Aqueous Sol−Gel Process for Protein Encapsulation

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Porous silica materials made by low-temperature sol−gel process are promising host matrixes for encapsulation of biomolecules. To date, researchers have focused on sol−gel routes using alkoxides such as tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS) for encapsulation of biomolecules. These routes lead to formation of alcohol as a byproduct that can have a detrimental effect on the activity of entrapped biomolecules. We have developed a novel aqueous sol−gel process to encapsulate biological molecules (such as enzymes, antibodies, and cells) that uses neutral pH and room temperature and does not generate alcohol as a byproduct. The process uses sodium silicate as precursor and is carried out in two steps: preparation of a low-pH silicate sol followed by gelation at neutral pH with a suitable buffer containing biomolecules. Two enzymes widely used in biosensing applications, horseradish peroxidase (HRP) and glucose-6-phosphate dehydrogenase (G6PDH), were used to prepare enzyme-doped silica monoliths and to investigate the effect of silica as host matrix on enzyme kinetics. The yield of the encapsulation process was close to 100% for both enzymes, and no significant leaching of enzyme molecules was observed over time. Encapsulated enzymes followed Michaelis−Menten kinetics and maintained good catalytic activity, the specific activity of encapsulated HRP and G6PDH being 73% and 36% of the specific activities of the free enzymes, respectively. The values of the Michaelis constant (K_m) of the encapsulated enzymes were higher than those of the free enzymes, indicating the presence of partitioning and diffusional effects in the pores of the sol−gel matrix. The encapsulated enzymes also exhibited a different pH dependence of catalytic activity; the pH maxima for enzymatic activity for encapsulated enzymes were higher by 0.5−1 pH unit than those for enzymes in solution. These novel enzyme-doped silica matrixes provide promising platforms for development of biosensors, affinity supports, and immobilized enzyme reactors.

Introduction

Supports with immobilized biological receptors are finding ever-increasing application in a wide variety of fields such as biosensing, affinity chromatography, and enzyme reactors. One of the most challenging aspects in development of these matrixes is immobilization and integration of biological molecules in the host matrix and retaining the functionality of the biomolecules. Numerous techniques such as physical adsorption, covalent attachment, entrapment, and encapsulation in polymer and inorganic matrixes have been explored over the years to achieve a high-yield, reproducible, and robust immobilization technique that preserves the activity of the biological molecule. No single method or material has emerged as the standard for every application, and ongoing efforts strive to optimize these methods to render them adequate for specific applications. Silica host matrixes, made by the sol−gel process,[1] have emerged as a promising platform for encapsulation of biomolecules such as enzymes,[2−4] antibodies,[5] and cells.[6] Enzymes find a more stable environment upon encapsulation in a silica host, because the polymeric framework grows around the biomolecule, creating a cage and thus protecting the enzyme from aggregation and unfolding. These silica matrixes are chemically inert, hydrophilic, and inexpensive to synthesize. They also exhibit higher mechanical strength, enhanced thermal stability, and negligible swelling in organic solvents compared to most organic polymers. The silica matrix can also be tailored to act as a reservoir for water, thereby enhancing the ability to maintain the biological activity of entrapped enzymes, antibodies, and cells.

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Other advantages of silica supports include biocompatibility and resistance to microbial attack.

In biosensing applications, silica matrixes offer advantages over organic polymers from another viewpoint as well—ease of integration into the transduction platform. Their optical transparency makes them ideal for transduction methods that rely on transmission of light for detection, such as absorbance or fluorescence. Silica films can be made relatively quickly and can be cast as thin layers on sensor surfaces. For silicon oxide/nitride-based sensors, such as field-effect transistors or optical fibers, formation of silica films results in minimum alteration in optical, chemical, and physical properties of the base material.[7,8] The hydrophilic nature of silica should also allow unrestricted transport of water and other hydrophilic molecules such as substrates and products of an enzymatic reaction.

In most of the reported applications, silica monoliths containing encapsulated proteins or cells have been prepared by hydrolysis and condensation of an orthosilicate such as tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS).[9,10] First, TMOS is partially hydrolyzed in an acidic medium by addition of a controlled amount of water. Next, the biological species is introduced in a suitable buffer to facilitate gelation. The buffer pH is chosen so as to allow the final solution to be close to neutrality in order to avoid denaturation of proteins. However, use of TMOS (or TEOS) as starting material leads to generation of methanol (or ethanol), the presence of which in large quantities can be detrimental to the activity of proteins and cells. In low-temperature processing, typically used for encapsulation of biological species, the problem is compounded because aging and, hence, generation of alcohol can proceed for prolonged periods.

