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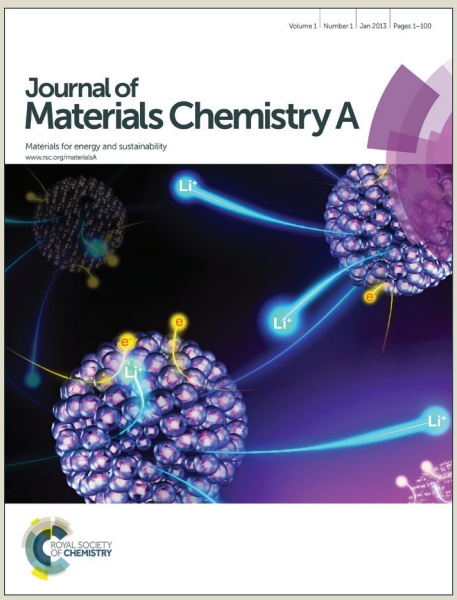

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Silk fibroin hydroxyapatite composite thermal stabilisation of carbonic anhydrase.

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J. H. Lopes[a], M. Guilhou[a], B. Marelli[b], F. G. Omenetto[b], D. L. Kaplan[b], J. E. Barralet[a,c]‡ and G. Merle[a]‡

Carbonic anhydrase was entrapped in a matrix of ultrasonically bonded hydroxyapatite microparticles coated with β-sheet structured silk fibroin. Transfer of the reactant and product between the enzyme and the assembly surface was evident and the system showed a remarkable operational, storage and thermal stability, with enzymatic activity almost unchanged after a one hour's treatment at 110°C and the assembly retained 45% of its initial activity after 3 weeks of continuous heating at 80°C in an amine solution. This thermal stability was excellent compared with described CA immobilization systems and indicates that silk fibroin may limit thermally induced enzyme conformation changes and prevent desorption.

Many biotechnological applications of enzymes are often hampered by a poor operational and long-term stability [1]. Immobilization methods to maximise operational and thermal stability and minimise the high costs by virtue of reusability[2] are therefore critical. Several approaches to enzyme immobilisation have been established, including inorganic and organic substrates for adsorption, lyophilisation, entrapment and covalent grafting. Generally enzyme immobilization offers an improved stability by limiting enzyme mobility that can occur with hydrophobic hydration changes and thus aggregation and loss of activity[3]. There have been considerable accomplishments in enzyme immobilisation[4], nevertheless they represent a compromise between maintaining a high catalytic activity with accessibility of the reactants to the active site of the enzyme, while providing thermal stability, reuse etc. Despite the large number of enzyme materials combinations investigated to enhance the enzyme stabilization, only few of them can be widely applicable, easily processed or do not substantially increase the price and even fewer can be used at temperatures other than in ambient conditions[5]. In a previous study we developed a facile technique to immobilise enzyme without altering its active conformation, preventing desorption and allowing its reuse[6]. Carbonic anhydrase (CA), a zinc metalloenzyme well known to activate the capture and conversion of CO_2 into protons and bicarbonate ions [7], was used as model enzyme. It is of interest to accelerate CO_2 scrubbing from flue gases by overcoming the low solvation rate of CO_2. However the rate of solvation is still limited because CA is not thermally stable and denatures at around (50°C)[6, 8].

In a prior proof of concept study[9] we ultrasonically bonded low solubility hydroxyapatite (HA) (-log K_{sp} 55-58[10]) nanoparticles to create high surface area (>100 m^2 g^{-1}) microparticles to allow a significant CA adsorption. We prevented desorption [11], with a PEI alginate polyelectrolyte. However, despite demonstrating the feasibility of the approach, PEI polyelectrolytes are generally unstable at high temperature [12]. Only strong polyelectrolytes with high charge density and a number of at least 8 layers are required to fabricate thermally stable coatings but this drastically reduces the accessibility to the enzyme and retards mass transfer[12].

