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Biomimetic CO_2 Sequestration: 1. Immobilization of Carbonic Anhydrase within Polyurethane Foam

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Bovine carbonic anhydrase (CA) was immobilized within polyurethane (PU) foam for biomimetic CO_2 sequestration. The catalytic activities for the free and immobilized CA were estimated using *para*-nitrophenyl acetate (*p*-NPA) as the substrate. Because the *p*-NPA has limited solubility in the aqueous phase, the activities were estimated in Tris buffer containing 10% acetonitrile. A Lineweaver−Burk relationship was employed to estimate the Michaelis−Menten kinetic parameters for the free and immobilized CA. The k_{cat}, K_m, and k_{cat}/K_m values for the free CA were found to be 2.02 s^{-1}, 12.2 mM, and 166.4 M^{-1} s^{-1}, respectively. The K_m value for the immobilized CA was estimated to be 9.6 mM at the same conditions. The immobilized CA was stable and did not lose any activity over seven consecutive washings and activity tests. While the free CA lost its activity in 45 days stored at 4 °C in refrigerator, the immobilized CA maintained 100% of its activity over a 45 day period stored in Tris buffer at ambient conditions. It was concluded that the immobilized CA as a very stable biocatalyst could be employed in biomimetic CO_2 sequestration.

1. Introduction

The atmospheric concentration of carbon dioxide (CO_2) has been increasing because of anthropogenic activities.[1] To stabilize CO_2 in the atmosphere, a number of CO_2 sequestration methods have been proposed.[2] In these sequestration options, CO_2 has to be pure, for which CO_2 has to be captured from the flue gases, compressed to the desired pressures, transported to a sequestration site, and finally, injected within the sequestration area for long-term storage.[3] All of these processes increase the sequestration cost.[4,5] The sequestration cost could be reduced if CO_2 is captured and sequestered directly at the production sites. The production of valuable products from CO_2, such as nanosized $CaCO_3$, which is extensively used as fillers or pigment in paper, paints, and plastics industries, could make the sequestration process economically feasible.[6,7] In this case, the CO_2 should undergo a number of transformations, such as the dissolution in an aqueous phase (eq 1), hydration by water (eq 2), ionization (eq 3), carbonate formation (eq 4), and finally, the precipitation by the available Ca^{2+} ions (eq 4).[8] Among these, the hydration of CO_2 (eq 2) is the slowest step.[9]

$$CO_2(g) \rightleftharpoons CO_2(aq) \quad (1)$$

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \quad (2)$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (3)$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+ \quad (4)$$

$$Ca^{2+} + CO_3^{2-} \rightleftharpoons CaCO_3 \quad (5)$$

When the pH is higher than 10, eq 6 dominates for the carbonate formation.[10] When the pH is less than 8, eq 6 is negligible.

$$CO_2(aq) + OH^- \rightleftharpoons CO_3^{2-} + H^+ \quad (6)$$

Attempts have been made to enhance the hydration of CO_2 in the presence of a biocatalyst, carbonic anhydrase (CA).[8,11,12] The CA (EC 4.2.1.1) is a zinc-containing metalloenzyme that mainly catalyzes the reversible hydration of carbon dioxide (CO_2).[13] The CAs can also catalyze the dehydration of various aldehydes and the hydrolysis of esters, which are very useful for activity assays.[14−16] There are at least

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five classes of CA, structurally characterized as α, β, γ, δ, and ε classes.[17−19] These CAs are found in most eukaryotic and many microbial organisms. α-CA belongs to mammals and divided into four subgroups: cytosolic, mitochondrial, secreted, and membrane bound.[20] There are 16 different isozymes in mammals, and at least 10 of them are human isozymes.[21] β-CAs belong to prokaryotic and plant chloroplast.[22,23] γ-CAs are from methane-producing bacteria that grow in hot springs.[20] Recently, δ- and ε-CA have also been identified.[24] The catalytic active site of CAs consists of a Zn^{2+} ion, which forms coordination bonds with the nitrogen atoms of three histidine residues.[19] There are 18 lysine groups in the amino acid sequence of CA, and most of them are at the surface of the enzyme.[25] The lysines containing amine groups provide the enzyme-efficient immobilization. The mechanism of the hydration of CO_2 by the CA enzyme is initiated by a nucleophilic attack on the carbon atom of CO_2 by the zinc-bound OH group to produce bicarbonate, which is then displaced from zinc by a water molecule.[8] The diverse classes of CA have different activities for CO_2 and other substrates.[15−17,25−28]