We have developed a new aqueous route for synthesis of silica monoliths with encapsulated biological entities that uses sodium silicate as a precursor.[11] This approach completely avoids generation of alcohol and, at the same time, allows encapsulation to be carried out at neutral pH to preserve biological activity. We have modified conventional sodium silicate processing[12] to develop a two-step method that makes the processing compatible with addition of enzyme. In the first step a low-pH sol is prepared using sodium silicate as the silica precursor. Enzyme is then introduced in a suitable buffer solution and added to the low-pH sol to form a doped silica matrix. This study focuses on encapsulation of the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and horseradish peroxidase (HRP). Both HRP and G6PDH are widely used enzymes in bioanalytical applications, as they are compatible with a multitude of detection methods including absorbance, fluorescence, chemiluminescence, and electrochemical detection. G6PDH catalyzes the oxidation of glucose-6-phosphate (G6P) in the presence of nicotinamide adenine dinucleotide phosphate ($NADP^+$), which in turn gets reduced to NADPH.[13,14] NADPH can be detected by measuring absorbance at 340 nm or by measuring fluorescence at 465 nm. HRP is a heme protein from horseradish that reduces H_2O_2 to H_2O while oxidizing a chromophore such as ABTS (azinobis-ethylbenz-thiazoline-sulfonic acid) to generate a colored product.[15]

Proteins encapsulated in pores of a sol−gel matrix can exhibit a behavior significantly different from that of free enzyme. It has been reported that in some instances enzymes only retain 1−2% of their specific activity upon encapsulation in sol−gel supports.[16,17] In the present work, the effect of the encapsulation process on proteins was evaluated in regard to retention of enzymatic activity as well as diffusional resistance to enzymatic catalysis. Surfaces in a sol−gel matrix are highly charged at neutral pH, and hence, the pH in nanopores of the matrix can be significantly different than the pH of the bulk solution. The catalytic activity of an enzyme generally is a strong function of pH,[18] and hence, altered pH in the vicinity of the encapsulated enzyme can lead to suboptimal reaction rates. Although a large number of researchers have explored encapsulation of proteins by a sol−gel process, surprisingly few studies have been reported on changes in the pH-dependent behavior of encapsulated proteins. We determined the pH dependence of enzyme activity for encapsulated HRP and G6PDH and compared it to that of free enzymes. The effectiveness of a protein-quantitation assay (BCA assay) was also evaluated to estimate the amount of proetin encapsulated in sol−gel matrixes.

Methods and Materials

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (suspension in 3.2 M ammonium sulfate), HRP, NADP, NAD, G6P, ABTS, and phosphate-buffered saline (PBS) were obtained from Sigma (St. Louis, MO). Sodium silicate was obtained from Fisher Scientific (Pittsburgh, PA). Dowex 50WX8-100 ion-exchange resin was purchased from Aldrich (Milwaukee, WI). A bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL). Low-binding 96-well microtiter plates were obtained from Rainin Instruments (Woburn, MA). Deionized water at 18.2 MΩ (Mili-Q-Plus, Millipore, Marloborough, MA) was used to prepare all reagents. Enzymatic activities were measured spectrophotometrically with an EL340 biokinetics microplate reader (Bio-Tek Instruments, Winooski, VT) equipped with a temperature-controlled chamber.

Enzyme Encapsulation by Sol−Gel Process. Silica sols were prepared from sodium silicate [(3.25 SiO_2/Na_2O), H_2O] solutions. The ratio of 3.25 was selected to minimize the volume of acid required for neutralization of alkali. Polypropylene containers were used in all steps of sol−gel preparation. Sodium silicate solution (11.5 g) was combined with 34 mL of DI water. To this aqueous solution was added 15.4 g of strongly acidic cation-exchange resin with stirring to bring the pH of

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the solution to a value of 4. The resin was then removed by vacuum filtration and regenerated as needed with 500 mL of 4% acid per 100 g of used resin. Hydrochloric acid (0.3 mL of 2 M) was added to the filtrate to adjust the pH to 2.0. The low pH of the sol solution minimizes the rate of siloxane condensation, allowing it to be stored at room temperature for up to 48 h before it gels. A phosphate buffer (1 M, pH 7) containing enzyme at the desired concentration was added to the sol solution in a 1:5 (volume) ratio. After mixing, the solution was quickly transferred to cuvettes or wells of a microtiter plate. Gelation occurred in approximately 5 min at room temperature. Gels were aged at 4 °C for 24 h prior to use. Gels used for enzymatic reactions were aged in sealed containers to avoid dehydration of the encapsulated enzymes. Gels containing G6PDH were equilibrated with 0.2 M phosphate buffer (pH 7) by repeated buffer exchange before enzyme activity measurements. Gels containing HRP were equilibrated with 50 mM citrate buffer pH 4.2 prior to measurement of enzyme activity.

