Supplementary Material (ESI) for Soft Matter
This journal is (c) The Royal Society of Chemistry 2010

# Self-assembly hollow nanosphere for enzyme encapsulation

**Wei Ha[a,b], Xian-Wei Meng[c], Quan Li[b], Min-Min Fan[c], Shu-Lin Peng[b], Li-Sheng Ding[b], Xuan Tian[a], Sheng Zhang[c,*], and Bang-Jing Li[b,*]**

[a]*State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China*

[b]*Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China, Tel: +86-28-85223843. Fax: (+86)28-85223843. E-mail: libj@cib.ac.cn (B. Li)*

[c]*State Key Laboratory of Polymer Materials Engineering, Polymer Research Institute of Sichuan University, Sichuan University, Chengdu 610065, China, Tel, Fax: +86-28-85400266. E-mail: zslbj@163.com (S. Zhang)*

## Supplementary Information

**CONTENTS:**

1. Materials and Instruments.
2. Synthesis of the PEG-Branched Alginate.
3. Preparation of the Hollow Spheres, Encapsulation Procedure.
4. Determination the Activity of the Encapsulated and Free L-Asparaginase.
5. Determination of L-Asparaginase Encapsulation Efficiency.
6. Determination of L-asparaginase Leakage from the Hollow Spheres.
7. Determination the Activity Recovery of the Encapsulated L-asparaginase.
8. Measurement of the Michaelis Constant.
9. Detection whether the substrate L-asparagine can react with α-CD.
10. Detection the effect of Alg-PEG and α-CD on the activity of L-asparaginase.

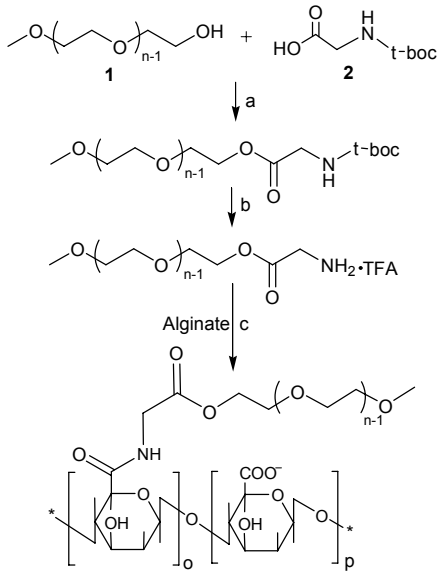
## 1. Materials and Instruments

Alginate was purchased from Tianjin Yuanhang Chemicals Co., Ltd., China. α-CD was purchased from TCI Co. Ltd., Tokyo. L-asparaginase from *E. coli* with absolute activity of 672±7 U/mg (the absolute activity of the enzyme was determined by literature method)[1] was purchased

from Changzhou Bio-Pharma Qianhong Co., China. PEG was purchased from Sigma-Aldrich Co., Inc. Other reagents were analytical pure and used directly without further purified.

$^1$H NMR were measured on a Bruker 600MHz spectrometer. The chemical shifts of $^1$H NMR are expressed in parts per million downfield relative to the internal tetramethylsilane (δ = 0 ppm) or chloroform (δ = 7.26 ppm). IR spectra were recorded on a Perkin Elmer spectrum one FT-IR spectrometer using KBr discs in the range of 400-4000 cm$^{-1}$ region. The centrifugations were taken on TGL-20M, Saite Centrifuge Co., Shanghai, China. The absorbance value of the solution at 436 nm was recorded on APL-UV-2000 spectrophotometer, Shanghai, China. The dynamic light scattering (DLS) were measured by BI-9000AT, BI-200SM, Brookhaven Instruments Co., USA. The TEM were measured by Jeol JEM -100CX, and observation was done at an accelerating voltage of 80 KV.

