Fabrication of Single Carbonic Anhydrase Nanogel against Denaturation and Aggregation at High Temperature

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A two-step procedure to encapsulate a single bovine carbonic anhydrase (BCA) molecule into a spherical nanogel was proposed. BCA was reacted first with *N*-acryloxysuccinimide to introduce surface vinyl groups, followed by in-situ aqueous polymerization. Characterization of the nanogel by dynamic light scattering, transmission electron microscopy, and atomic force microscopy confirmed that each nanogel contained a single BCA molecule. The encapsulated BCA maintained 70% of the activity of its free counterpart, but exhibited an increase in the molten temperature from 64 to 81 °C demonstrated by differential scanning calorimetry and an extension of the half-life from less than 3 to over 90 min at 75 °C. Circular dichroism spectroscopy indicated that the encapsulation and the multi-point covalent linkage between BCA and the polymer shell strengthened the secondary structure and thus inhibited the aggregation at high temperature. The uniform BCA nanogel with enhanced structural stability against denaturation and aggregation expands the applications of BCA catalysis, particularly those carried out at high temperatures.

Introduction

Carbonic anhydrase (CA, E.C. 4.2.1.1) is a zinc metalloenzyme that efficiently catalyzes the reversible hydration of carbon dioxide to bicarbonate and a proton with a maximum turnover rate of 10^6/s.[1,2] In-vivo CA facilitates the extraction of CO_2 from tissues and the subsequent transport to the vascular system as a bicarbonate, followed by the discharge of CO_2 by breathing.[3] In-vitro CA can accelerate the conversion of CO_2 to bicarbonate 2000-fold as compared to that performed without CA[4] and accomplish the extraction of CO_2 from air at a dilute concentration below 0.04%.[5] Growing efforts have been directed to the exploration of CA catalysis in, for example, CO_2 adsorption in a space shuttle, CO_2 hydration in an artificial lung, CO_2 reduction in the atmosphere, and the enrichment of natural gas.[6−10]

Bovine carbonic anhydrase (BCA) is a globular protein fabricated by a single polypeptide chain without disulfide bridges. When the temperature gets above its unfolding temperature of 63 °C, BCA starts to unfold and then aggregate irreversibly.[11,12] The aggregation occurs even at ambient temperature due to intermolecular hydrophobic interactions.[13] Addition of PEG,[14] grafting polysaccharide,[15,16] and surfactants/cyclodetrins[17] as artificial chaperones for BCA refolding is effective in depressing protein aggregation but cannot stabilize BCA at high temperature. Encapsulation of BCA in silica does not improve the stability of BCA,[18] whereas coating BCA with a Michael-adduct-base membrane could retain only 7% of the original activity.[19] Immobilization of BCA on Sepharose 4B[20] and methacrylic acid polymers,[21] although effective in stabilizing BCA, is not suitable for the hydration of CO_2 that occurs at the gas−liquid interface due to the extra mass transport resistance caused by the presence of the porous matrix. These practices indicate that a suitable modification method should (1) strengthen the molecular structural stability so to expand the applications of BCA catalysis, and (2) enhance the catalytic performance so to enhance the process catalyzed with BCA.

Recent efforts in fabricating a single enzyme into a nanoparticle[22,23] or nanogel[24] have given a promising way to realize the above objective. The thin nanocoating, while enhancing the stability of encapsulated enzyme via multiple covalent attachments with coating material, generates an insignificant or minor increase in the mass transfer resistance. In our efforts in encapsulating a single horseradish peroxidase (HRP) into a nanogel, we found the encapsulated HRP exhibited a significantly improved stability over its free counterpart at high temperature or in the presence of water-miscible organic solvents. The porous nanocoating enabled an efficient contact of the substrate to the encapsulated enzyme and thus had a minor effect on the enzymatic catalysis. Moreover, the nanogel was prepared in the aqueous phase and is suitable to encapsulate an enzyme that is fragile to organic solvents.[24]

BCA aggregation driven by intermolecular hydrophobic interaction has been observed,[13] which is one problem that hindered the applications of BCA. The simulation of a caged protein shows that a hydrophilic cage favors the stability of the confined protein.[25] On the other hand, such a hydrophilic cage as a "shell" may also depress the aggregation of protein, which serves as the "core" of a single nanoparticle or nanogel. Thus, we directed our attention to fabricate a single BCA nanogel to enhance its structural stability against denaturation and aggregation at high temperature.

