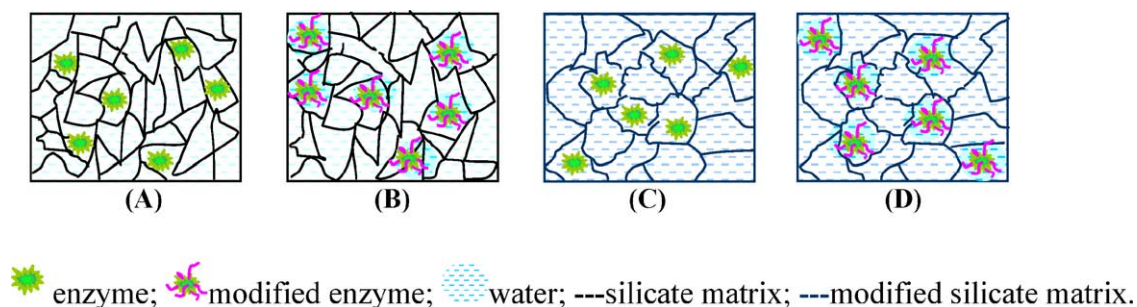

enzyme; modified enzyme; water; ---silicate matrix; ---modified silicate matrix.

Fig. 10. Structure sketch of the four types of the immobilized enzyme in silicate matrix.
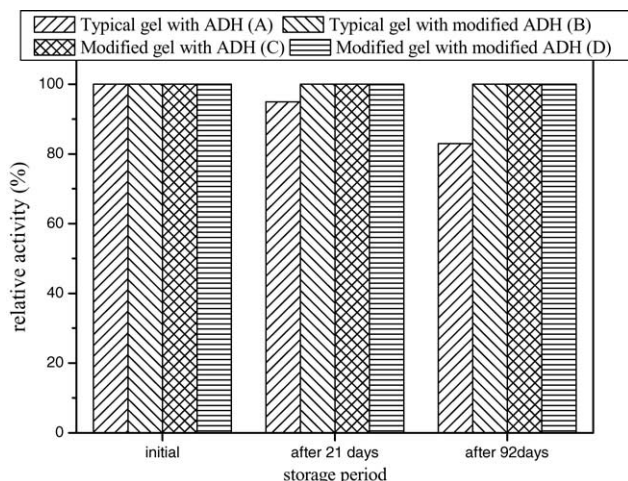
Fig. 11. Storage stability of immobilized enzymes.

4. Conclusion

CO_2 can be converted to formate, formaldehyde and methanol through a biochemical way catalyzed by dehydrogenases immobilized in silica gel. PEG600 and SC-PEG5000 were used for enlarging the pore size of the silica gel and improving the compatibility of ADH with its microenvironment. Six types of gels were prepared, including typical gel without enzyme, modified gel without enzyme, typical gel with ADH (A), typical gel with modified ADH (B), modified gel with ADH (C) and modified gel with modified ADH (D). The most probable gel pore size was enlarged from 7.8 to 9.3 nm with the mediation of PEG600, and the interaction between PEG and silica was through van der Vaal's force and hydrogen bond rather than chemical covalent bond. The efficiency of pegylation was evaluated by the Michaelis constants and initial reaction rates in the model reaction of HCHO reduction to CH_3OH catalyzed by ADH. The results indicated that both modification methods had favorable effects on the enzyme activity while the enzyme pegylation was more significant. The pegylated ADH in conventional gel (B) had the highest activity. The enhancement of immobilized enzyme activities came from the improvement in microstructure of the gel, the change in microenvironment for the immobilized enzyme, and the increase of the stability of ADH by PEG modification.

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