Characterization of Sol−Gel Matrixes. To obtain the pore size distribution of the silica gels prepared by the aqueous route, aerogels were obtained by supercritically drying the wet gels.[19] Eight milliliters of pH 7 phosphate buffer containing the enzyme was added to 40 mL of the low-pH sol, and the solution was quickly transferred to cylindrical vials. The gels were allowed to age at 4 °C for 3 days. The cylindrical wet gels were then washed with pH 7 phosphate buffer for 2 h to desorb any enzyme molecules not encapsulated in the pores of the gel. The buffer in the pores of the gels was then replaced with amyl acetate. The gels were placed in a Polaron critical point dryer (Structure Probe Inc., West Chester, PA) that was prefilled to half its volume with amyl acetate. The chamber was sealed, and liquid CO_2 (18 °C, 950 psi) was allowed to enter the chamber and displace the amyl acetate. The samples were flushed with liquid CO_2 three to four times to completely replace the pore fluid with CO_2. Between the flush cycles, the chamber was sealed to allow soaking in liquid CO_2. The temperature was increased to 36 °C over a period of about 1 h, causing the pressure to rise to 1150 psi, well above the critical temperature and pressure of 31 °C and 1050 psi. The chamber was held above the critical point for approximately 0.5 h, after which time the CO_2 was vented to obtain the aerogels. The nitrogen sorption technique was employed to characterize the aerogels using the Micromeritics ASAP (Accelerated Surface Area and Porosimetry) 2010 Instrument (Micromeritics Instrument Corporation, Norcross, GA). Prior to analysis, the aerogels were degassed overnight at 1 mmHg pressure. The BJH model was used to obtain the pore size distribution and the pore volume of the gels. The porosity of a sol−gel matrix was calculated as the ratio of the pore volume to the total volume, where the total volume is the sum of the pore volume and the volume occupied by solid $\{=1/(2.2\ \mathrm{g\ cm^{-3}}) \times$ weight of matrix$\}$.

Enzyme Kinetics for Free and Encapsulated Enzymes. Kinetic parameters for the free and entrapped enzymes G6PDH and HRP were determined using Michaelis−Menten kinetics, given by

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

where V is the reaction rate, S is the substrate concentration, V_{max} is the maximum reaction rate, and K_m is the Michaelis constant.[20] The kinetics parameters K_m and V_{max} were determined from a plot of reaction rate versus substrate concentration using a nonlinear least-squares fit of the experimental data. The maximal reaction rate, V_{max}, can also be written as

$$V_{max} = k_p E_0 \tag{2}$$

where k_p is the enzyme specific activity (also called k_{cat} or the catalytic constant) and E_0 is the enzyme concentration. In all experiments performed to measure enzyme activity, the enzyme concentration used was at least 1000-fold lower, typically 10^5 to 10^6-fold lower, than the substrate concentration. This was done to satisfy the quasi-steady-state assumption in Michaelis−Menten kinetics.

HRP undergoes a two-substrate reaction, the substrates being H_2O_2 and ABTS. The concentration of H_2O_2 used in determining the kinetic parameters was kept constant at a saturation value (5 mM), and only the concentration of colorimetric substrate, ABTS, was varied. This allows us to use the Michaelis−Menten equation for a one-substrate reaction to determine the kinetic parameters of HRP. The enzyme concentration used was 0.1 nM, and the buffer used was 50 mM citrate at pH 4.2. The kinetic runs were performed at room temperature by measuring the rate of change of absorbance at 405 nm in a UV−vis plate reader. The plate was shaken initially and between readings to ensure good mixing during the kinetic measurements. For kinetic measurements on HRP encapsulated in the silica host, enzyme-doped silica matrixes were cast in wells (50 μL/well) of a microtiter plate at a final enzyme concentration of 1 nM. The sol−gel disks used for kinetic assays had a thickness of approximately 0.18 mm after 1 day of aging. After equilibrating with the desired buffer, solutions of ABTS and H_2O_2 were dispensed into the wells at the desired concentration and the plate was read in a plate reader in kinetic mode to determine the initial rate. It should be emphasized that the k_p and K_m values determined are not true constants; they are functions of the concentration of the second substrate H_2O_2, that was kept constant for all the experiments.

G6PDH catalyzes conversion of G6P to gluconolactone in the presence of NAD^+ or $NADP^+$. Kinetic parameters with respect to each substrate were determined by varying the concentration of that substrate, while keeping the concentration of the second substrate constant at the saturation level. Initial reaction rates were determined by monitoring the rate of change of absorbance at 340 nm (due to production of NADPH) at room temperature. The buffer used in all measurements was 0.2 M phosphate buffer at pH 7.0. Encapsulated G6PDH was cast in wells of a microtiter plate for kinetic measurements, as described above. The concentrations of enzyme used were 1 nM and 13.33 nM for free and encapsulated G6PDH, respectively.

Effect of pH on Enzyme Activity. The effect of pH on the catalytic activity of both HRP and G6PDH, in free as well as encapsulated form, was evaluated by measuring initial rates of reaction in buffers of the same ionic strength but different pH. For HRP, the buffer used was citric acid/sodium citrate at a concentration of 50 mM (in citrate) at pH values (at 25 °C) of 2.6, 3.16, 3.74, 3.93, 4.44, 4.8, 5.2, 5.51, and 5.85. For G6PDH, the buffer used was monobasic sodium phosphate/dibasic sodium phosphate at a concentration of 100 mM (in phosphate) at pH values (at 25 °C) of 5.78, 6.19, 6.61, 6.98, 7.39, 7.82, 8.29, and 9.2. The enzyme-doped sol−gel monoliths cast in microtiter plates were equilibrated with the buffer of desired pH for 21 h before kinetic measurements. All the reactions were done at saturation concentrations of substrates to measure the maximal reaction velocity (V_{max}).