We present here a two-step procedure to encapsulate a single BCA into a nanogel. The first step was to react BCA with *N*-acryloxysuccinimide to introduce surface vinyl groups. The second step was the in-situ polymerization at room temperature using acrylamide as the monomer, *N*,*N*′-methylene bisacrylamide as the cross-linker, and ammonium persulfate/*N*,*N*,*N*′,*N*′-tetramethylethylene diamine as the initiator. The BCA nanogel was characterized by Fourier transform infrared spectrum (FTIR), transmission electron microscopy (TEM), atomic force microscopy (AFM), and dynamic light scattering (DLS). The CO_2 hydration activities of the free BCA and the BCA nanogel

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were compared. The esterase activity of the BCA nanogels was also determined to establish a more comprehensive picture of the effect of encapsulation on BCA catalysis. The thermal stability of the BCA nanogel was examined by both differential scanning calorimetry (DSC) and enzyme activity assay. The secondary structure of the free BCA and the BCA nanogel at high temperatures was analyzed by circular dichroism (CD) to elucidate the stabilization of BCA by the encapsulation into nanogel.

Experimental Section

Materials. The main chemicals used in this study including bovine carbonic anhydrase II (BCA), acrylamide, *N*,*N*′-methylene bisacrylamide, *N*-acryloxysuccinimide, fluorescamine, and *p*-nitrophenylacetate were purchased from Sigma-Aldrich. Sephadex G-25 and G-75 column was purchased from Amersham Pharmacia Biotech, Sweden. Other chemicals were analytical grade.

Acryloylation of BCA. One hundred milligrams of BCA was dissolved in 38 mL of pH 9.3 100 mM boric acid buffer. Next, 40 mg of *N*-acryloxysuccinimide dissolved in 2 mL of DMSO was slowly added, and the reaction was carried out for 2 h at room temperature. Finally, the reaction solution was desalted with a G-25 column.

In-Situ Polymerization. A solution containing a specific weight of the acryloylated BCA of 3.5 mL was added to a vial and purged with nitrogen. Radical polymerization from the surface of the acryloylated BCA was initiated by adding 3 mg of ammonium persulfate dissolved in 30 μL of deoxygenated and deionized water and 3 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine into the test tube. Next, a specific amount of acrylamide and *N*,*N*′-methylene bisacrylamide (molar ratio = 10:1) dissolved in 0.5 mL of deoxygenated and deionized water was dropped into the test tube within 60 min. Similar dropwise procedures were applied recently in the practice of coating inorganic nanoparticles and was proven to be effective to reduce particle size distribution.[37,38] After reaction for another 60 min in nitrogen atmosphere, gel filtration by Sephadex G-75 was applied to remove unreacted proteins, monomers, and initiators. The yield of polymerization is defined as the amount of the polymer over that of the monomer, which is calculated by weight of lyophilized nanogels after dialysis against deionized water. The yield of enzyme encapsulation is defined as the amount of encapsulated enzyme over that added in the polymerization, which is determined according to the protein concentration assay.

Determination of the Reacted Amine Groups in BCA.[26] 1.5 mL of fluorescamine dissolved in acetone at a concentration of 0.1 mg/mL was first added to 0.5 mL of pH 8.0, 0.1 M phosphate buffer containing the acryloylated BCA. After 7 min reaction at 25 °C, the fluorescence intensity was determined at an excitation wavelength of 390 nm and an emission wavelength of 483 nm, using a Shimadzu spectrofluorometer. The free BCA was used as a control to determine the unreacted amine groups of the acryloylated BCA.

