Enhanced carbonation reaction using chitosan-based carbonic anhydrase nanoparticles

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The objective of the present study was to develop a single enzyme nanoparticle of carbonic anhydrase (SEN-CA), formed by modifying the surface of CA with a thin layer of organic/inorganic hybrid biopolymer such as chitosan. SEN-CA enhances the rate of CO_2 hydration further, for subsequent fixation into stable mineral carbonates, compared to free CA. SEN-CA shows CO_2 sequestration capacity of 147 mg of $CaCO_3$/mg of CA, which is far better than CO_2 sequestration capacity of 35 mg of $CaCO_3$/mg for free CA under limiting concentration of CO_2 (14.5 mg of CO_2/10 ml). SEN-CA showed improved stability compared to free enzyme assayed by carbonation reaction.

Keywords: Carbonation reaction, carbonic anhydrase, single enzyme nanoparticle, stability.

CARBON DIOXIDE (CO_2) is a vital greenhouse gas whose atmospheric level has increased from 280 to 387 ppm since the Industrial Revolution, and is a major contributor to global warming. Biological sequestration of CO_2 is one of the proposed methods to capture anthropogenic CO_2. Carbonic anhydrase (CA) is used to hydrate CO_2 into bicarbonate and then into stable and environmentally benign calcium carbonate ($CaCO_3$)[1]. Each molecule of CA, a zinc metalloenzyme, can catalyse 1.4×10^6 molecules of CO_2 in one second[2–4].

$$CO_2 + H_2O \xleftrightarrow{CA} H^+ + HCO_3^-.$$

However, the short half-life of CA limits its usefulness. Different methods have been used to improve catalytic stability of enzymes such as immobilization, modification, protein engineering and genetic modification. In the recent past, considerable attempts have been made regarding enzyme stability, in the form of single enzyme nanoparticles (SENs)[5]. In SENs, an enzyme molecule is modified by enclosing it in a cage formed with a porous organic/inorganic structure less than a few nanometres thick. SENs of chymotrypsin and trypsin have been endorsed with great advantages such as increased stability and activity, high surface area, performance at high temperatures, stability to denaturation and aggregation[6], minimal mass-transfer limitation on substrates and improved half-life[7].

We have developed a protocol for the synthesis of single enzyme nanoparticle of carbonic anhydrase (SEN-CA) by modifying the surface of a single molecule with a hybrid organic/inorganic biopolymer silica network using biopolymers such as chitosan[8]. Chitosan, a natural polysaccharide, with reactive amino ($–NH_2$) and hydroxyl (–OH) groups appears to be a good and green substitute to the vinyl polymer, used by Kim and Grate[5], by virtue of its non-toxic nature and protein affinity. The present study reports the carbonation reaction using partially purified CA and SEN-CA.

Extracellular CA from *B. pumilus* TS1 was isolated by centrifuging the culture broth at 8000 rpm for 20 min, and concentrating CA from the supernatant by acetone (20–60% saturation) precipitation. The precipitate was then lyophilized. The lyophilized powder contained 6840 units/g of CA. This partially purified CA was provided by Department of Microbiology, University of Delhi, South Campus, New Delhi.

The SEN-CA synthesis has been reported in our earlier work[8]. It was prepared by dissolving CA according to the protocol given in Figure 1. The yield of CA activity in the form of SEN-CA was 50–70%. In the case of reagent blank we have followed the same synthesis protocol, but with no addition of CA. In the synthesis of SEN-CA, chitosan was used as such and it was modified with glutaraldehyde (GR grade, Merck) and hexadecyltrimethylammonium bromide (HDTMABr; GR grade, Merck) surfactant. Chitosan was modified using glutaraldehyde and surfactant to enhance its binding to CA.

The enzyme activity of CA was estimated spectrophotometrically using *p*-nitrophenyl acetate (*p*-NPA) as a substrate according to the method described by Armstrong *et al.*[9] with slight modification[8].