Carbon dioxide was shown to be used in biomimetic processes. Bond et al.[10] developed an integrated system for biomimetic CO_2 sequestration, which employed the enzyme CA to accelerate the rate of CO_2 hydration for the subsequent fixation into stable mineral carbonates. Mirjafari et al.[8] investigated the application of the CA to enhance the hydration of CO_2 in the solution. Liu et al.[12] studied the precipitation of $CaCO_3$ from produced waters in the presence of the CA enzyme. However, there are some disadvantages using the free enzyme in solution, such that the stability of the enzyme is low, its repeatable usage is limited, and recovery from the reaction environment generally will not be possible. These disadvantages can be eliminated by immobilizing the enzyme. There are some enzyme immobilization techniques, such as entrapment in matrices, adsorption on the solid surfaces, covalent bonding, and cross-linking within polymeric networks.[29] CA has been immobilized on some solid and polymeric supports. Drevon et al.[25] immobilized CA in coatings by covalent attachment. Liu et al.[12] immobilized CA on chitosan−alganite beads by encapsulation. Jovica and Kostic[30] immobilized CA within silica monoliths by the sol−gel method. Cheng et al.[31] immobilized CA within the poly(acrylic acid-co-acryamide) hydrogel by entrapment. Hosseinkhani and Gorgani[32] immobilized CA on a hydrophobic adsorbent of Sepharose 4B by adsorption. The immobilization in coatings as well as entrapment within sol−gels and alginate beads would yield the diffusion limitation, whereas the adsorption on the solid adsorbents would yield a detachment of the enzyme during the process. Therefore, more efficient immobilization methods are needed.

Polyurethanes (PUs) have been shown to be widely used supports for immobilization of enzymes and cells.[33] Wood et al.[34] patented a method of irreversibly immobilizing enzymes within PU in 1982. Today, their method is still used in enzyme and cell immobilization and includes mixing of a HYPOL prepolymer with the aqueous enzyme or cell solution.[33−37] Figure 1a shows a chemical structure of the HYPOL as the prepolymer of poly(ethylene glycol) capped with isocyanate-terminated end groups.[38] Figure 1b shows a possible mechanism of immobilization of enzymes within the PU foam and the polymerization of the prepolymer in the presence of water, which are deduced from the isocyanate chemistry.[39] The polymerization is initiated by a nucleophilic attack by an OH^- at the carbonyl group following a protonation and deprotonation and a release of CO_2 from an unstable intermediate, thus converting one isocyanate group into an amine group (NH_2). The produced $-NH_2$ groups react immediately with a neighboring isocyanate group, resulting in a cross-linking between two prepolymer chains. This process continues until all of the isocyanate groups were consumed. As a result, the generated CO_2 causes a porous and sponge-like polymeric matrix of the PU foam. Because the amine and/or hydroxyl groups are readily available on the surface of the enzyme, the enzyme is preferentially cross-linked by the isocyanates of the prepolymer. Consequently, a covalently immobilized enzyme containing biocatalytic material can be obtained in a cross-linked form. The advantages of this assay are that the process is faster and higher activity retention could be obtained.[33−36,40]

Although PUs are widely used supports for immobilization of enzymes and cells,[33,35−37,40] to the best of the author's knowledge, the CA has not been immobilized within PU foam. In the present work, the CA was immobilized within PU foam covalently by cross-linking for the biomimetic CO_2 sequestration purposes.