Protein Quantification in Silica Gels Using the BCA Assay. The concentration of encapsulated enzyme was determined using the BCA assay. This assay is a colorimetric determination of protein concentration in aqueous solutions. BCA, in the water-soluble sodium salt form, is a highly specific reagent for Cu^{1+}. Certain amino acids and peptide bonds in a protein reduce Cu^{2+} to Cu^{1+} in an alkaline environment (Biuret reaction). Cu^{1+} then complexes with two molecules of BCA to form a purple complex that is water-soluble and has a strong absorbance at 562 nm. In the presence of excess BCA, the color formation (monitored by a spectrophotometer) is directly proportional to the concentration of protein in a sample.

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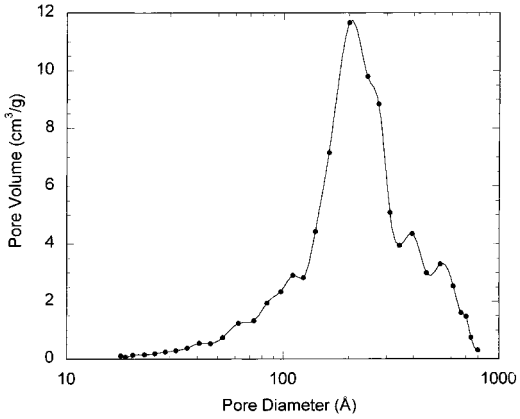

Figure 1. Pore size distribution of enzyme-doped silica obtained from nitrogen sorption measurements.

Known amounts of a protein are used to form a calibration curve, and the amount of protein in an unknown sample is determined from the calibration curve. However, because the biuret reaction is sensitive to the amino acid content of a protein, the protein used to generate the calibration curve should be the same as the one whose concentration is being determined. Various concentrations of G6PDH were encapsulated in sol−gel matrixes cast in wells of a microtiter plate. One hundred microliters of free G6PDH ranging in concentration from 0 to 1 μM were added to another set of wells to generate a standard curve. The BCA assay was performed according to the manufacturer's instructions. Briefly, reagents were mixed to obtain a final concentration of 0.08% BCA and 2% cupric sulfate in carbonate/bicarbonate buffer. One hundred fifty microliters of the mixture was then added to the wells containing silica casts, free enzyme, or blanks, and the plate was incubated at 37 °C. After 2 h of incubation, 100 μL of solution was removed from each well and transferred to another plate. The absorbance of the transferred solution at 562 nm was determined using a UV−vis plate reader.

Results and Discussion

Characteristics of Enzyme-Doped Sol−Gel Monoliths. The pore size of an enzyme-doped silica matrix needs to meet two requirements. Pores should be large enough to allow unrestricted transport of molecules including buffer ions, substrates and products of the enzymatic reaction, and analytes. At the same time, pores should be small enough to prevent leakage of encapsulated macromolecules as well as exclude hostile agents such as microbes routinely found in the environment. Figure 1 shows the pore size distribution of enzyme-doped aerogel matrixes obtained from BJH desorption experiments. The distribution is relatively large, with pores as small as 20−30 Å and as large as 800 Å being present. The majority of the pore volume lies in pores having diameters in the range 120−400 Å, and the mean pore size of the matrix is approximately 200 Å. The pores are sufficiently large to allow transport of small molecules such as substrates and products but exclude large particles such as bacteria. Assuming a globular structure, HRP and G6PDH have diameters of 64 and 88 Å, respectively.[21] Hence, they should be able to diffuse, although at a rate slower than that in solution, in most of the pores of the silica matrix. However, no significant leaching of entrapped enzymes was observed over time, or during repeated washes,

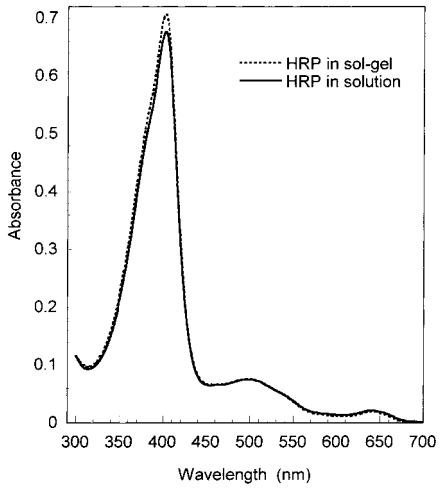

Figure 2. Absorbance spectrum of HRP (horseradish peroxidase) in solution and in a silica matrix. The peak at 403 nm is attributed to the heme group.

suggesting that most of the enzyme molecules were sterically confined in smaller pores. Since the protein is added prior to gelation, it is possible that a sol−gel structure is formed around the protein with the protein molecule acting as a template. This is indirectly corroborated by the fact that surfactant micelles and other organic molecules, as small or smaller than proteins, have been successfully used in our laboratory as templates for forming mesoporous sol−gel matrixes.[22,23] Above pH 4, silanols in a silica matrix are negatively charged and strong adsorption of cationic proteins due to electrostatic attraction can be another mechanism of retention.[24,25] At pH 7, the final pH during gelation, HRP has approximately no net charge (pI = 7.2) and G6PDH has a net negative charge (pI = 4.6).[26] Hence, neither protein should adsorb strongly to the negatively charged silica matrix. The high ionic strength (1 M phosphate) of the buffer used for gelation should further minimize the electrostatic interaction.