Wilbur–Anderson (WA) assay[10] was performed in a vessel maintained at 4°C using crushed ice with water-jacket and constant-temperature circulator. The vessel was sealed with a rubber-stopper and fitted with a pH electrode. Next, 50 µl sample was added to 3 ml of 20 mM Tris buffer solution, pH 8.3. The reaction was started by addition of 2 ml of CO_2 saturated water at about 4°C. CA activity was indicated by the time required for the pH to change from 8.3 to 6.3.

Carbonation study was carried out by the method reported by Favre *et al.*[4], with slight modification. In our procedure, 1 ml of Tris buffer (1 M, pH 8.3) was added to 10 ml of CO_2-saturated water (pH 3.57). The mixture was shaken at 25°C and then 10 ml of 2% $CaCl_2$ (pH 6.41) was added along with 1 ml (1 mg/ml loading) of the enzyme in phosphate buffer (0.1 M, pH 7.0). The final pH of the mixture was 6.85. The time required for the forma-

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tion of carbonate with respect to the onset of the reaction was monitored in the sample as well as control (without enzyme) by the turbidometric method. The carbonate obtained was filtered using Whatmann filter paper-42 and dried at room temperature. The results were also confirmed by gas chromatography (GC).

The precipitated carbonate was quantified using GC method[11] coupled with thermal conductivity detector (TCD). This eliminated the interference of other precipitates like calcium phosphate. The precipitated carbonate was treated with 0.5 M HCl and the CO_2 evolved was collected and analysed in GC/TCD using Porapak Q column.

For storage stability study, free CA and SEN-CA were prepared in bulk and stored as aliquots at 4°C for 30 days, and the carbonation capacity was determined at an interval of 5 days.

Transmission electron microscope (TEM) image of lyophilized SEN-CA was recorded using Tecnai-F20 equipped with an energy dispersive X-ray (EDX) analyser (FEI, The Netherlands). The lyophilized SEN-CA was dispersed in ethanol, and after 5 min, clear supernatant was dropped on a Cu grid covered with carbon. Fourier transform infrared analysis (FTIR) spectra of $CaCO_3$ precipitate (1 wt%) mixed with KBr pellets, which were obtained from free CA and SEN-CA, were recorded on a Bruker Vertex-70 by diffused reflectance accessory technique. Scanning electron microscope (SEM) study of the carbonate precipitate of SEN-CA was carried out using a JEOL JED-2300 SEM. The $CaCO_3$ precipitate was evaluated using GC (Model no. Perkin-Elmer Clarus 500).

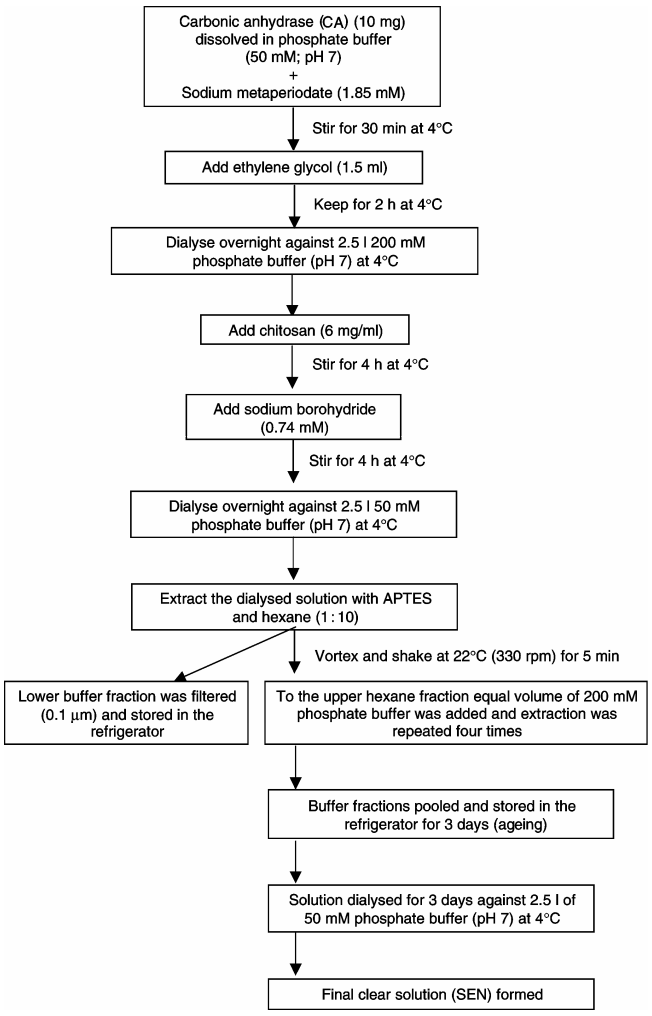


Figure 1. Protocol for single enzyme nanoparticle synthesis.