## 2. Synthesis of the PEG-Branched Alginate




***Scheme 1.*** Synthesis of the PEG-Branched alginate. Reagents and conditions: a) DCC, DMAP, $CH_2Cl_2$, 0℃; b) $CH_2Cl_2$/TFA( 1:1), 0℃; c) NHS, EDCI, $H_2O$, 25℃

The PEG-Branched alginate was synthesized by following a literature procedure.[2] In our case, poly (ethylene glycol) methyl ether ($M_n$=2000) was utilized. Poly (ethylene glycol) methyl ether (**1**, 2.5 mmol , M=2000) was reacted with N-*t*-Boc-glycine (**2**, 2.7 mmol) and DMAP (0.75 mmol) in 25 ml anhydrous $CH_2Cl_2$. Subsequently, 3 mmol of DCC was added, and the reaction mixture was stirred at 0 °C for 24 h. The dicyclohexylurea (DCU) was filtered, and the filtrate was concentrated in a vacuum at room temperature. The resultant was dissolved in minimal amount of acetone and cooled overnight, and the precipitated DCU was filtered off. For the removal of a *t*-Boc group, PEG-*t*-Boc-amino acid diester (**3**) was dissolved in a mixture of $CH_2Cl_2$/TFA (1/1, v/v). The reaction mixture was stirred at 0 °C for 3 h and then evaporated to dryness. The deprotected derivative (**4**) was dissolved in a 15 % NaCl solution, and the pH was adjusted to 5. The solution was filtered to remove water insoluble *tert*butyl salts. The filtrate was extracted three times with chloroform; the organic phases were combined and dried over $Na_2SO_4$. The $Na_2SO_4$ was filtered, the solvent was evaporated, and the oily residue was dried under vacuum at room

temperature.

**4:** $^1$H NMR (600 MHz, $CDCl_3$, ppm): δ 3.33 (s, O**CH$_3$**), 3.90 (s, N**CH$_2$**CO), 4.32 (m, COO**CH$_2$**CH$_2$), 3.47- 3.72 (m, O**CH$_2$CH$_2$**).

A 2% (w/w) sodium alginate solution was prepared in a buffer solution of 0.1 M MES and 0.5 M NaCl, and the pH was adjusted to 6. The molar ratio of EDC: NHS: $COO^-$ was 1: 0.5: 1. A (0.76 mmol) sample of NHS and (1.52 mmol) of EDC were added to a 2 % (w/w) alginate solution to activate the carboxylic acid groups on the polymer backbone. The solution was agitated for 10 h to obtain a homogeneous solution followed by the addition of PEG-amine. The solution was dialysed by bag filter (MWCO: 8000-14000) at 37 °C for 36 h, the solvent was evaporated, and dried under vacuum at room temperature.

**Alg-*g*-PEG**: $^1$H NMR (600 MHz, $D_2O$, ppm): δ 3.33 (s, O**CH$_3$**), 3.53-3.76 (m, O**CH$_2$CH$_2$**) 3.91 (s, N**CH$_2$**CO), 4.37 (m, COO**CH$_2$**CH$_2$), 3.85, 4.00, 4.06, 4.29 (**CH**-OH of alginate).

FT-IR (KBr): 3429 (-OH), 2888 (C-H), 1624 (COOH), 842-1281 (C-O-C of PEG) cm$^{-1}$.