Silk fibroin is a relatively new biomaterial for enzyme immobilisation and there have been only few studies mainly concerned with glucose oxidase[13], chymotrypsin[14], lipase[13b] and horseradish peroxidase[13b, 15]. This natural protein extracted from the caterpillar *Bombyx mori*[16] exhibits a copolymeric structure with large hydrophobic domains and small hydrophilic spacers[17]. One feature of this macromolecule is that during the assembly process, these domains and spacers form organized crystalline domains of beta sheets and less organized more flexible domains[18]. This extensive network of beta sheets, creates mechanically strong biopolymers that are inherently very resistant to changes in temperature and moisture[19]. The mild preparation conditions (ambient temperature and aqueous solvent) of silk are conducive to processing the enzyme during immobilisation without detrimentally affecting its activity. In this study we examined the use of silk on the thermal stability of CA in an amine-based solvent at 80˚C and 110˚C

HA microparticles were formed from aqueously precipitated HA nanoparticles as starting materials. By applying ultrasound the

[a] *Faculty of Dentistry, McGill University, Montreal, H3A 2B2, (Canada).*
[b] *Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, Massachusetts 02155, USA*
[c] *Department of Surgery, Faculty of Medicine, McGill University, Montreal, (Canada)*
‡ *These authors contributed equally.*
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initial HA nanoparticles formed mechanically robust HA microparticles (**Figure 1A**) that were separated from the unbounded nanoparticles by washing and sieving. As observed by scanning electron microscopy, the HA microparticles had a linear dimension of ~75-125µm (**Figure 1A**) and a nanoporosity of less than 100nm (**Figure 1B**). BET analysis shows only a small reduction in surface area between the constituent HA-nanoparticles (120 m^2 g^{-1}) and the assembled HA-microparticle (107 m^2 g^{-1}). At pH 6.8, a maximum loading of 44.5 ± 9 wt% of CA was determined with Bradford assay after 6 hours through of electrostatic adsorption. Silk fibroin was then deposited onto the enzyme loaded microparticles. As a direct consequence, much of the nanoporosity of the HA microparticles appeared to be have been occluded by the polymer enabling the prevention of enzyme leakage over the time (**Figure 1C)**. Successful deposition of the coatings was further confirmed with thermogravimetric (TGA) and FTIR analyses. TGA of HA microparticles without the silk coating (**Figure 2**) showed a 5% weight loss up to 250°C attributed to its dehydration and loss of adsorbed water. Above 250°C no additional loss was observed. Besides an important weigh loss of up to 250°C due to the dehydration, water and CA degradation[20], TGA analysis of the same microparticle covered with silk exhibited a one-stage degradation process of 10% weight loss from 300°C to 500°C related to silk degradation[20-21].

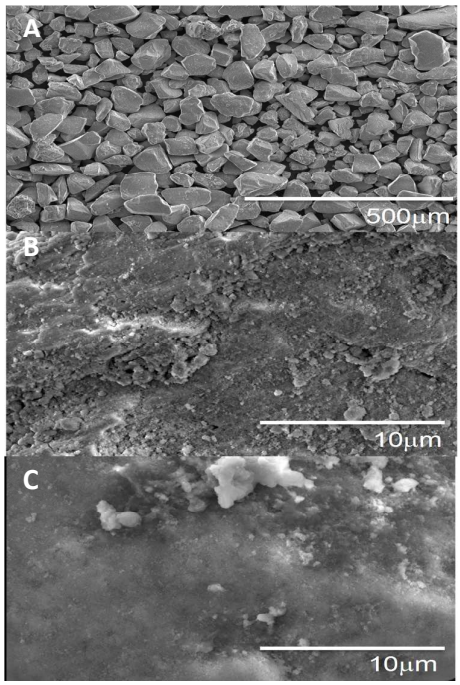

Figure 1. Scanning electron micrographs of (A) HA microparticle made from HA nanoparticle, (B) high magnification view of the surface of HA microparticle and (C) high magnification view of the surface of HA-microparticle with silk coating.