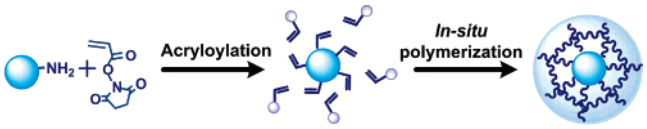
Protein Concentration Assay. The protein content in the nanogel was determined by bicinchoninic acid (BCA) colorimetric protein assay.[28]

Fourier Transform Infrared Spectrometry (FTIR). FTIR spectra of the free BCA and the BCA nanogel were recorded on a Nicolet 560 FTIR spectrometer using KBr pellets.

Transmission Electron Microscopy (TEM). TEM of the free BCA and the BCA nanogel was determined using a Hitachi JEOL200CX high-resolution TEM at 100 000×. During a run, the sample was diluted in water to give concentrations of 20 μM for the free BCA and the BCA nanogel. Carbon-coated grids were prepared by adding a drop of the protein solution, removing the excess, and applying 1%, pH 7.0 sodium phosphotungstate.[27] The sample was then subjected to TEM measurement.

Atomic Force Microscopy (AFM). A new mica surface was prepared by fresh cleaving and then exposed to a BCA nanogel solution of 3 μM for 30 s. Next, the AFM measurement of the sample was performed with a Nanoscope III (Digital Instruments, Santa Barbara, CA) operated in the tapping mode in air.

Scheme 1. Encapsulation of a Single BCA into a Nanogel



Dynamic Light Scattering Measurements (DLS). DLS was performed using a DynaPro-801 dynamic light scattering instrument (Protein Solutions, Co.). Data were collected and analyzed using the AutoPro data software for the DynaPro-801 instrument (Protein Solutions, Co.).

Circular Dichroism Spectroscopy (CD). Protein solution was dialyzed by a 10 mM pH 7.5 sodium phosphate buffer for 12 h at room temperature. CD measurements (180−250 nm) were performed with a Jasco-715 instrument in a quartz cuvette with a 1 mm path length. For the measurements, the protein concentration was adjusted to 5−7 μM. Each sample was scanned three times at 20 nm/min with a step length of 0.5 nm.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry was conducted with a VP-DSC calorimeter (Microcal, Inc., North Hampton, MA). BCA of 5 μM (0.6 mL per cell) was prepared with a 25 mM, pH 7.5 sodium phosphate buffer. The data were recorded when the temperature was raised from 30 to 90 °C at a rate of 1 °C per min.

Hydrase and Esterase Activity Assay. Hydrase activity was determined by the calorimetric method proposed by Wilbur and Anderson.[29] 50 μL of enzyme solution of appropriate concentration and 2 mL of a 2 °C saturated CO_2 solution were added into 2 mL of 20 mM Tris sulfate, pH 8.3 at 2 °C. The time required for the pH to change from 8.3 to 6.3 was recorded by a pH meter (59003-05 Cole Parmer). The hydrase activity was determined according to the following equation,

$$U = 20(T_0/T - 1)/(\text{mg protein})$$

in which T_0 and T represent the time required for the pH to change from 8.3 to 6.3 in the reaction without and with BCA, respectively.

Esterase activity assay was performed according to the method proposed by Pocker and Stone.[30,31] A stock solution of *p*-nitrophenylacetate was prepared with acetonitrile, while the free BCA or the BCA nanogel was dissolved in a 25 mM, pH 7.5 phosphate buffer. The initial rate of product formation was determined with a Shimadzu MultiSpec 1501 UV−vis spectrometer at 348 nm (ϵ348 nm = 5000 M^{-1} cm^{-1}), with the blank solution as the control. The Michaelis−Menten equation was applied for both the free and the BCA. The kinetic parameters were obtained via the Lineweaver−Burk plot.

For the thermal deactivation experiment, the BCA nanogel of 70 μM was incubated at 75 or 37 °C for a given time and then sampled out at specific interval into vials. After 2 h incubation at 4 and 25 °C, respectively, the sample was subjected to hydrase activity assay.