High porosity and surface area is another advantage silica matrixes provide over organic polymers. The porosity of a silica matrix prepared by our method is as high as 0.92 (the range being 0.81−0.92), compared to a porosity of 0.3−0.5 for typical polymeric supports. This should allow one to encapsulate higher amounts of enzymes without significantly reducing their accessibility. The pore size and porosity of a silica matrix are a strong function of pH during gelation. Over the pH range 4−7, the lower the pH of the buffer used to gel the network, the lower the mean pore size and volume fraction porosity.[11] This provides a controllable parameter to tune the mean pore size of a silica matrix, even though the pore size distribution remains fairly wide.

Figure 2 shows the spectra of HRP in solution and in the silica matrix. The concentration of HRP used was 0.8 mM in both cases. The two spectra are almost

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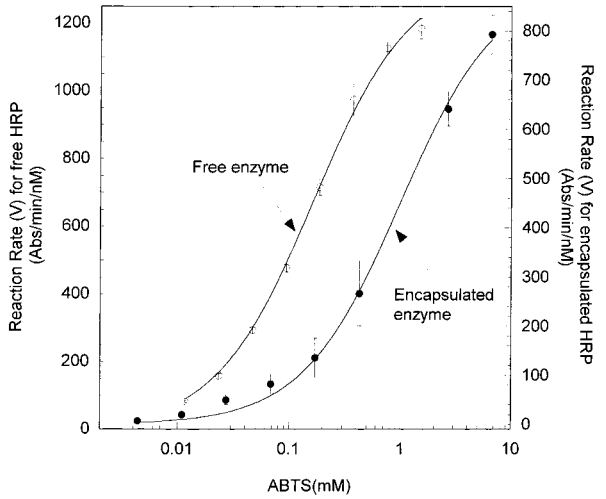

Figure 3. Reaction kinetics for free (○) and encapsulated (●) HRP at a fixed concentration of H_2O_2 and varying concentrations of ABTS. The solid lines represent the best fit to the Michaelis−Menten equation using nonlinear least-squares regression. The curve for the encapsulated enzyme is shifted to the right, indicating an increase in the K_m of the enzyme upon encapsulation.

identical, indicating that the enzyme has not undergone major conformational change upon encapsulation. The peak at 402 nm is due to the presence of the heme group[27] and is quite sensitive to the conformational state of the enzyme. Denaturation of the enzyme can lead to loss of the complexed iron, resulting in a decrease in absorbance at 402 nm. The identical spectra of the two forms of the enzyme also indicate that the yield of the encapsulation process is close to 100%. In other words, the entire amount of enzyme added gets encapsulated. Similar results were observed with encapsulation of G6PDH. We also monitored the loss of encapsulated enzyme in pH 7 phosphate buffer at room temperature over time and did not observe any measurable loss of enzyme over a period of 30 days.

Catalytic Behavior of Encapsulated HRP. The most important issue in immobilization of a biomolecule is retention of its biological activity upon immobilization. The immobilization or encapsulation process has to be mild enough to retain most of the activity of an encapsulated molecule. For enzymes encapsulated in silica, catalytic activity was determined by measuring the initial rate of product formation. Figure 3 shows the rate of reaction V, expressed as the rate of change of absorbance due to product formation, as a function of substrate (ABTS) concentration for both free and encapsulated enzyme. The experimental data were fit with eq 1 using nonlinear least-squares regression (the R^2 values of the fits were 0.9988 and 0.9987 for free enzyme and encapsulated enzyme, respectively), and the kinetic constants are listed in Table 1. Comparing the k_p values of free and encapsulated enzyme, it can be observed that HRP lost only 27% of its activity upon immobilization, underscoring the mildness of the sol−gel process we have developed. Comparing the plots for free and encapsulated HRP, it can also be noticed that the rate versus substrate concentration curve is shifted to the right (higher substrate concentration) for the encapsulated enzyme. In other words, the rate reaches its maximum value V_{max} at a higher substrate concentration when enzyme is in the encapsulated form. This indicates that K_m is higher (approximately 6-fold) for encapsulated enzyme compared with free enzyme. An increase in K_m upon encapsulation can result from a number of factors, including partitioning of substrates between solution and support, and diffusional resistance to the transport of substrates to the enzyme.[28] The presence of small pores, or bottlenecks even in big pores, can reduce the diffusion coefficients of substrates and products significantly in a silica matrix. This can lead to a diffusion-limited regime in the interior of a silica matrix where overall reaction rate is determined not by the turnover rate of the enzyme but by the rate of transport of substrate to the enzyme. If the dimensions of the matrix are large or if there are a large number of very small pores, enzyme molecules buried inside the matrix encounter a substrate concentration significantly lower than that at the surface. If the diffusion rate of the substrate is sufficiently slow compared to enzymatic catalysis, the enzyme molecules close to the surface can use up most of the substrate molecules entering the matrix, effectively making the substrate concentration zero in the interior of the matrix. In designing immobilized enzyme matrixes, it is imperative to choose dimensions of the support matrix so as to minimize diffusional resistance, thereby making the entire population of encapsulated enzyme participate in catalysis.