2. Materials and Methods

2.1. Materials. CA from bovine erythrocytes (MW, 29 000; 89% pure in protein as dialyzed and lyophilized powder), *para*-nitrophenyl

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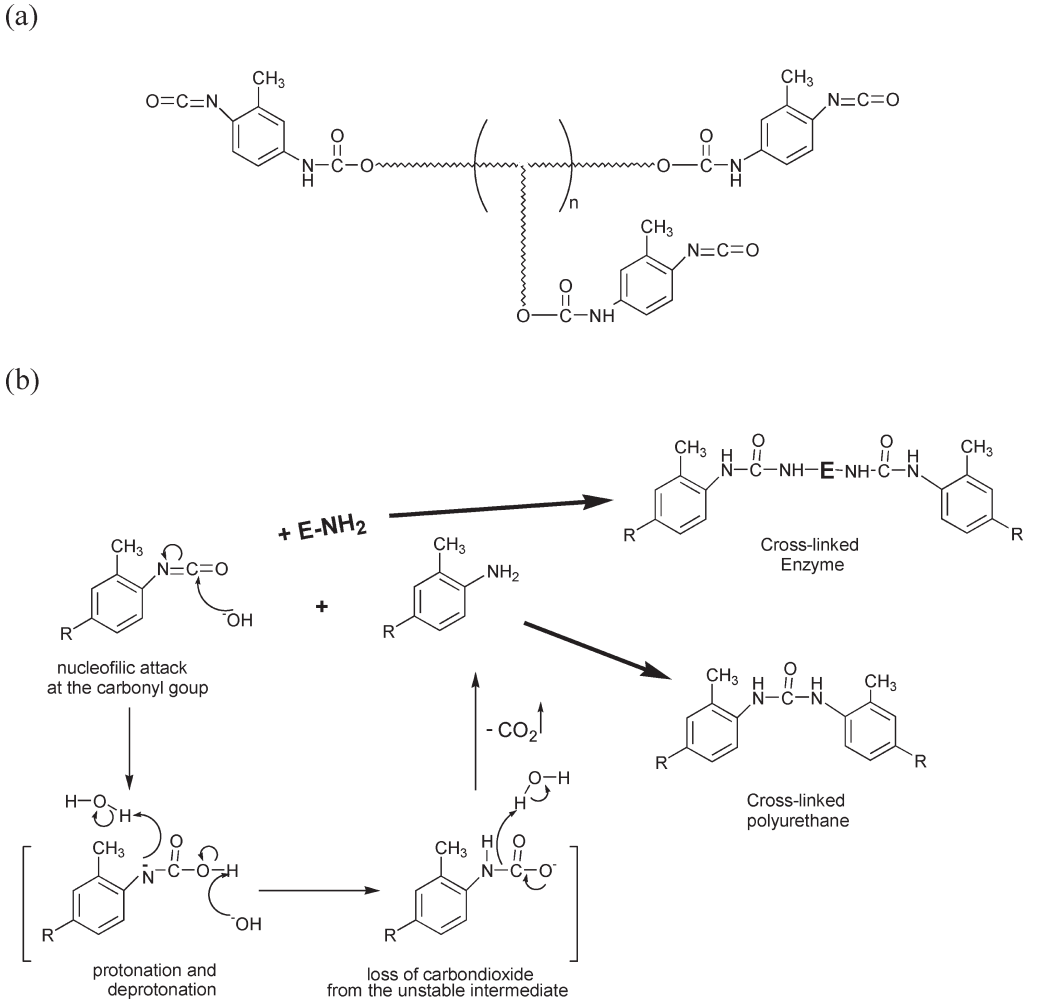

Figure 1. (a) Chemical structure of the HYPOL prepolymer and (b) mechanism of enzyme immobilization by cross-linking within PU foam deduced from isocyanate chemistry.[39]

acetate (*p*-NPA), and *para*-nitrophenol (*p*-NP) were all purchased from Sigma-Aldrich. Acetonitrile (99.9%, v/v), sodium hydroxide (97%, w/w), and hydrochloric acid (35%, v/v) were purchased from Merck. PU prepolymer, HYPOL-2060, was provided as a kind gift from Dow Chemical Co., Turkey.

2.2. Immobilization of CA within PU Foam. The immobilization of CA within PU foam was achieved in a similar fashion as described in refs 35 and 36. Briefly, a known amount of CA enzyme in powder form was dissolved in 3 mL of DI water and poured onto about 3 g of viscous HYPOL2060 prepolymer in a 50 mL falcon tube. The two-phase system was mixed vigorously for 30 s to achieve a homogeneous distribution of the enzyme within the prepolymer. The level of the white polymeric solution started to increase as a result of a CO_2 release during the polymerization. The level and, thus, the polymerization settled in 3−5 min. Another 10 min were allowed for curing. After completion of the enzyme immobilization, a piece of foam was cut from the middle of the whole product and assayed for immobilized CA activity.