Results and Discussion

Formation of Single BCA Nanogels. A two-step procedure including surface acryloylation and in-situ aqueous polymerization to encapsulate a single enzyme in nanogel[24] was applied to fabricate a single BCA nanogel. As shown by Scheme 1, after generating vinyl groups on the protein surface by reacting with *N*-acryloxysuccinimide in alkali buffer, an aqueous in-situ polymerization of a protein is carried out, encapsulating one acryloylated protein molecule into a nanogel. The polymerization step can be repeated conveniently by adding monomers and cross-linkers to react with the primary nanogel so to give

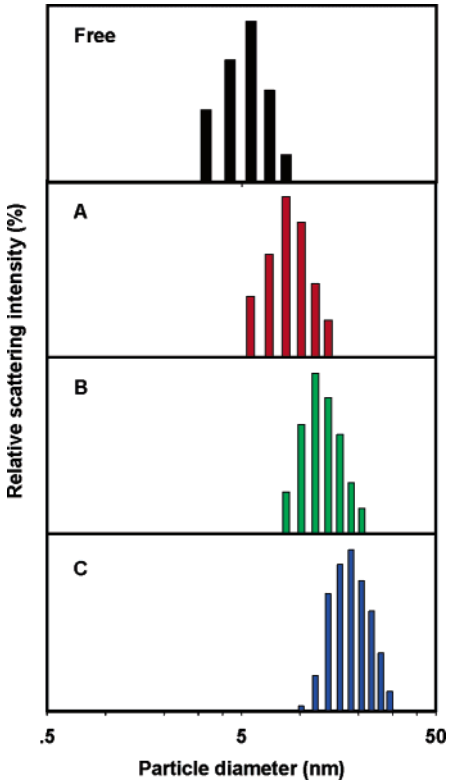


Figure 1. DLS of BCA nanogels (free − free BCA; A, B, C − BCA nanogels A, B, C).

the final product that contains a single enzyme of an expected size and shape.

Acryloylation of the ϵ-amino lysine groups of BCA into the acryloylated form was performed according to Scheme 1. The amount of residual ϵ-amino lysine groups of BCA, as determined by the fluorescamine method described above, was less than 5%, indicating that 17 of the 18 lysine residues of BCA were acryloylated.

BCA nanogels were prepared at the molar ratio of monomer to the acryloylated BCA of 300 (hereafter noted as nanogel A), 600 (hereafter noted as nanogel B), and 900 (hereafter noted as nanogel C), respectively. The yield of enzyme encapsulation is 94.7%, 93.4%, and 92.1% for nanogels A, B, and C, respectively. The yield of polymerization is 89.6%, 92.3%, and 92.7% for nanogels A, B, and C, respectively. The protein nanogel was then subjected to gel fitration on Sephadex G-75 to remove the impurities with molecular weight below 70 kDa. However, it is also possible that the impurities with molecular weight over 70 kDa exist in the product, leading to an overestimation of the polymerization yield.

As shown in Figure 1, the average size of nanogels A, B, and C, as determined by DLS, was 9.1, 13.7, and 18.2 nm, and the polydispersity index was 1.18, 1.22, and 1.26, respectively. The average diameter of the free BCA determined by DLS is 5.6 nm, which agrees with the BCA dimension, 5.2 nm $\times$ 5.2 nm $\times$ 4.6 nm (Protein Data Bank, www.rcsb.org). Therefore, we arrive at the conclusion that each nanogel A contains a single BCA molecule. Recall that the diameter of nanogel A is 9.1 nm, and each nanogel B with diameter 1.7 nm can contain only one nanogel A and, consequently, one BCA. Similarly, each nanogel C contains only one nanogel B and one BCA as its core. However, the relatively large PDI of nanogels B and C indicates that it is also possible that a small portion of nanogels B and C contain more than one BCA.