Table 1. Kinetic Parameters for Free and Encapsulated HRP and G6PDH

	K_m (mM)	k_p ($\times 10^9$ Abs/(min M))
free HRP, varying ABTS, fixed H_2O_2	0.163	1348
encapsulated HRP, varying ABTS, fixed H_2O_2	0.985	977
free G6PDH, varying $NADP^+$, fixed G6P	0.185	39.6
encapsulated G6PDH, varying $NADP^+$, fixed G6P	1.07	12.4
free G6PDH, varying G6P, fixed $NADP^+$	0.837	33.3
encapsulated G6PDH, varying G6P, fixed $NADP^+$	1.98	11.8

Catalytic Behavior of Encapsulated G6PDH. Figure 4 shows the reaction kinetics of free and encapsulated G6PDH at varying concentrations of $NADP^+$ while keeping G6P concentration constant, and Figure 5 depicts the kinetics at varying concentrations of G6P while keeping $NADP^+$ concentration fixed. The experimental data were fit by eq 1, and in all instances the fits were excellent, indicating that Michaelis−Menten kinetics were followed. The kinetic parameters for free and encapsulated enzyme are listed in Table 1. Comparing k_p values for free and immobilized G6PDH, we observe that the specific activity of encapsulated enzyme is 31−36% of that of free enzyme. Comparing specific activities of encapsulated HRP and G6PDH, it can be concluded that HRP retains its specific activity to a greater extent than G6PDH as a result of the encapsulation process. In other words, HRP appears to be a more robust protein than G6PDH with respect to the sol−gel encapsulation process. Similar results have been reported for immobilization in membranes, where re-

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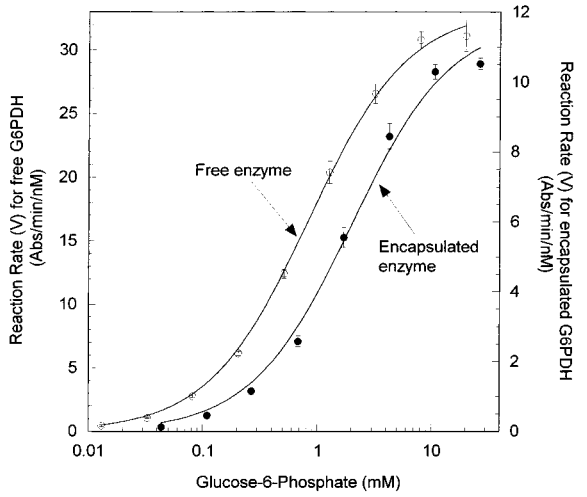

Figure 4. Reaction kinetics for free (○) and encapsulated (●) G6PDH at a fixed concentration of $NADP^+$ and varying concentrations of G6P. The kinetic data were fit to the Michaelis−Menten equation using nonlinear least-squares regression.

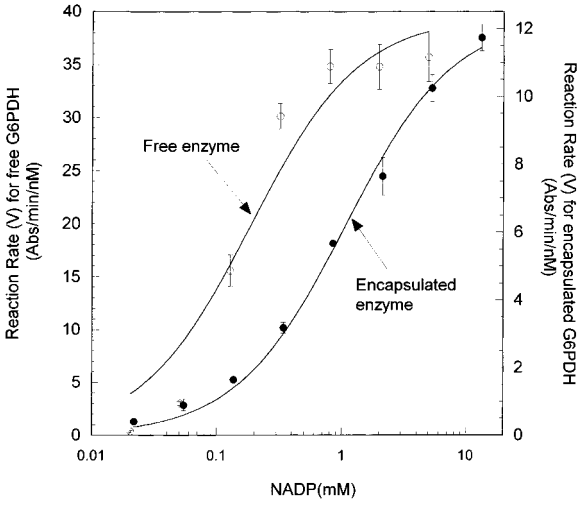

Figure 5. Reaction kinetics for free (○) and encapsulated (●) G6PDH at a fixed concentration of G6P and varying concentrations of $NADP^+$. The solid lines represent the best fit to the Michaelis−Menten equation using nonlinear least-squares regression.

searchers found that, while HRP conserved 60% of its activity, G6PDH retained only 10% of its activity.[29] Most proteins lose their biological activity to some degree during a physical or chemical modification step such as encapsulation.[30] However, each protein, due to its unique tertiary and quaternary structure, responds differently to the modification steps and alterations in its environment. Consequently, in any application requiring immobilized protein, the immobilization process has to be optimized for the specific protein with respect to preserving its biological activity. In general, proteins prefer conditions such as physiological pH (close to neutrality), low temperature, and moderate ionic strength. The conditions that adversely affect the activity of most proteins are high temperature, dilution, and the presence of organic solvents. In the sol−gel encapsulation technique presented here, gelation is done