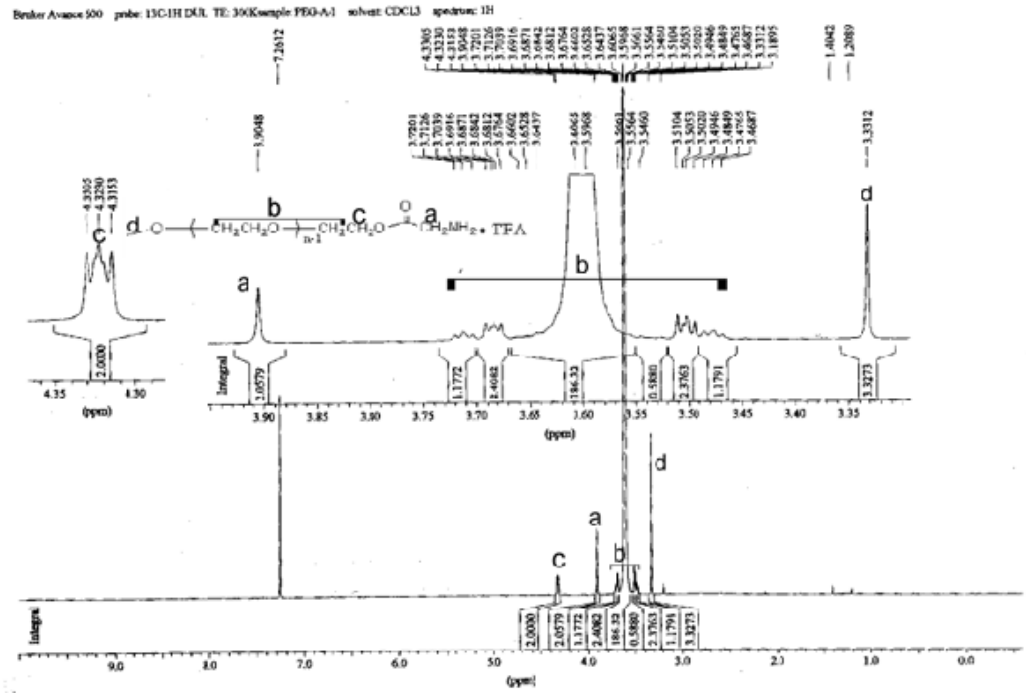



Figure S1. $^1$H NMR spectra of **4**

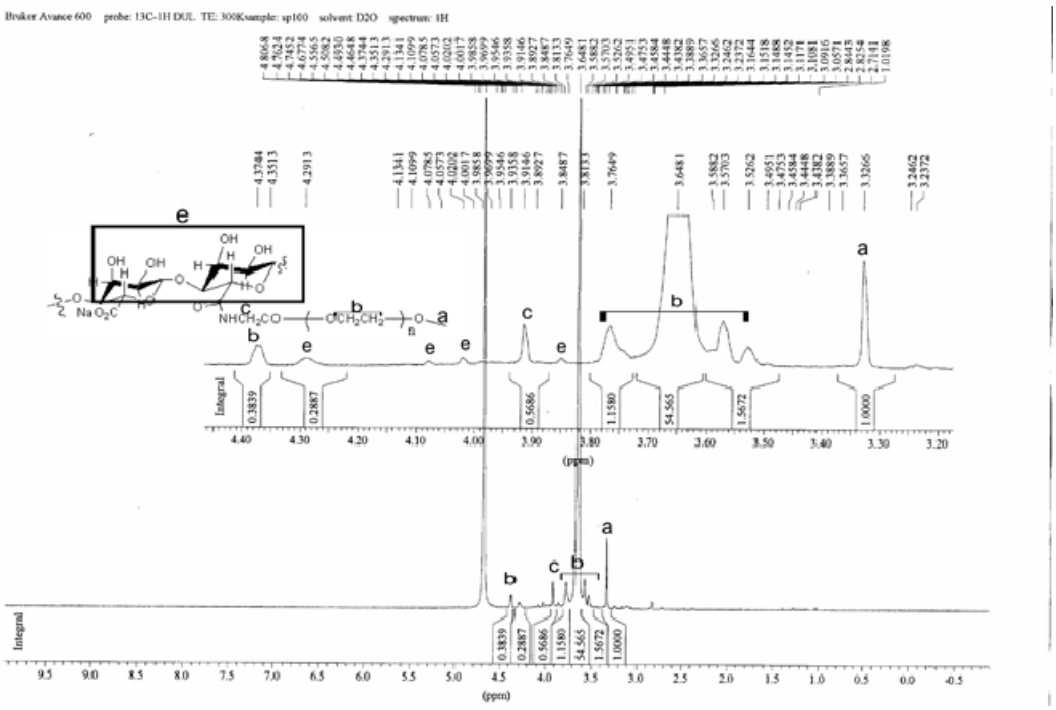


Figure S2. $^{1}$H NMR spectra of alginate-*g*-PEG.