This procedure was repeated for the immobilization of various amounts of CA within about 3 g of HYPOL prepolymer.

2.3. CA Activity Assays. The enzyme activities were measured at 25 °C by monitoring the changes in the concentration in *p*-NP, which is one of the hydrolysis products of *p*-NPA. For the free CA, the activity assay was performed in a 1 mL UV cuvette. The reaction mixture, composed of 0.8 mL of Tris buffer (50 mM, pH 7.5), 0.1 mL of substrate solution (*p*-NPA dissolved in acetonitrile), and 0.1 mL of enzyme solution (the CA in Tris buffer), was mixed in the cuvette by a micropipet. Thus, the final mixture contained 10% of acetonitrile. The enzyme activity was measured in a Perkin-Elmer UV/vis spectrophotometer at 400 nm for 3 min. Different concentrations of substrate when the enzyme concentration was constant as well as different concentrations of enzyme when the substrate concentration was constant were tested to determine the free enzyme activity. Blank experiments were also conducted to estimate the self-dissociation of *p*-NPA in each assay solution.

The immobilized CA activity was estimated in a similar fashion as described in refs 35 and 36. Briefly, a piece of CA-immobilized PU foam was washed by squeezing several times in a fresh Tris buffer. In a 30 mL vial, 1 mL of substrate solution (*p*-NPA dissolved in acetonitrile) was mixed with 9 mL of Tris buffer (50 mM, pH 7.5) on a magnetic stirrer. Therefore, the final solution contained 10% acetonitrile. The reaction started once the initially washed and soaked foam was added into the substrate-containing mixture. In every minute, 1 mL of sample was withdrawn from the reaction mixture, its absorbance was measured in the UV/vis spectrophotometer at 400 nm for a short time, and the sample was returned back to the reaction mixture. This procedure was repeated at 1 min intervals for 25−30 min. At the end of the assay, the foam was washed and dried at 80 °C under vacuum, after which it was weighed for analysis. Different amounts of foam when the substrate concentration was constant as well as different concentrations of substrate when the same foam was used were tested to determine the immobilized enzyme activity. For the latter, the same foam was washed by squeezing several times in Tris buffer, and the next assay was initiated by adding it to a fresh reaction mixture.

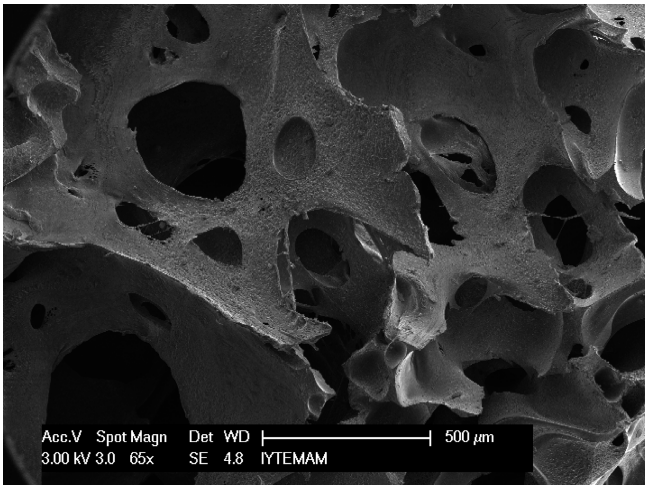

Figure 2. Scanning electron microscopy (SEM) image of the PU foam.

2.4. Reusability of Immobilized CA. A stock solution of *p*-NPA was prepared in acetonitrile. A slice of enzyme-immobilized foam was washed in Tris buffer 5 times and then soaked in fresh Tris buffer. The reaction was initiated by adding the enzyme-immobilized foam into the reaction mixture composed of 1 mL of stock solution of *p*-NPA and 9 mL of Tris buffer (50 mM, pH 7.5). The absorbance of the solution was measured at 400 nm in a UV/vis spectrophotometer at 1 min intervals for 25−30 min. This procedure was repeated 7 times for the same slice of the enzyme-containing foam sample.