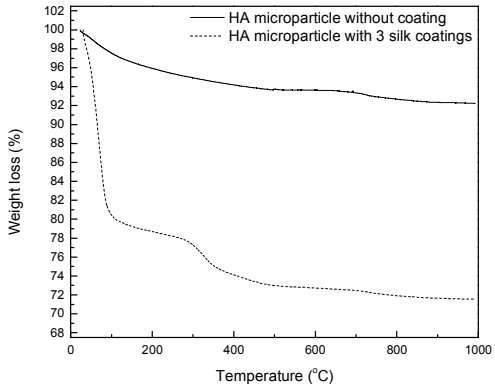

Figure 2. TGA curves of the microparticles before and after three successive deposition of silk layers

This indicated that ~8.5 wt% silk was deposited after three coatings and based on the mass of silk and the specific surface area and assuming a density of 1.4 g cm^{-3},[22] an average thickness of ~0.6nm, i.e. 0.2nm per layer, could be estimated. Therefore we estimated an average thickness of ~ 1.2nm for the 6 layer coating.

The silk polymer was treated with ethanol to make it not only water insoluble but to induce a stacked β-sheet structures after to obtain a film with greater strength and water- insolubility[23]. Besides the four peaks at 580, 960, 300 1450 $cm^{-1}$and 3600 cm^{-1} attributed to the phosphate, carbonate and hydroxyl groups respectively of the HA (**inset Figure 3**), two additional peaks at around 1,627cm^{-1} corresponding to the amide I group and 1,515 cm^{-1} for amide II (**Figure 3**) were observed, characteristic of the structural conformation of silk in β-sheet[24].

To assess the effect of the silk β-sheet structure on the CA, storage, operational and thermal stabilities were studied. The enzymatic activities were checked for 30 days period at 3 days intervals and stored at 4°C in a Tris-HCl buffer (50 mM, pH 6.4) to minimise any activity loss of the enzyme itself due to storage (**Figure 4**).

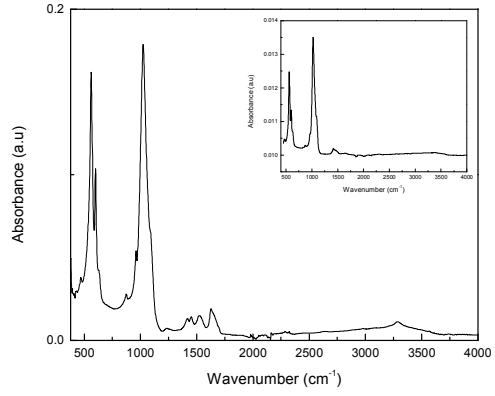

Figure 3. FTIR spectra of silk- modified hydroxyapatite microparticles (Inset. FTIR spectra hydroxyapatite nano microparticles)

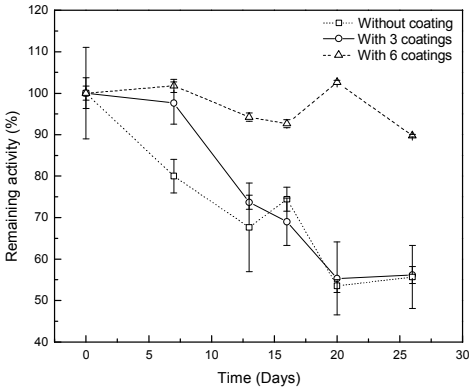

Figure 4. Remaining activity of immobilised enzyme on HA-microparticle with 3 (open circles), 6 (open triangles) and without (open squares) silk layers over time at 4°C

Figure 4 shows that the residual activity for the 6 layer-coated materials remained high after 30 days (90% of initial activity, i.e. 0.101 μmoles min^{-1} g^{-1} of HA) whereas the uncoated microparticles or 3 layer-coated resulted in a decrease of 50% initial activity after 30 days storage. This loss of enzymatic activity was then essentially the consequence of the desorption of the enzyme from the HA substrate over time but it appeared that 6 layers of prevented this desorption. Following this experiment the microparticle prepared with a 6 layer deposition was chosen as lead candidate for temperature stability evaluation.