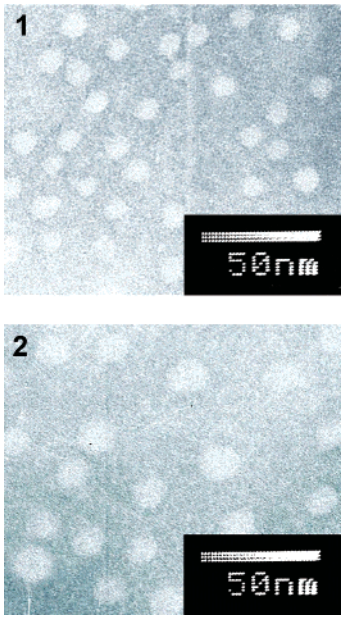


Figure 2. TEM of BCA nanogels (1, 2 − BCA nanogels B, C).

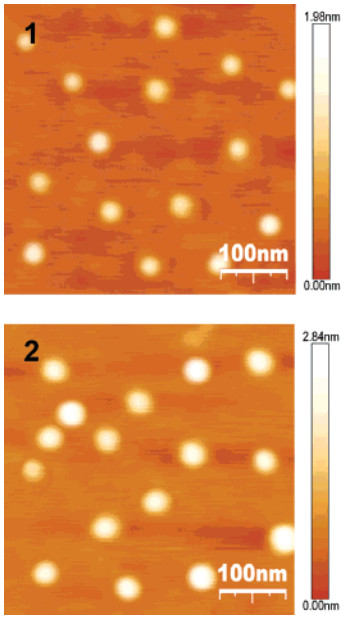


Figure 3. AFM of BCA nanogels (1, 2 − BCA nanogels B, C).

Figure 2 shows TEM images of the negatively stained nanogels B and C,[27] in which nanogel B appears spherical in shape with an average diameter of 11 nm. From the TEM picture, we assumed that nanogel B was nearly spherical and the encapsulated BCA maintained its original dimension. Thus, the depth of the polymer shell was interpreted as 2−3 nm. Further addition of the monomer to the reaction solution gave nanogel C with an average diameter of 16 nm. As compared to that determined by TEM, the DLS measurements gave a larger diameter for nanogels A, B, and C. This was due to their different sample treatment procedure in which the nanogel was swollen in the DLS measurement.

Figure 3 gives AFM images of nanogels B and C, in which the diameter is shown to be 33 and 40 nm, respectively. Because the radius of curvature of the AFM tip is 20 nm, the diameters of nanogels B and C are thus interpreted as 13 and 20 nm, respectively, being close to those determined by DLS and TEM.

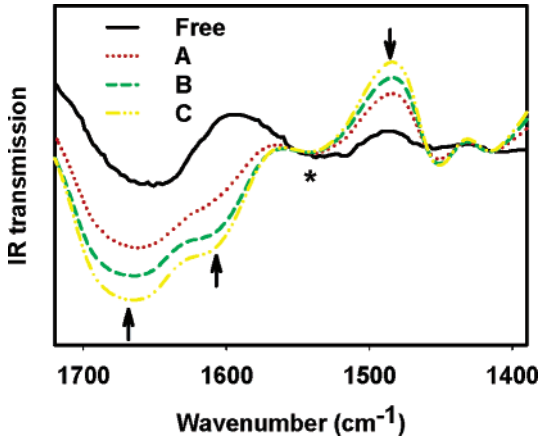


Figure 4. FTIR spectra of BCA nanogels (free − free BCA; A, B, C − BCA nanogels A, B, C; ↑ − characteristic peaks of polymer; * − characteristic peaks of protein).

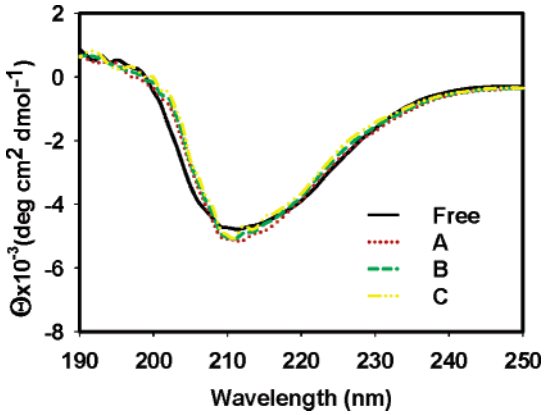


Figure 5. CD spectra of free BCA and BCA nanogels (free − free BCA; A, B, C − BCA nanogels A, B, C).