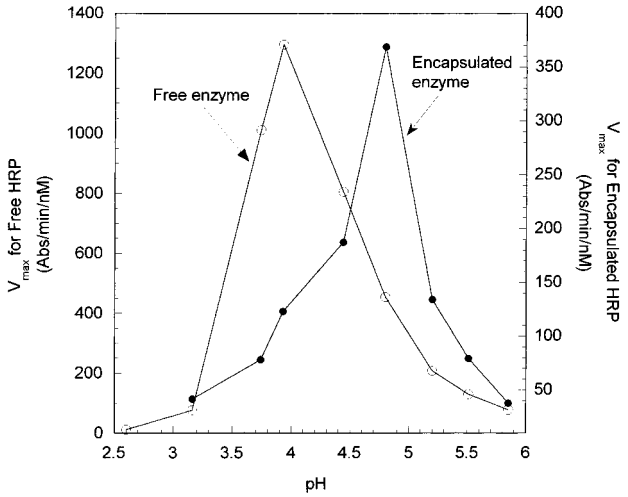

Figure 6. Effect of pH on V_{max} of free (○) and encapsulated (●) HRP. The pH where V_{max} is the maximum is higher by approximately 1 pH unit for the encapsulated enzyme.

at neutral pH and room temperature in an all-aqueous environment to minimize loss of activity, and both enzymes were able to conserve a significant portion of their activity upon encapsulation. It is also worth noting that although encapsulation may lead to an initial loss of specific activity, the long-term stability of encapsulated or immobilized enzymes has been shown to be far superior to those in solution.[6,31] Similar to encapsulation results for HRP, we also observe an increase in the K_m of G6PDH upon encapsulation, indicating the presence of diffusional resistance to transport of substrates in pores of the sol−gel matrix. The increase in K_m is 5.8-fold with respect to $NADP^+$ and 2.4-fold with respect to G6P upon encapsulation.

Effect of pH on Enzyme Activity. pH variations in pores of a sol−gel, or any support matrix, are important because they can affect the stability as well as the biological activity of encapsulated biological molecules. For most enzymes the catalytic activity is strongly dependent on pH. Figures 6 and 7 show the effect of pH on the maximal catalytic rate (V_{max}) for HRP and G6PDH, respectively, for both free and encapsulated forms of the enzymes. In both cases, V_{max} versus pH curves are shifted to higher pH values, when enzyme is in the encapsulated form. The shift is approximately +1 pH unit for HRP and +0.5 pH unit for G6PDH. One possible reason for the apparent shift in pH_{max} (pH where V_{max} is largest) can be that the encapsulated enzyme is experiencing a local pH which is lower than that of the bulk solution. With immobilized enzymes, pH effects can be manifested due to a number of reasons, including partitioning of hydrogen ions between the solution and the surface. At neutral pH, silanol groups in a silica matrix are negatively charged and hence the electrical double layer consists of primarily cationic buffer ions and hydrogen ions. An excess of H^+ makes the pH in the double layer lower than that of the bulk solution. Since proteins are confined in pores that match their dimensions, these pores have diameters (assuming cylindrical shape) of approximately 10

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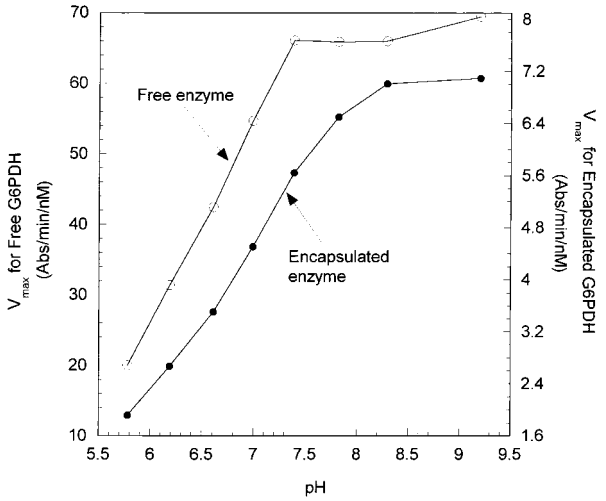

Figure 7. Effect of pH on V_{max} of free (○) and encapsulated (●) G6PDH. The pH at which V_{max} attains saturation is higher by approximately 0.5 pH unit for the encapsulated enzyme.

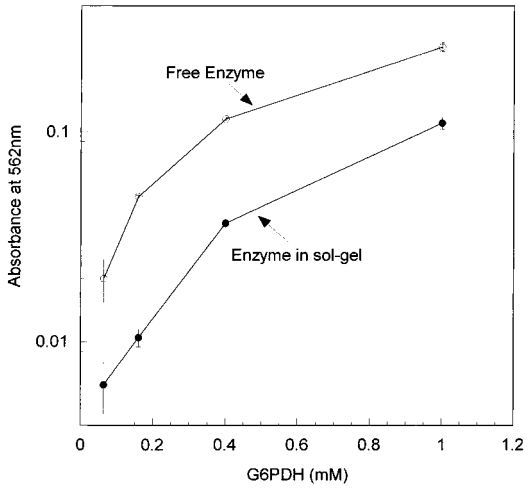

Figure 8. Quantitation of G6PDH by the BCA protein assay. (○) represents the signal (absorbance at 562 nm) in the BCA assay for G6PDH in solution, and (●) represents the signal for encapsulated G6PDH.

nm or lower. At these dimensions, there will be significant double-layer overlap in the pores, leading to the presence of excess H^+ and hence lower pH. Even in the bigger pores, if protein is immobilized on the surface, protein will be contained within the double layer and exposed to a pH lower than that in the bulk solution. On the basis of the preceding arguments and the observation that pH_{max} for encapsulated enzyme is higher than that of free enzyme, it appears that pH is 0.5−1 unit lower in pores than in the buffer solution. This compares well with the titration results of Dunn and Zink,[32] indicating the pH to be up to 1 unit lower in pores than in the bulk buffer.