2.5. Stability of Free and Immobilized CA. The stability of the free CA was estimated by the free enzyme activity assay. The known amount of powder CA was dissolved in Tris buffer (50 mM, pH 7.5) and stored in the refrigerator at 4 °C. Aliquots from this stock solution were used in the free enzyme activity assay every 3−4 days over 45 days. The stability of the immobilized CA was estimated using the same piece of foam sample stored in Tris buffer (50 mM, pH 7.5) at room temperature. The foam sample was washed by squeezing several times in Tris buffer and assayed for the immobilized enzyme activity. At the end of the enzyme activity, the foam was washed again and stored in the Tris buffer until the next activity assay. The activity tests were repeated in every 3−4 days over 45 days.

3. Results and Discussion

3.1. Immobilization of CA within PU Foam. CA was immobilized within PU foam. When isocyanate groups were reacted with water, a gas, carbon dioxide, was released during the immobilization, which resulted in local gas bubbles in the polymeric network. These bubbles created large pores, which made the PU foam a sponge-like material. Those pores were as large as 500 μm in size, as shown in Figure 2, which were much larger than about 4 nm of the size of an enzyme with a molecular weight of 29 000.[41] The larger pores did not affect the enzyme immobilization because the enzyme was immobilized within the polymeric network covalently with multiple attachments rather than by an entrapment or adsorption mechanism. Also, the hydrophilic nature of the PU sponge and large porosity made the PU foam advantageous for enzyme immobilization, where the substrates could easily access the hydrophilic foam and the substrates or the products could easily diffuse in and out of the sponge containing the enzyme.[36] Therefore, the PU foam was found to be a good support material for the CA immobilization.

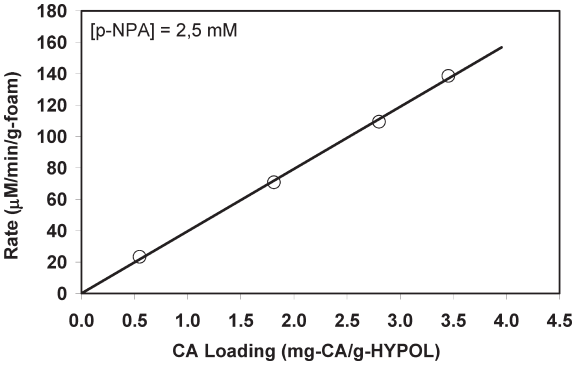

Figure 3. CA loading within PU foam.

How much CA could be immobilized within PU foam was tested by mixing different amounts of enzyme with about 3 g of HYPOL prepolymer. The activities were measured with the immobilized enzyme activity assay on a piece of sample from each loading. The amount of CA enzyme per gram of HYPOL versus the activity as micromolar *p*-NP produced per minute per gram of foam were reported, as shown in Figure 3. As shown in the figure, the enzyme-loading capacity was linear up to 4 mg of CA/g of HYPOL. This capacity could be higher when considering the total reactive groups available, such as a 6.2×10^{-7} mol of lysine/mg of CA and 1.5×10^{-3} mol of isocyanate/g of HYPOL when there are 18 lysines on a CA with a molecular weight of 29 000[25] and 3 isocyanate groups per a HYPOL prepolymer with a molecular weight of 2060.[38] It is hard to tell theoretically how much capacity could be achieved because there are multiple functional groups involved in the cross-linking. The linear trend would indicate the efficiency of immobilization, which would be the same for each immobilization up to 4 mg of CA loading/g of HYPOL.