High-resolution TEM images show a distinct layer with the hollow centre being attributed to the presence of enzyme in the individual nanoparticles. The hollow transparent layer of the core protein is surrounded by the contrasting outer dark structure (Figure 2 *a*). EDX (Figure 2 *b*) confirmed the presence of silicon in the contrasting outer dark structures. The presence of CA in the transparent hollow centre has been confirmed by EDX (Figure 2 *b*) through the presence of Zn, which is used as a marker to confirm the presence of CA. Average size of these SEN particles appears to be in the range 70–80 nm.

Figure 3 shows the comparison of enzyme activity of different chitosan-based nanoparticles with respect to free CA by *p*-NPA assay. SEN-CA showed more specific activity compared to modified SEN-CA.

WA assay for free CA and SEN-CA was also performed to substantiate the research findings. The results presented in Table 1 correlate with the activities obtained for free and SEN-CA using *p*-NPA and carbonation reaction.

In the precipitation reaction, the time recorded for the onset of carbonate precipitate in SEN-CA is 37 s compared to free CA which is 20 s, whereas the same for SEN-CA treated with glutaraldehyde, surfactant and reagent blank is 40, 43 and 170 s respectively (Table 2). The results establish that SEN-CA is instrumental in accelerating carbonation reaction. Further studies are in progress to optimize the conditions for carbonation reaction elucidating the kinetics and mechanistic aspects. Table 3 shows the FTIR peaks obtained for the precipitated carbonate, which is compared with standard $CaCO_3$. Two prominent peaks of precipated carbonate obtained from SEN-CA, modified SEN-CA and free CA were observed at 712 and 874 cm^{-1} respectively, which coincided with the spectra of standard $CaCO_3$. Figure 4 shows the SEM image of $CaCO_3$ obtained from SEN-CA depicting well-defined faceted, rhombohedral structures characteristic of calcite crystals. Chitosan used in the synthesis protocol without modification (SEN-CA), showed reasonably good CO_2 sequestration capacity of 147 mg $CaCO_3$/mg CA, compared to CO_2 sequestration capacity of 35 mg $CaCO_3$/mg free CA under limiting concentration of CO_2 (14.5 mg CO_2/10 ml), as shown in Table 4 and Figure 5. SEN-CA modified with glutaraldehyde and surfactant showed CO_2 sequestration capacity of 52 mg $CaCO_3$/mg CA and 49 mg $CaCO_3$/mg CA respectively.