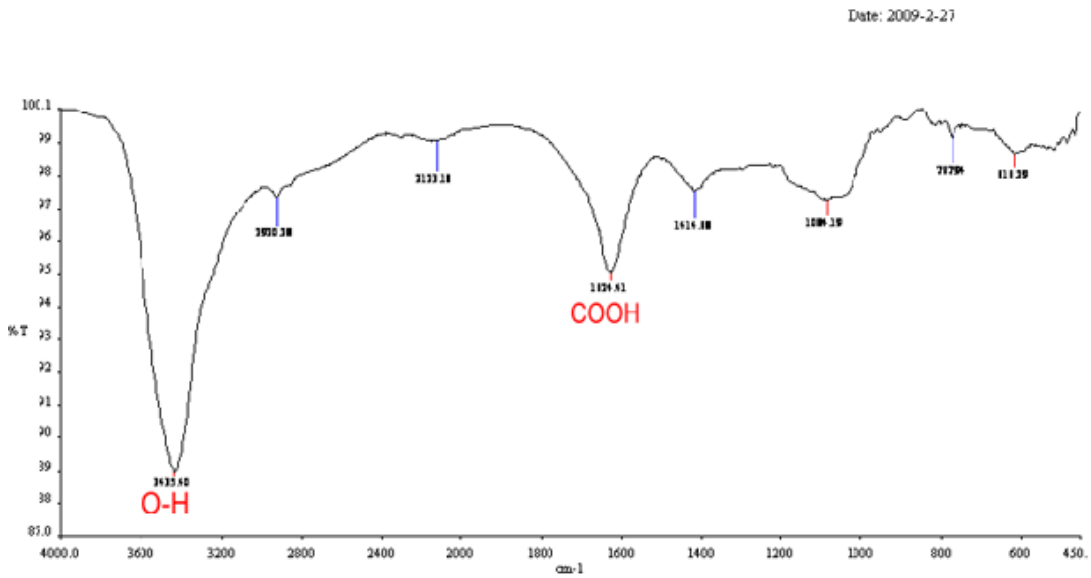



Figure S3. FT-IR spectra of alginate-*g*-PEG.

## 3. Preparation of the Hollow Spheres, Encapsulation Procedure

The novel building block of rigid chains of (α-CD)-PEG was found to be able to self-assemble easily into hollow spheres when PEG-Branched alginate solutions were added dropwise to the α-CD solutions in water. And if the enzyme was added into PEG-Branched alginate solutions, enzyme could be entrapped while the hollow spheres were formed. Brief procedures: 2 ml branched alginate solution in different concentrations [0.065, 0.125, 0.1875, 0.25, 0.375, 0.5 %

(w/v)] (with 1 mg L -asparaginase ) were added dropwise into 6 ml α-CD[6 % (w/v)] solutions, stirred for 2 h, the solution turned to muddy and slightly blue, the hollow spheres were collected by centrifugation at 16,000 rpm. The supernatants of the centrifugation were collected for measurements of the encapsulation efficiency. 2 ml 0.25% (w/v) branched alginate solution (with 1 mg L -asparaginase ) were added dropwise into 6 ml α-CD solution in different concentrations[1.8, 3.6, 5.4, 6 % (w/v)] solutions, stirred for 2 h, the solution turned to muddy and slightly blue, the hollow spheres were collected by centrifugation at 16,000 rpm. The supernatants of the centrifugation were collected for measurements of the encapsulation efficiency.