Table 1. Hydrase and Esterase Activity of Free BCA and BCA Nanogels

	hydrase activity		esterase activity		
BCA	units/mg	(%)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat}/k_m (%)	K_m (μM)
free BCA	2540 ± 154	100	513 ± 25	100	1804 ± 89
BCA nanogel A	1980 ± 86	78	368 ± 14	72	1845 ± 74
BCA nanogel B	1800 ± 92	71	356 ± 13	69	1865 ± 37
BCA nanogel C	1600 ± 103	63	336 ± 18	66	1878 ± 62

Moreover, the measurements by DLS, TEM, and AFM all confirm that each single nanogel contained only one BCA molecule.

Figure 4 shows the FTIR of the BCA nanogels, in which four characteristic peaks of polyacrylamide, 1670, 1610, 1430, and 1460 cm^{-1}, appear. The first three are the characteristic peaks of the amide group of polyacrylamide, and the last one is the characteristic peak of the methylene backbone of polyacrylamide.[32] Moreover, an increase in the polymer content gave a larger peak. The yield of the polymerization, determined according to the weight of lyophilized nanogels, was over 85% for A, B, and C.

Figure 5 displays the CD spectra of the free and the BCA nanogels, which shows that BCA nanogels retained the secondary structure of the free BCA.

Catalytic Activity of the Free BCA and the BCA Nanogels. Table 1 lists the hydrase activity of the free BCA and the BCA nanogels, as determined by the method described in the Experimental Section. Nanogels A, B, and C retain 78%, 71%, and 63% of the specific activity of the free BCA, respectively. The esterase activity of the free BCA and the encapsulated BCA is also shown in Table 1. The Michaelis−Menten parameters for the hydrolysis of *p*-nitrophenylacetate, k_{cat} and K_m, were determined. As shown in Table 1, k_{cat}/K_m of the BCA nanogels A, B, and C are 72%, 69%, and 66% of free BCA, while the K_m is very close to that of the free BCA. These suggest that the polyacrylamide porous gel does not hinder significantly the access of the substrate to the encapsulated BCA, while the encapsulated BCA can reproduce essentially the catalytic behavior of its free counterpart.

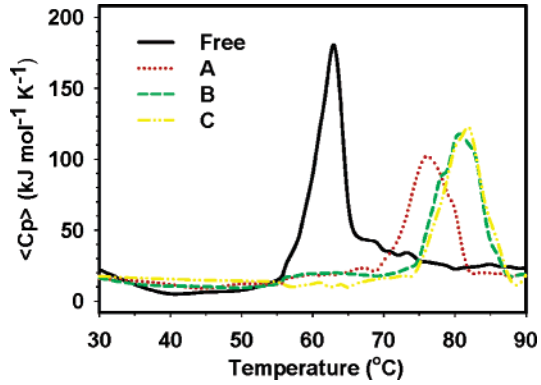


Figure 6. DSC curves of free BCA and BCA nanogels (free − free BCA; A, B, C − BCA nanogels A, B, C).

A significant reduction in apparent enzyme activity was often observed in enzyme immobilization or encapsulation, which was also seen with the BCA in the case of its immobilization on membrane surface or encapsulation in silica monolith, in which the residual activity was 7% and 1% of the original activity,[18,19] respectively. The reduction in enzyme activity is mainly due to the rigid matrix as an envelope that hinders (1) the access to the enzyme by its substrate, that is, the mass transport resistance, and/or (2) the enzyme conformation change required in the reaction, that is, the steric hindrance. In comparison, the encapsulation in the polyacrylamide nanogel had only a minor effect on the apparent activity of the enzyme. This is the major advantage of the present procedure that enables (1) the encapsulated protein to maintain its native conformation, as shown by the CD spectra (Figure 5), and (2) the accessibility to the encapsulated enzyme by its substrates, as shown by the K_m values listed in Table 1.