After consecutive heat treatments of one hour at temperatures increasing from 50°C to 140°C (**Figure 5**), the free enzyme degraded at 50°C with no catalytic activity measureable whereas the enzyme simply adsorbed on HA exhibited a gradual loss of activity with 30% of remaining activity at 90°C. However the thermal stability was much greater when the silk was incorporated with 80% of its initial activity retained up to 110°C.

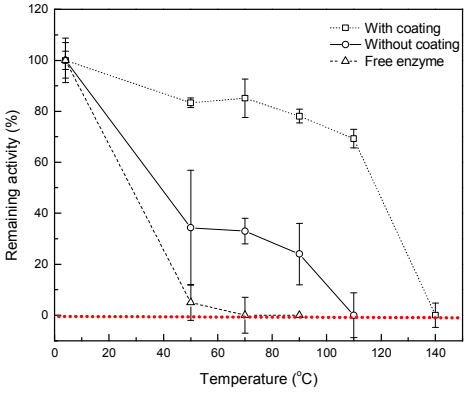

Figure 5. Remaining enzymatic activity after one-hour treatment at increasing temperatures in tertiary amine solvent (MDEA), free enzyme (open triangles), immobilised enzyme without coating (Open circles) and immobilised enzyme with 6 layers (Open squares)

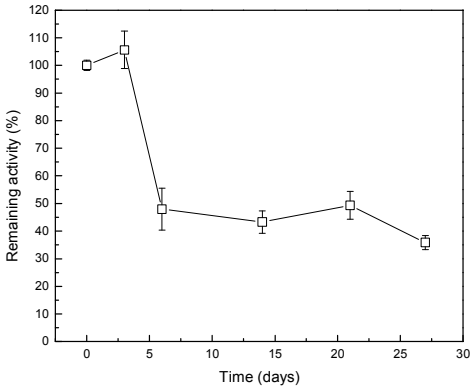

Figure 6. Effect of ageing time in MDEA at 80°C on remaining enzymatic activity of immobilised enzyme

Catalytic activity losses may occur for several reasons such as: enzyme inactivation, enzyme partial desorption, conformational changes while still remaining adsorbed on the HA or complete CA desorption from the microparticles. The CA stability inside the silk fibroin matrix was outstanding as compared to the stability of the enzyme in solution or adsorbed only.

The block copolymer structure of silk is able to form hydrogen bonds with the CA, promoting protein stability[23a]. Moreover, the predominantly hydrophobic nature of silk proteins excludes water from the crystallized beta sheet domains, while also limiting the water content in the less crystalline yet still hydrogen bonded spacers or more hydrophilic regions. To better characterize this thermal stability, we also recorded the enzymatic activity in an amine solvent, MDEA at 80°C. The enzymatic activity remained unchanged up to 3 days, decreased to 45% and then remained stable for 3 weeks (**Figure 6**).

Clearly both immobilisation material and nanoenvironment affect enzymatic stability but approaches have been rather empirical with results being largely similar in the range 50-70°C. CA immobilised in PU foam was stable up to 50°C and its activity started to decrease as the temperature was increased further and when the temperature is higher than 56°C, the immobilized CA lost its activity faster than the free enzyme[25]. Another study showed that after 30 days, the CA immobilised on silica nanoparticles retained up to 27% of its initial activity at 50°C[26]. CA immobilised on porous poly(acrylic acid-*co*-acrylamide)/hydrotalcite nanocomposite hydrogel, lost 30% of its original activity after only 60 min at 50°C[27] and on a chitosan/SiO_2-Fe_2O_3 composite[28] only □30% activity retention was achieved at 70 °C after a 4 hour incubation. In our proof of concept study into the feasibility of a polymer coated HA microparticles, we demonstrated that CA immobilised on HA microparticles coated with a polyelectrolyte complex of alternative layers of PEI and alginate retained only 40% of activity after 4 weeks at 50°C. A