The turbidity experiments were carried out to demonstrate the enzyme can not form aggregates with Alg-*g*-PEG or be included in α-CD molecules when the enzyme was mixed Alg-*g*-PEG solution or α-CD solution separately. Brief procedures: 1 mg L-asparaginase was mixed with 4 ml 6 % α-CD solutions, 4 ml 0.25 % Alg-PEG solutions and 4 ml the mixture of 6 % α-CD and 0.25 % Alg-PEG solutions, respectively. The absorbance value of the corresponding solutions at 436 nm was recorded on a spectrophotometer (Fig. S4). It can be seen that the turbidity shows no obviously increased when the enzyme was mixed with Alg-PEG or α-CD solutions, which indicates that the enzyme can not form aggregates with Alg-PEG or α-CD molecules. However, after mixed Alg-PEG solutions with α-CD solutions, the turbidity of solution increased significantly, indicating the formation of aggregations.

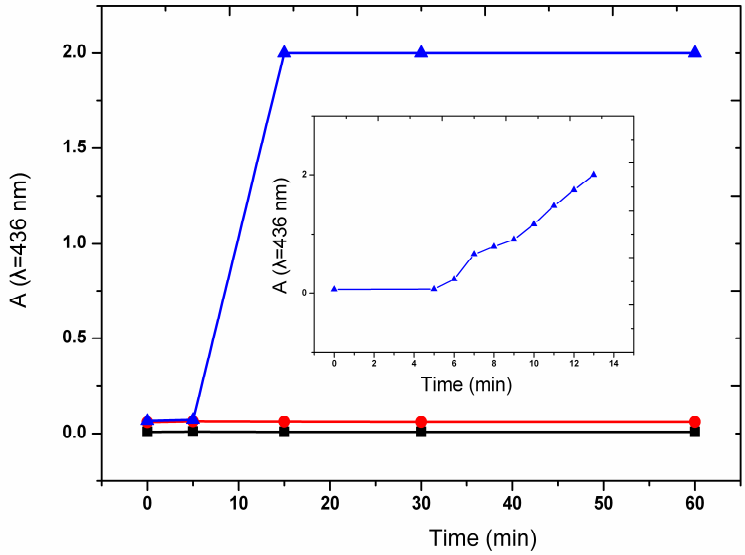



Figure S4. turbidity plot at λ=436 nm. (The mixture of α-CD and enzyme, squares; the mixture of Alg-PEG and enzyme, circles; the mixture of Alg-PEG, α-CD and enzyme, triangles)

## 4. Activity of the Encapsulated and Free L-asparaginase

The activities of the encapsulated and the free L-asparaginase were performed with a slightly modified method according to literature [1] described previously. This concise procedure contains the following steps as described next. Step 1: after 0.1 ml free L-asparaginase or encapsulated enzyme was added to the temperature-stabilized mixture containing 0.1 ml 189 mM L-asparagine solution, 1.0 ml buffer and 0.9 ml deionized water in a container, the mixture was incubated at the assigned temperature for 30 min. Then 0.1 ml 1.5 M trichloroacetic acid was added to interrupt the

enzymatic reaction. Step 2: 0.2 ml of supernatant from step 1, 4.3 ml deionized water and 0.5 ml Nessler's reagents were added to a suitable container. The absorbance value of the solution at 436 nm was recorded on a spectrophotometer. The absolute activities of the encapsulated and free enzyme were calculated with the standard curve plotted by the $\Delta A_{436nm}$ of the standard versus the ammonia concentration (the standard curve was plotted using a standard $(NH_4)_2SO_4$) solution) from the equation below:

$$\text{The absolute activity of the enzyme (Units/mg)} = \frac{(\ \mu\text{mole of } NH_3 \text{ liberated})\times 2.20}{0.2\times 30\times 0.1\times(\text{used enzyme in mg/ml})}$$