3.2. Activities of Free and Immobilized CA. The activity of the free CA was estimated using *p*-NPA in the liquid phase instead of gaseous CO_2 as the substrate. Although the CA activity was defined as 1 Wilbur−Anderson (W−A) unit, which causes the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3/min at 0 °C,[42] this method uses a CO_2-saturated solution within 20 mM Trizma buffer and measures the pH drop from 8.3 to 6.3 at 0 °C. With this respect, Mirjafari et al. investigated the hydration of CO_2 by pH variation and they could capture only 2−4 pH data in 3 s time intervals.[8] They estimated the hydration rate in these scattered and limited data points. Similar data were reported by Bond et al., where the pH drop occurred "stepwise" in less than 25 s.[10] The enzyme activity estimated from such data could bring large uncertainties. Therefore, the *p*-NPA was selected as the substrate for the free and immobilized CA enzyme activity determination. Also, the selection of *p*-NPA resulted in repeatable experimental results. The self-hydrolysis of the *p*-NPA was investigated in a separate study, and it was found that the rate of self-hydrolysis was concentration- and temperature-dependent, for which it obeyed a first-order kinetic. Here, those data were not reported, but all of the self-hydrolysis rates were subtracted from the raw data of the

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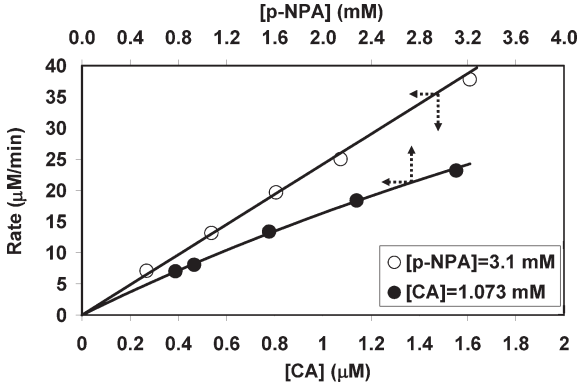

Figure 4. Effect of the substrate concentration as well as the enzyme concentration on the free CA activity.

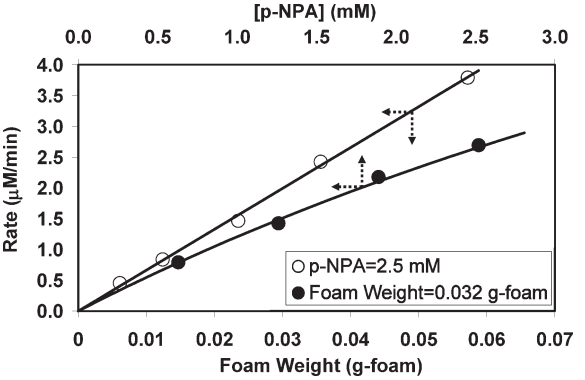

Figure 5. Effect of the substrate concentration as well as the foam weight on the immobilized CA activity.

enzyme-catalyzed hydrolysis rates and reported solely as the CA activity in the given conditions.

The activity of the free CA was estimated at different substrate concentrations while keeping the enzyme concentration constant. The substrate concentration of *p*-NPA could only be used up to 3.1 mM because of the limited solubility of *p*-NPA in the aqueous phase. The activity of the free CA was also estimated at various enzyme concentrations while keeping the substrate concentration constant. Figure 4 shows the activity of the free CA in Tris buffer (50 mM, pH 7.5) in the presence of 10% acetonitrile. The continuous lines shown in the figure are the curve fit of the experimental data to the Michaelis−Menten equation (eq 7) using the estimated kinetic parameters

$$R = \frac{k_{cat}[E][S]}{K_m + [S]} \tag{7}$$

where R is the rate for *p*-NP generation, R_{max} ($=k_{cat}[E]$) is the maximum rate when the substrate concentration is infinite, k_{cat} is the catalytic rate constant or the turnover number, [E] is the enzyme concentration, [S] is the substrate concentration, K_m is the substrate concentration when the rate is equal to $R_{max}/2$, which also shows the affinity of the enzyme for the substrate, and k_{cat}/K_m is the kinetic constant when $[S] \ll K_m$. The kinetic parameters were obtained from the Lineweaver−Burk equation of the double-reciprocal (eq 8).