major source of protein activity loss particularly in response to heating and organic solvents is unfolding of the protein structure. Adsorption and entrapment of CA would be expected to increase protein rigidity and reduce conformational mobility compared with free proteins, thereby improving thermal stability. Moreover, precipitated silk provides an appropriately "molecularly crowded" environment[23a] where hydrogen bonds can be formed between entrapped CA and the silk fibroin protein reducing the mobility and promoting protein stability. Furthermore, silk predominantly consists of hydrophobic domains and because the active site of the CA is highly hydrophobic one can envision that hydrophobic interactions between CA and silk occur enhancing further the enzymatic stability. Finally, the fibroin stacked β-sheets is able to create a space where a degree of buffering can be expected from changes that occur in the bulk solution. The fibroin matrix microenvironments may contribute to increased stabilization against pH inactivation when protons are formed and/or in organic solvents. The combination of HA as support to adsorb CA and embedding in silk fibroin demonstrated extremely high temperature stability in an organic solvent. To the best of our knowledge this is the first report of such a degree of stability of carbonic anhydrase. Although previous work has established that silk fibroin stabilises and reduces the mobility of enzymes[13b, 29], only one study tested the thermal stability of the glucose oxidase at 80°C[13a, 30]. They proved that less than 10% of the activity remained after 30 minutes of thermal treatment in 0.1M phosphate buffer, pH 7.0. Surprisingly, although there are many studies on enzymes adsorbed on inorganic substrates there are relatively few on the effect of the substrate on thermal stability. For example at 55-60˚C, lipase adsorbed in vesicular silica, that maintained 59% of its initial activity after 1.5 h of incubation [31], hydroperoxide lyase on HA[32] for 30 minutes maintained 70% of residual activity and carbonic anhydrase immobilized on mesoporous aluminosilicate[33] had 20% of relative activity. There is consensus therefore on neither thermal stability and adsorption substrate nor silk encapsulation, but our study tentatively suggests a cumulative effect conferring stability to the CA up to 110°C. Further studies will unravel the nature of the interactions. Such a system may have application in accelerating industrial enzymatic reactions such as CO_2 sequestration.

Experimental

Hydroxyapatite nanoparticle synthesis and ultrasonic self-assembly Solutions of calcium nitrate tetrahydrate (200 mL, 0.1 M) and of ammonium phosphate dibasic (120 mL, 0.1M) were dissolved in water. The pH of the calcium nitrate tetrahydrate solution was adjusted to 11 by adding some drops of ammonium hydroxide. Under continuous stirring, a precipitate was formed after addition of ammonium phosphate dibasic solution in the calcium nitrate tetrahydrate solution. During the reaction the pH was maintained at 11 with ammonium hydroxide. The resulting solution was stirred for 10 min and then placed on a hot plate until complete evaporation of ammonium hydroxide. The particles were collected by centrifugation (4000 rpm, 2 min) and washed 4 times with water to remove the supernatant. The particles were then placed in a vacuum oven at 50°C until completely dryness. After that, the solid was gently crushed to break the agglomerates and self-assembled into nanoporous microparticles by a technique developed by Bassett et al.[9], Na_3PO_4 solution at 90 mM was added to the powder (2 mL per gram of solid) and the whole was placed under vacuum until complete drying. The mixture was dispersed in isopropanol and ultrasonic treatment was applied for one hour. Finally, the suspension was filtered through a 75□m sieve and the particles ≥75 µm diameter were collected and dried.

Adsorption of Carbonic Anhydrase Carbonic anhydrase was diluted in a potassium phosphate buffer (0.1 M, pH 6.8) in order to obtain a final concentration of 0.2 mg mL^{-1}. HA and carbonic anhydrase exhibit an isoelectric points of 7.0 and 6.4-6.7 respectively. In order to create opposite charge thereby enhancing the adsorption process between the two materials, a pH of 6.8 was chosen. Both enzyme solution and HA microparticles (1 mL per 0.1 g of HA) were stirred for 2 hours at room temperature and then kept in the fridge for 12 hours.