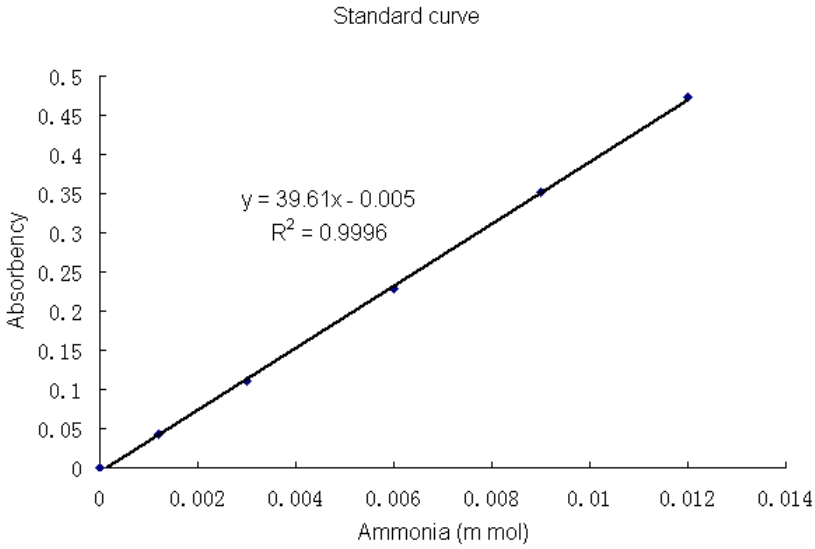



Figure S5. The standard curve plotted by the $\Delta A_{436nm}$ of the standard versus the ammonia concentration

## 5. L-Asparaginase Encapsulation Efficiency

The free and encapsulated L-asparaginase were separated by centrifugation of the suspension at 16,000 rpm for 10 min. In order to determine the encapsulation efficiency of the nanocapsules, it was necessary to detect the free enzyme in the supernatant. Measurements were carried out by the standard method.[1] The supernatant was diluted to 25.0 ml and measured by UV spectrometer. The amount of the free L-asparaginase in the clear supernatant was determined. The L-asparaginase encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation efficeiency (\%)} = \frac{\text{mass of L-asparaginase used in formulation} - \text{mass of free L-asparaginase}}{\text{mass of L-asparaginase used in formulation}}\times 100$$

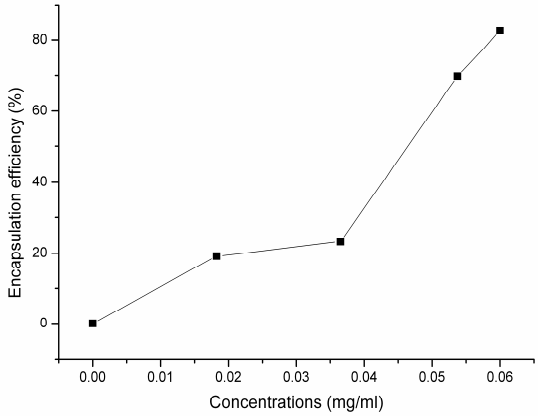



Figure S6. The effect of α-CD concentrations on the encapsulation efficiency of enzyme.

## 6. Determination of L-asparaginase Leakage from the Hollow Spheres

In order to evaluate L-asparaginase diffusion out of the hollow spheres, the procedure is designed as follow: the hollow spheres which contain enzyme prepared from section **5** were kept in 2.0 ml tris buffer at 4 $^{o}$C for 48 h. Then, the mixture was separated by centrifugation at 16,000 rpm. The clear supernatant was collected and the mass of L-asparaginase it contained was determined. The percentage of leakage from the nanocapsules was calculated using the follow equation:

$$\text{Percentage of leakage of the nanocapsules (\%)} = \frac{\text{mass of L-asparaginase detected in the supernatant}}{\text{mass of L-asparaginase encapsulated}} \times 100$$

## 7. Activity Recovery of the Encapsulated L-asparaginase

To determine the effect of the encapsulation process on the enzyme, the activity recovered enzyme was conducted. The loading amount of the enzyme was fixed at 1.0 mg. The absolute activity of the encapsulated enzyme was calculated. The activity recovery of the encapsulated enzyme was calculated from the equation listed below:

$$\text{Activity recovery (\%)} = \frac{\text{The absolute activity of encapsulated L-asparaginase used in formulation}}{\text{The absolute activity of the native L-asparaginase } (672 \pm 7 \text{ U/mg})} \times 100$$