From Figures 6 and 7, we can also observe the difference in pH behavior of the two enzymes, HRP and G6PDH. The maximum catalytic activity (V_{max}) of HRP as a function of pH is a bell-shaped curve, while the V_{max} of G6PDH increases monotonically with pH before saturating at a sufficiently high pH. Enzymes that exhibit pH-dependent activity have prototropic (ionizable) groups in their active sites participating in catalysis.[33] Only one state of the ionized enzyme (or enzyme−substrate complex) is catalytically active, and hence, the enzyme shows a strong dependence on pH. If the enzyme has only one prototropic group involved in catalysis, and the unprotonated (or less protonated) state of this group is catalytically active, then the catalytic rate increases with pH before reaching a maximum (at a pH greater than the pK_a of the ionizable group). G6PDH is an example of this case. If the protonated state of the ionizable group is catalytically active, then the reaction rate is maximum at low pH (at pH < pK_a) and decreases with increasing pH. If the enzyme has two prototropic groups in the active site involved in catalysis, the enzyme activity versus pH curve is bell-shaped and passes through a maximum as pH is increased. HRP is an example of this case. pH_{max} for an enzyme with two prototropic groups is the average of the pK_a's of the two ionized states.

Protein Quantitation Using the BCA Assay. In the majority of applications such as biosensors, protein encapsulated in sol−gel supports will be present in concentrations too small to be measured accurately by UV absorption. For protein concentrations in microgram per milliter range, protein quantitation assays such as BCA or Bradford assays are routinely employed. We evaluated a modified BCA assay to estimate the concentration of encapsulated proteins in sol−gel monoliths. Figure 8 shows the results of the BCA assay for encapsulated as well as free G6PDH for the same concentrations of the enzyme. It can be observed that the BCA assay underestimates the concentration of enzyme in the encapsulated form by an average of 68% for the concentrations tested. Considering the fact that almost 100% of added protein gets encapsulated in the silica matrix (Figure 2), the underestimation of protein concentration is most likely due to hindered diffusion of either the reactants or products of the biuret reaction. The diffusing species in a BCA assay consist of ionic species such as Cu^{2+}, Cu^{1+}, and BCA (doubly negatively charged). Anomalous transport behavior of ionic species in pores of a sol−gel matrix has been observed before. It was reported that, at pH values at which the silica matrix was negatively charged, anions were only partially taken up, whereas cations were preferentially taken up.[16] The reason for this behavior is the strong attraction and repulsion exhibited toward cations and anions, respectively, by the silica walls of the porous matrix. The interaction is amplified exponentially when ionic species carry multiple charges. Although Cu^{2+} and BCA are present in large molar excess over protein concentration, preferential adsorption or exclusion of a species in the pores can lead to reduced reaction rates in the assay. Although, in the absence of a means to determine the local concentrations of each ionic species in the porous volume, the exact reason for anomalous results in the BCA assay cannot be determined, it is important to emphasize that ionic species may exhibit complex behavior in a highly charged porous silica structure. Adsorption of positively charged molecules on silica surfaces is well documented and is of great concern in the fields of capillary electrophoresis,[24]

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chromatography using silica-based stationary phases,[35] and silica-based biosensors such as fiber optic biosensors.[36] Sol−gel matrixes, due to the presence of small pores, can have exceedingly large surface areas where preferential adsorption of cations and exclusion of anions can occur. For example, a sol−gel matrix with 100 nm diameter pores will have roughly an area of 25 m^2/g and a matrix with 10 nm pores will have a 250 m^2/g surface area. This behavior is of great concern in applications such as biosensors, affinity chromatography, and immobilized enzyme reactors, where enzymes are immobilized or encapsulated in porous charged supports and analytes or substrates are frequently ionic species.

Conclusions

A novel, aqueous-based process for synthesis of bioinorganic composites for potential applications in biosensing has been demonstrated. The process uses sodium silicate as a precursor to avoid generation of alcohol, a potential denaturant for biomolecules. The encapsulation is carried out at room temperature and neutral pH to further minimize denaturation of biomolecules. Two widely used enzymes in the field of immunodiagnostics and biosensors, horseradish peroxidase and glucose-6-phosphate dehydrogenase, were used for encapsulation to determine the effect of the sol−gel process on enzyme activity. The average pore diameter of the enzyme-doped material is 200 Å, and the pore-size distribution is relatively large. HRP retained as much as 73% of its activity after immobilization, and G6PDH exhibited up to 36% remaining specific activity upon immobilization. Encapsulated enzymes also exhibited a pH-dependent behavior that is different from that of free enzymes. These silica matrixes offer a number of advantages over conventional organic polymers as immobilization platforms for biosensors owing to their superior mechanical strength, chemical inertness, hydrophilic nature, and, above all, optical transparency. We are evaluating the long-term stability of biomolecules encapsulated in sol−gel matrixes, a key issue in the development of biosensors.

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