$$\frac{1}{R} = \frac{1}{R_{max}} + \frac{K_m}{R_{max}}\frac{1}{[S]} \tag{8}$$

The kinetic parameters for the free CA were estimated as $k_{cat} = 2.02\ s^{-1}$, $K_m = 12.2$ mM, and $k_{cat}/K_m = 166.4\ M^{-1}\ s^{-1}$. Because different types of CAs have different activity with different kinetic constants, it was hard to make an easy comparison between the present data and the literature. For instance, the K_m values for the bovine CA II,[26] the human CA II,[16] and the human CA I[16] were 76.9, 30.53, and 3.02 mM, respectively, and the k_{cat}/K_m values for the same enzymes were 960, 2607, and 753 $M^{-1}\ s^{-1}$, respectively, for *p*-NPA as the substrate. The k_{cat}/K_m value for the bovine CA was estimated to be 166.4 $M^{-1}\ s^{-1}$, which is in good agreement with the available literature of 153.3 $M^{-1}\ s^{-1}$.[15] Also, because the [S] versus rate graph resulted in a linear-like trend at low substrate concentrations, some kinetic date, such as k_{cat} and K_m, were not reported in the literature; instead, k_{cat}/K_m values were reported as the slope.[15,16,25,26,28,43] This is somehow correct if $[S] \ll K_m$. However, this would be not the case for the bovine CA, where the substrate concentration was up to 3.1 mM and was not much lower than the K_m value of 12.2 mM.

The catalytic activity of the immobilized CA was estimated from the liberation of *p*-NP over time by the hydrolysis of *p*-NPA as the substrate. The self-hydrolysis rate for the *p*-NPA was also subtracted from the catalytic hydrolysis rates. Figure 5 shows the experimental data and the curve fit to the Michaelis−Menten model (eq 7). The immobilized enzyme assay was employed for a single foam sample at various substrate concentrations, where the foam contained a fixed amount of CA. Before and after the assay, the piece of foam was washed by squeezing in Tris buffer and used in the assay for the subsequent substrate concentrations up to 2.5 mM. In another catalytic assay, the foam samples were cut at various weights and washed in Tris buffer and then each piece of foam sample was assayed at a constant substrate concentration of 2.5 mM. As can be seen in the figure, a linear trend was seen between the foam weight and rate, whereas a nonlinear trend was obvious between the substrate concentration and the catalytic rate. The experimental data were evaluated by employing a double-reciprocal Lineweaver−Burk equation (eq 8). From the data, the K_m value for the immobilized CA was found to be 9.6 mM for *p*-NPA in Tris buffer (50 mM, pH 7.5) in the presence of 10% acetonitrile. Here, the only amount of CA originally mixed with about 3 g of HYPOL was known during the immobilization. However, how much enzyme was immobilized and how much of it was active in the sponge were not known. In other words, the enzyme and the foam became a composite polymeric material, and the amount of enzyme that was essential for the calculation of k_{cat} and k_{cat}/K_m values could not be estimated accurately within the foam. Therefore, the k_{cat} and k_{cat}/K_m values could not be reported for the immobilized CA. The K_m value of 9.6 mM for the immobilized CA was slightly lower than the K_m value of 12.2 mM for the free CA. On the other hand, these values were very close when comparing the K_m values of 76.9 and 30.5 mM for the bovine CA-II[26] and human CA-II,[16] respectively, for the same substrate, *p*-NPA. The difference could be due to the diffusion effect and/or the partition of *p*-NP between the foam and buffer.[36]

3.3. Reusability of Immobilized CA. The most important feature of a covalent attachment of an enzyme is that the immobilized enzyme can hold a constant activity when it is

(43) Pocker, Y.; Stone, J. T. *Biochemistry* **1968**, *7* (11), 4139–4145.

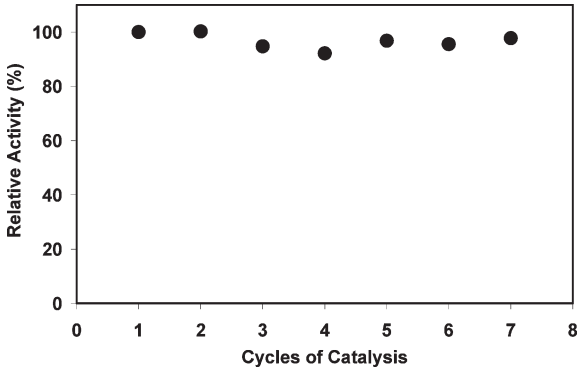

Figure 6. Cycle of usage for CA immobilized within PU foam.

used again and again. High reuse capacity is also crucial for the industrial applications because the production and purification of native enzymes are expensive. Figure 6 shows the reuse capacity of the CA enzyme immobilized within the PU foam for 7 cycles. Between each cycle, the piece of foam was washed by squeezing several times in the Tris buffer and assayed for the immobilized enzyme activity. As shown in the figure, the immobilized enzyme maintained 100% of its activity during the whole cycles and it was anticipated that the activity would be maintained for subsequent cycles. The strong stability during the cycles of usage indicated that the CA has been covalently attached within PU foam and did not lose its activity by either denaturing or detachment from the polymeric structure.