## 8. Measurement of the Michaelis Constant

The Lineweaver-Burk plots based on the results obtained at pH 8.6 at 37 $^{o}$C, the substrate L-asparagine concentration was varied from 0.11 to 1.87 mM. The time intervals for sample collection were 30 min. Assuming Michaelis–Menten type reaction kinetics for both the free and encapsulated enzyme systems, data were plotted according to the Lineweaver–Burk double reciprocal model as follows:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{S} + \frac{1}{V_{\max}}$$

where $V$ is the apparent initial catalytic rate, $V_{\max}$ is the maximum apparent initial catalytic rate, $S$ is the substrate concentration, and $K_m$ is the apparent Michaelis–Menten constant.

## 9. Detection whether the substrate L-asparagine can react with α-CD

This concise procedure contains the following steps as described next. Step 1: after 0.1 ml free L-asparaginase (1) or 5 % α-CD solutions (2) was added to the temperature-stabilized mixture containing 0.1 ml 189 mM L-asparagine solution, 1.0 ml buffer and 0.9 ml deionized water in a container, the mixture was incubated at the assigned temperature for 30 min. Then 0.1 ml 1.5 M trichloroacetic acid was added to interrupt the enzymatic reaction. Step 2: 0.2 ml of supernatant from step 1, 4.3 ml deionized water and 0.5 ml Nessler's reagents were added to a suitable container. The absorbance value of the solution at 436 nm was recorded on a spectrophotometer (**Table S1**).

**Table S1** The absorbance value of the corresponding solution.

| Entry | A (λ=436 nm) |
|---|---|
| (1) | 0.405 |
| (2) | 0 |

## 10. Detection the effect of Alg-PEG and α-CD on the activity of L-asparaginase

The first procedure contains the following steps as described next. Step 1: 0.1 ml free L-asparaginase added to the temperature-stabilized mixture containing 0.1 ml 189 mM L-asparagine solution, 1.0 ml buffer and 0.9 ml deionized water (1), 0.1 ml 189 mM L-asparagine solution, 1.0 ml buffer and 0.9 ml 0.25 % Alg-PEG solutions (2) and 0.1 ml 189 mM L-asparagine solution, 1.0 ml buffer and 0.9 ml 6 % α-CD solutions (3), respectively. The mixture was incubated at the assigned temperature for 30 min. Then 0.1 ml 1.5 M trichloroacetic acid was added to interrupt the enzymatic reaction. Step 2: 0.2 ml of supernatant from step 1, 4.3 ml deionized water and 0.5 ml Nessler's reagents were added to a suitable container. The absorbance value of the solution at 436 nm was recorded on a spectrophotometer (**Table S2**). Another experimental procedure contains following steps, Step 1: 0.1 ml free L-asparaginase was added to the temperature-stabilized mixture containing 0.1 ml 0.25 % Alg-PEG solution, 1.0 ml buffer and 0.9 ml deionized water (1) and 0.1 ml 6 % α-CD solutions, 1.0 ml buffer and 0.9 ml deionized water (2) in a container, respectively. The mixture was incubated at the assigned temperature for 30 min. Then 0.1 ml 1.5 M trichloroacetic acid was added to interrupt the enzymatic reaction. Step 2: 0.2 ml of supernatant from step 1, 4.3 ml deionized water and 0.5 ml Nessler's reagents were added to a suitable container. The absorbance value of the solution at 436 nm was recorded on a spectrophotometer (**Table S3**).

**Table S2** The absorbance value of the corresponding solution.

| Entry | Absolute activity (U/mg) |
|---|---|
| (1) | 672±7 |
| (2) | 660±4 |
| (3) | 681±7 |

**Table S3** The absorbance value of the corresponding solution.

| Entry | A (λ=436 nm) |
|---|---|
| (1) | 0.001 |
| (2) | 0.001 |

**Reference**

1 S. Shifrin, C. L. Parrott and S. W. Luborsky, *J. Biol. Chem* 1974, **249**, 1335-1340.

2 P. Eiselt and D. J. Mooney, *Macromolecules* 1999, **32**, 5561-5566.