Thermal Stability of the Free BCA and the BCA Nanogels. The unfolding of the free BCA and the BCA nanogels was monitored via DSC. As shown in Figure 6, the melting temperature, T_m, is 63 °C for the free BCA in agreement with that reported elsewhere[11,12] and 76, 81, and 82 °C for the BCA nanogels A, B, and C, respectively. The BCA nanogels show a significant increase in T_m. Moreover, the higher is the molecular weight of the nanogel, the higher is the T_m.

The deactivation curves of the free BCA and the BCA nanogels were determined at 37 and 75 °C, respectively. As shown by Figure 7, the BCA nanogels exhibit significantly improved stability, particularly at 75 °C, at which the free BCA loses almost all of its activity in 5 min, while the activity of the BCA nanogels A, B, and C decreases to one-half of its original activity after 40, 90, and 100 min, respectively. At 37 °C, as shown in Figure 7b, the BCA nanogels maintain over 85% of their original activity after 48 h, whereas the free BCA loses 50% of its original activity in 24 h. Here, nanogels B and C show a similar thermal stability that is better than nanogel A. This indicates that there is an optimal content of the polymer

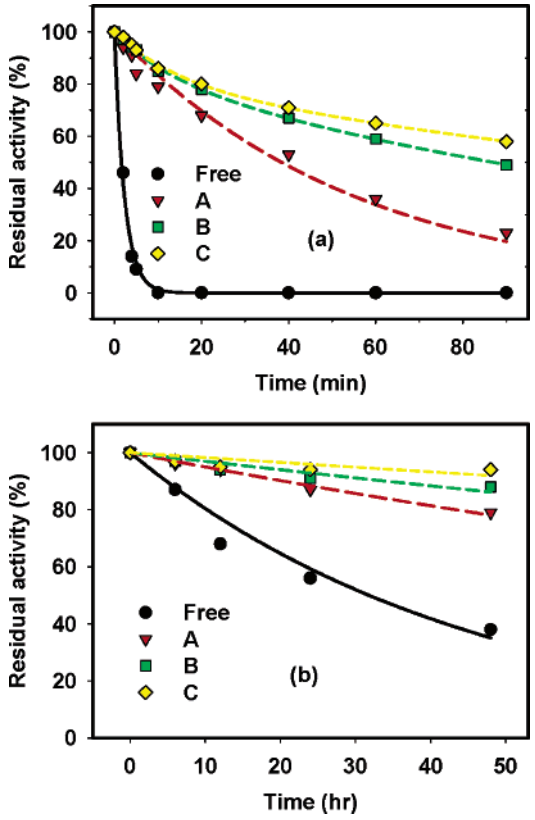


Figure 7. Thermal deactivation of free BCA and BCA nanogels at 75 °C (a) and 37 °C (b) (free − free BCA; A, B, C − BCA nanogels A, B, C).

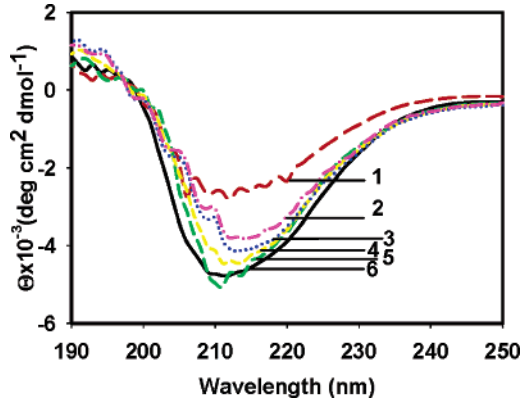


Figure 8. CD spectra of free BCA and BCA nanogel at 70 °C (1, 6 − free BCA at 10, 0 min; 2, 3, 4, 5 − BCA nanogel B at 30, 20, 10, 0 min).

in the conjugate. The same conclusion can also be drawn from the DSC diagram shown in Figure 6. From the above thermal stability and activity assay, it is concluded that nanogel B is the best among the three BCA nanogels.