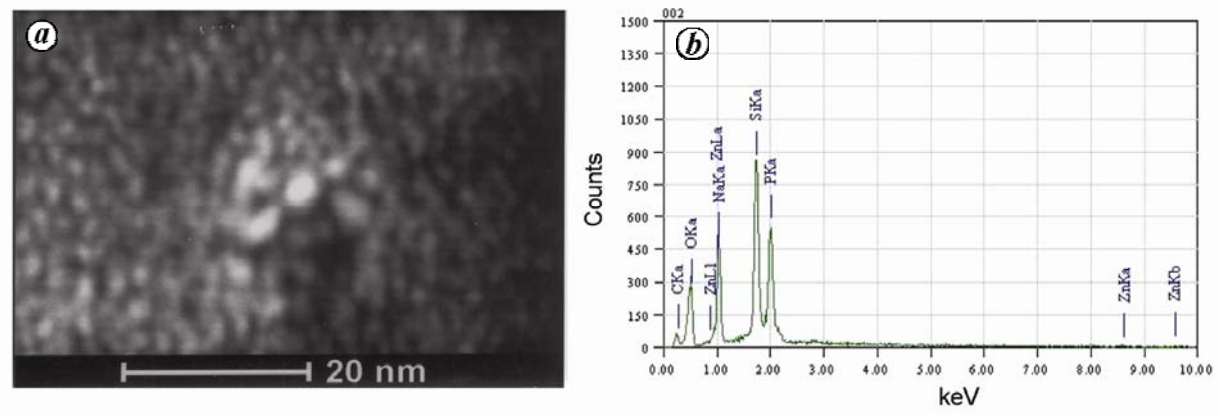


Figure 2. Transmission electron microscope (***a***) and energy dispersive X-ray (***b***) images of SEN-CA.

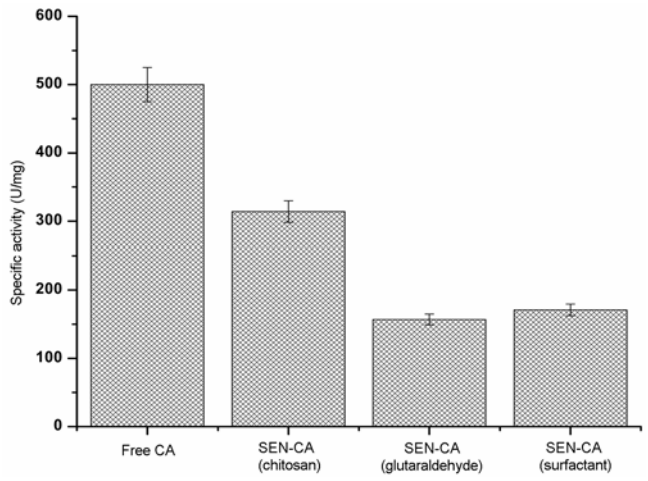


Figure 3. Comparison of enzyme activity of different chitosan-based nanoparticles with respect to free CA by *p*-nitrophenyl acetate assay.

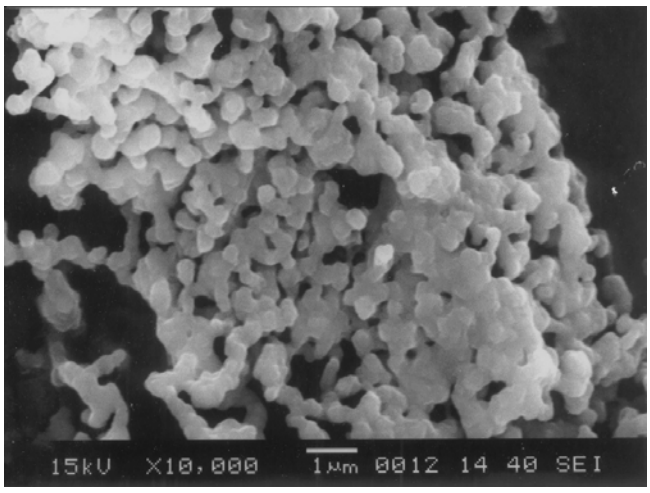


Figure 4. Scanning electron microscope image of carbonate precipitate of SEN-CA.

Table 1. Comparison of enzymatic activity of carbonic anhydrase (CA) obtained from different sources with single enzyme nanoparticle of carbonic anhydrase (SEN-CA) by Wilbur–Anderson (WA) assay

Sample	Source	WA activity (U)
CA (partially purified)	*Bacillus pumilus*	1600
SEN-CA (chitosan)	*Bacillus pumilus*	969
CA (ref. 12)	*Bacillus subtilis*	1560
CA (ref. 13)	*Chlamydomonas reinhardtii*	1200

Table 2. Summary of the time required for the onset of precipitation of calcium carbonate reaction

Sample	Time (s)
CA (partially purified)	20
SEN-CA (chitosan)	37
SEN-CA (glutaraldehyde)	40
SEN-CA (surfactant)	42
Blank without CA (reagent blank)	170
Blank without CA and chitosan (reagent blank)	230