3.4. Stability of Free and Immobilized CA. The storage stabilities of the free and immobilized CA were estimated simultaneously. The free enzymes can be stored in aqueous solution at low temperatures in a refrigerator to maintain their stability. On the other hand, the immobilized enzymes can be stored as dry or in aqueous solution at either low temperatures in a refrigerator or at laboratory conditions. It is certain that the enzymes are more stable when they were stored at low temperatures in a refrigerator. Here, the free CA was dissolved in Tris buffer (50 mM, pH 7.5) and stored at 4 °C in the refrigerator. The CA-immobilized PU foam was also placed in the Tris buffer but stored at ambient temperature in the laboratory. The activities of the free and immobilized enzyme were estimated by the free and immobilized enzyme assays using the same substrate concentrations in Tris buffer in the presence of 10% acetonitrile. Figure 7 shows the relative activities of the free and immobilized CA over time, where the rates were normalized with respect to the rates obtained for the first day. As shown in the figure, there was a dramatic loss in the activity for the free CA. The free CA has lost all of its activity in less than 45 days, although it was stored at 4 °C in the fridge. The stability of the free CA would have been much shorter if it was stored at room temperature. However, the immobilized CA maintained 100% of its activity up to 45 days even at room temperature. It can be deduced from the data that the

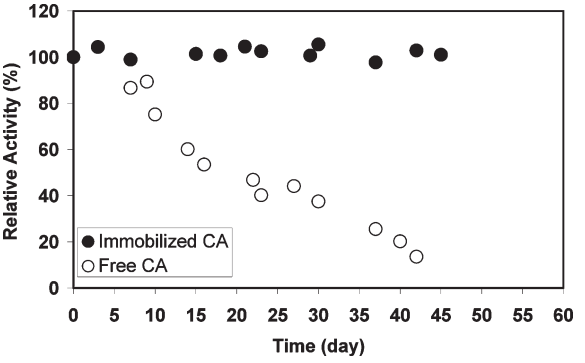

Figure 7. Stability of the free (at 4 °C) and immobilized (at room temperature) CA in Tris buffer.

immobilized CA could retain all of its activity for the subsequent duration of time. Improvement of the protein stability is important for enzymes for industrial processes. As shown in the figure, the half-life of the CA was prolonged noticeably by immobilization within the PU foam, which provided a better and convenient biocatalyst for the CO_2 sequestration applications.

4. Conclusion

The CA can enhance the hydration of CO_2 in aqueous solution. However, the free enzyme can lose its activity in solution in short times, and the repeatable usage of the free CA will not be possible when employed in CO_2 sequestration purposes. Also, the enzymes are expensive because of their purification costs, and they need to be immobilized for their repeatable usage. There are various methods for enzyme immobilization, such as adsorption on surfaces, entrapment within matrices, or cross-linking within polymeric scaffolds; however, there are some disadvantages, such as detachment from surfaces as in adsorption, mass-transfer limitations as in entrapment, and activity loss as in cross-linking. It was shown that the CA was successfully immobilized within PU foam. The PU foam was found to be a suitable support material for the CA immobilization because the PU foam was a highly porous polymeric material and was highly hydrophilic and the enzyme immobilization was easy and fast. The immobilized CA showed an excellent reuse capacity in aqueous solution, indicating that the enzyme was covalently bound within the polymeric matrix. The immobilized CA was maintained at 100% of its activity over time, and the excellent stability of the immobilized CA was astonishing. It was concluded that the immobilized CA could be used to accelerate the hydration of CO_2 in biomimetic CO_2 sequestration in an aqueous solution.

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