Structure Stability of the Free and Encapsulated BCA at High Temperature. The changes in secondary structure of the free and encapsulated BCA at high temperatures were monitored by the CD spectra shown in Figure 8. BCA is a protein with a β-sheet structure, which has a characteristic adsorption of 217 nm.[11] At 70 °C, the CD spectrum of free BCA is significantly altered after 10 min, whereas that of the BCA nanogel B remains unchanged for at least 30 min. This enhanced stability is attributed to the encapsulation by multipoint covalent linkage[22,24,33] between 17 vinyl groups of the acrylated BCA and the polyacrylamide network that strengthened BCA structure.

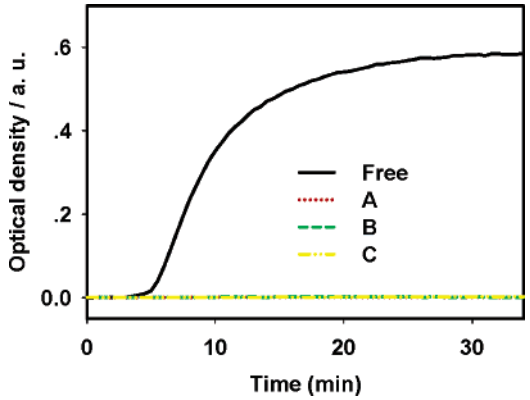


Figure 9. Optical density changes of free BCA and BCA nanogels at 75 °C (free − free BCA; A, B, C − BCA nanogels A, B, C).

Inhibition of BCA Aggregation at High Temperatures. Akiyoshi have confirmed the heat-induced aggregation of the free BCA (0.05 mg/mL), using the light scattering method at 500 nm.[15] As shown in Figure 9, the free BCA incubated at 75 °C aggregates within 5 min, whereas the BCA nanogel B remains stable until 300 min. Moreover, we have observed the aggregation of the free BCA at 37 °C after 24 h and at 25 °C after 72 h (data not shown here), as determined by HPLC,[13] which has not occurred to the BCA nanogels. This suggests that the hydrophilic polyacrylamide shell has also effectively inhibited the aggregation driven by intermolecular hydrophobic interactions, in addition to strengthening the molecular structure of BCA. This also agrees with our previous experiments and simulation that hydrophilic polymer can prevent the protein aggregation[34,35] and stabilize the enzyme in catalysis.[36] It was reported that, in the hydration of CO_2, BCA aggregation occurred when it was applied at a concentration over 50 μM.[6] Thus, the inhibition of BCA aggregation by encapsulating into a nanogel, as shown in Figure 9, is of practical interest because this allows a higher density load of BCA as the catalyst and a longer operation period of the enzyme reactor.

Conclusion

Encapsulation of a single bovine carbonic anhydrase II (BCA) into a polyacrylamide nanogel was accomplished by a two-step in-situ polymerization in aqueous solution, in which the first step was to generate vinyl groups on the surface of BCA. The nanogels were spherical and had a narrow size distribution, as characterized by DLS, TEM, and AFM. The encapsulation into nanogels had a very minor impact on both the catalytic behavior of BCA and its accessibility to the substrate, as suggested by the minor changes in K_m, but significantly increased the thermal stability, as shown by the increase in the molten temperature from 64 to 81 °C and the extension of the half-life at 75 °C from less than 5 to over 90 min. CD spectra showed that the loss of the secondary structure that occurred to the native BCA in free form at high temperatures was essentially depressed by the encapsulation. Aggregation of the free BCA in aqueous solution, driven by intermolecular hydrophobic interaction and being intensified at high temperatures and high concentrations of BCA, was also significantly depressed by the encapsulation into a polyacrylamide nanogel. The multi-point covalent linkage with the hydrophilic and porous polyacrylamide gel enables the encapsulated BCA to reproduce the catalytic properties of the native BCA in free form, strengthen significantly the structural stability, and effectively inhibit the formation of protein aggregate occurred to free BCA, particularly at high tempera-

tures and high concentrations. All of these are of fundamental and practical importance for BCA-catalyzed processes.

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