Modification with glutaraldehyde and surfactant has not resulted in any improvement compared to the usage of chitosan as such (Table 4). In case of glutarldehyde, the bonding between glutaraldehyde and the amino group of chitosan (Scheme 1) resulted in less amino group being available for binding with the aldehyde (–CHO) group of the enzyme. In the case of surfactant (HDTMABr), the positively charged ion ($HDTMA^+$) interacts with chitosan which is a cationic polymer. This interaction seems

Table 3. Comparison of Fourier transform infrared spectra of different chitosan-based nanoparticles with respect to free CA

Peak for $CaCO_3$ (cm^{-1})	Free CA (cm^{-1})	SEN-CA (chitosan; cm^{-1})	SEN-CA (glutaraldehyde; cm^{-1})	SEN-CA (surfactant; cm^{-1})
712	712	714	711	713
874	874	869	870	871

Table 4. Summary of precipitation of calcium carbonate ($CaCO_3$) reaction

Sample	Weight of $CaCO_3$ (mg/ml)	SEN-CA – reagent blank* (mg of $CaCO_3$/ml of SEN-CA)	mg $CaCO_3$/ mg enzyme
Blank	12	–	–
Free CA	35	–	35
SEN-CA–chitosan	17.1	9.7	147
Reagent blank–chitosan	7.3	–	–
SEN-CA–chitosan–glutaraldehyde	7.7	3.5	52
Reagent blank–chitosan–glutaraldehyde	4.3	–	–
SEN-CA–chitosan–surfactant	6.7	3.3	49
Reagent blank–chitosan–surfactant	3.5	–	–
Reagent blank–without CA and chitosan	6.2	–	–

*Difference obtained by subtracting reagent blank from SEN-CA = 17.1 mg/ml of SEN-CA – 7.3 mg/ml of reagent blank = 9.7 mg of $CaCO_3$/ml of SEN-CA. 9.7 mg of $CaCO_3$/0.066 mg of enzyme = 147 mg of $CaCO_3$/mg enzyme.

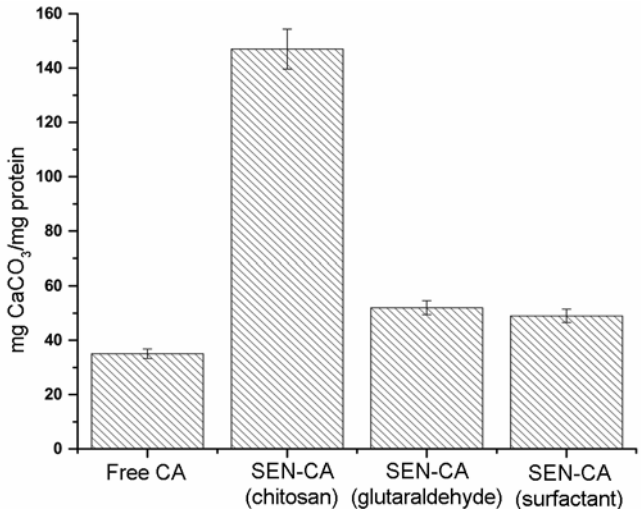


Figure 5. Comparison of CO_2 sequestration capacity of different chitosan-based nanoparticles with respect to free CA.

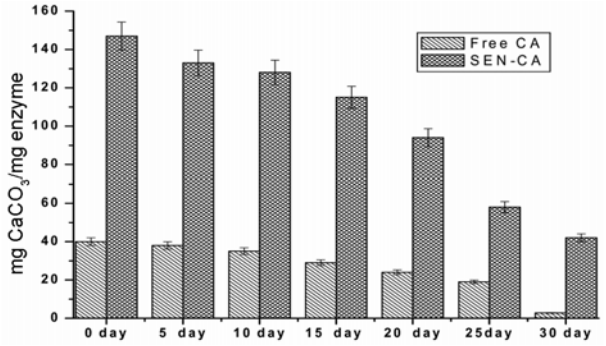
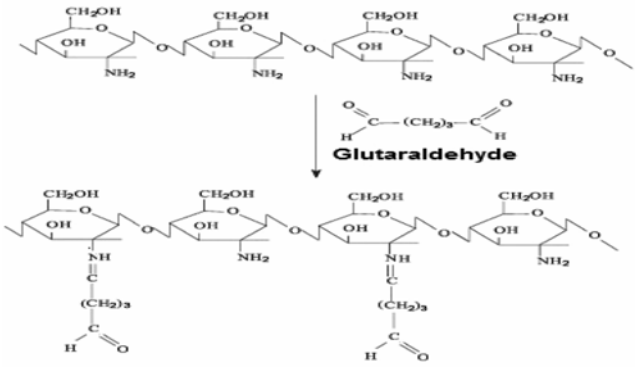


Figure 6. Storage stability in the form of carbonation capacity of SEN-CA and free CA.