Silk Fibroin preparation Silk fibroin solution was prepared according to a previously developed protocol[16], with some modifications. In brief, *B. mori* silkworm cocoons were boiled for 30 minutes in a solution of 0.02 M Na_2CO_3 to remove sericin, yielding a protein with an average molecular weight of ~120 kDa. The extracted fibroin fibers were rinsed in deionized water, set to dry for 24 h and then dissolved in a 9.3 M LiBr aqueous solution at 60°C for 4 h. The solution was dialyzed against deionized water using dialysis cassettes (Slide-a-Lyzer, Pierce, MWCO 3.5KDa) at room temperature for 2 days. The obtained fibroin solution (~50 mg/ml) was purified using centrifugation to remove processing debris. Silk fibroin layers were deposed on the surface of the enzymatic microparticles by alternating incubations cycles in 5wt% silk solution and ethanol solutions.

Characterisation Specific Surface Area (SSA) determination of the as-prepared hydroxyapatite was performed on a TriStar Surface area and porosity analyzer (Micromeritics) using the Brunauer-Emmett-Teller (BET) method of nitrogen adsorption/desorption. Thermogravimetric analyses (TGA) were done with a TA Intruments SDT Q600 TGA/DSC. The samples were heated from 25 °C to 1000°C in air at 10°C min^{-1} at flow rate of 100 mL min^{-1}. Absorbance measurements were realized using a Molecular Devices, Spectramax M2E spectrophotometer. The samples were placed in Suprasil quartz glass cells with a light path of 10 mm. During the adsorption process, a small amount of the supernatant was periodically removed and measured using the Bradford method[34] to measure the concentration of enzyme in solution over the time thereby the enzyme adsorption on the particles. The activity measurements were performed in para-nitrophenyl acetate (p-NPA) solution at 3 mM and monitored with a UV-Vis spectrophotometer at 400 nm. To study the stability of the enzyme on the microparticles without and with polymers coating, samples were kept in a refrigerator at 4 ° C in buffer solution at pH 6.8. Every 3 -4 days supernatant was removed, microparticles were rinsed and the absorbance of the supernatant was measured after 2 min, 10 min and 20 min after the addition of 2 ml of Tris-HCl buffer and 1 mL of p-NPA (3 mM). Once the measurements were completed particles were washed three times with the buffer at pH 6.8 and then returned to refrigerator. The evolution in absorbance was measured for 4 weeks. Investigating the thermal stability of the free and immobilised enzyme was performed in water and in an amine solution. Particles were heated during 1 hour at temperatures of 30, 50 70 and 90°C followed by the measurement of their enzymatic

activities. The thermal stability experiments were investigated in water and in a solution of N-methyldiethanolamine (MDEA) at 1 mol/L (only the heating was conducted in MDEA, the activity measurements were realized in water). Additionally enzymatic microparticles were place in a water bath set at 80°C and their enzymatic activities were checked periodically.

Acknowledgments

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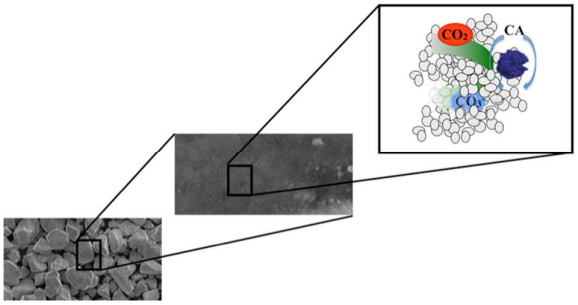
Table of contents

Hybrid organic-inorganic micro-particles have been prepared by a phosphate ultrasound treatment to immobilise carbonic anhydrase. These hierarchical structures show a noteworthy thermal stability in alkanolamine solvent used for CO_2 sequestration and allow an easy separation and re-use.



Keyword: Microparticles, Carbonic anhydrase, Biocatalysis, CO_2 capture, Thermal stability

J. H. Lopes, M. Guilhou, B. Marelli, F. G. Omenetto, D. L. Kaplan, J. E. Barralet‡ and G. Merle‡

Silk fibroin and hydroxyapatite thermal stabilisation of carbonic anhydrase