Scheme 1. Reaction of chitosan with glutaraldehyde.

to prohibit the reaction of amino group of chitosan with aldehyde (–CHO) group of the enzyme.

The storage stability in the form of carbonation capacity of SEN-CA and free CA is shown in Figure 6. The storage stability experiments were investigated at 4°C. From Figure 6, it can be observed that the percentage loss of the carbonation capacity in SEN-CA is 72 and for free CA it is 93 after 30th day. SEN-CA thus provides higher shelf-life compared to free CA, since there are multiple covalent attachment points within the nanostructure. From the above, we conclude that the stability of SEN-CA has improved and retained 28% initial carbonation capacity up to 30 days compared to free CA.

In conclusion, a simple process has been developed for stabilization of enzymes with minimal limitation of mass transfer compared to other entrapment techniques reported earlier. The soluble form of SEN-CA facilitates

its processing into other forms, including immobilized SEN-CA, etc. The synthesis protocol is versatile and can be employed for stabilizing other enzymes. This study illustrates the synthesis of SEN-CA by a simpler method and corroborates the findings of Kim and Grate[5] on armoured structures, with characteristics of enhanced stabilization of the enzymatic activity and reduced mass-transfer limitation relative to large-scale immobilization with the possibility of processability into additional forms, including hierarchical architectures. SEN-CA could be used to accelerate the hydration of CO_2 in biomimetic CO_2 sequestration in an aqueous solution. There is an improvement in the storage stability in the form of carbonation capacity of SEN-CA compared to free CA. It has been observed that SEN-CA retained 28% of its initial carbonation capacity up to 30 days.

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Detection of potential site for future human habitability on the Moon using Chandrayaan-1 data

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Chandrayaan-1, the maiden Indian lunar spacecraft, carried 11 different scientific payloads on-board. The Terrain Mapping Camera (TMC) having 5 m spatial resolution and three-dimensional viewing capability had better sensor parameters than other similar cameras flown to the Moon before this mission. TMC captured the lunar surface features with unprecedented clarity. A buried, uncollapsed and near horizontal lava tube was detected in TMC stereo images of the Oceanus Procellarum area on the Moon. A Digital Elevation Model was generated to view the feature in three-dimensional perspective. A couple of rilles have been found to be connected sub-surfacially by an undamaged lava tube, indicating that the roof of this section of the tube has remained intact since its formation. The lava tube has been analysed thoroughly in terms of morphometry, topography, surface composition and surface ages of the surrounding regions. Such a lava tube could be a potential site for future human habitability on the Moon for future human missions and scientific explorations, providing a safe environment from hazardous radiations, micro-meteoritic impacts, extreme temperatures and dust storms.

Keywords: Human habitability, lava tube, Moon, rille.

CHANDRAYAAN-1, the maiden Indian planetary mission to the Moon, carried 11 different and complementary sensors on-board. One of the sensors was a panchromatic camera – the Terrain Mapping Camera (TMC). TMC image in the panchromatic spectral range of 0.5–0.75 μm with a stereo view in the fore, nadir and aft directions of the spacecraft movement and with a high spatial resolution of 5 m at an orbital height of 100 km (ref. 1), enable three-dimensional viewing of the lunar surface with crisp and clear surface features and morphology. The Digital Elevation Model (DEM) generated from the three look angles enables morphometric study of various lunar features, thus furnishing topographic relief and dimensions of various morphological entities. Identifying sites for permanent base stations for possible human settlements on the Moon is important for long-term perspective of lunar exploration. The absence of an atmosphere and intrinsic magnetic field make the lunar surface vulnerable

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