Nicotinamide Adenine Dinucleotide Phosphate Fluorescence and Absorption Monitoring of Enzymatic Activity in Silicate Sol–Gels for Chemical Sensing Applications

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Sol–gel procedures can be used to encapsulate biomolecules with retention of activity in optically transparent silicate glass.[1–16] These new materials are of interest for their applications as optically based biosensors. The porosity of sol–gel glasses allows small analyte molecules to diffuse into the matrix while the large enzymes or other proteins remain physically trapped in the pores of the glass. The transparency of the matrix makes it feasible to monitor spectroscopically the reactions that occur in the pores of the glass.

Two characteristic optical changes that accompany the enzymatic reduction of nicotinamide adenine dinucleotide phosphate NAD^+ and its phosphate ester $NADP^+$ provide powerful methods for measuring the course of enzymatic reactions and thus for chemical sensor applications.[17–21] First, in their oxidized forms, these coenzymes show no absorption above 300 nm, but when they are reduced, a new absorption band appears at 340 nm, caused by the reduction of the aromatic pyridine ring of nicotinamide. Second, the reduced forms fluoresce strongly at ~440 nm, whereas the oxidized forms do not luminesce in solution. The fluorescence method of following the reaction is extremely sensitive. Previous work on enzyme activity in sol–gel materials generally used indirect methods involving dye formation.[13,15] If NAD and NADP are active in the pores of sol–gel materials, the optical detection of their absorption and luminescence changes would lead to a new direct method for biosensors.

In this paper we show that $NADP^+$ functions efficiently in the sol–gel glass as coenzyme for a typical dehydrogenase enzyme reaction, the oxidation of D-glucose-6-phosphate to D-glucono-δ-lactone-6-phosphate by glucose-6-phosphate dehydrogenase (G6PDH). The enzyme has a molecular weight

$$\text{D-glucose-6-phosphate} + NADP^+ \xrightleftharpoons{\text{G6PDH}} \text{D-glucono-}\delta\text{-lactone-6-phosphate} + NADPH \quad (1)$$

of 104 000 and an optimal pH of 9.1.[22] The reaction is quantified by monitoring the formation of NADPH by absorption and the disappearance of $NADP^+$ by luminescence spectroscopy.

The glass samples were prepared by mixing 6.45 mL of TMOS sol,[1,13] 6.45 mL of 0.01 M sodium phosphate buffer, pH 7.0, 1.80 mL of NADP stock solution, and 0.30 mL of G6PDH stock solution. The additions were made in the order above such that the sol was buffered prior to addition of the enzymes. The NADP stock solution was prepared by dissolving 15 mg of NADP (sodium salt, 98%, Sigma) in 5 mL of doubly deionized water. The G6PDH stock solution was prepared by dissolving 1 mg of G6PDH (Type XII from Torula Yeast, Sigma) in 1 mL of doubly deionized water. All additions were carried out on ice. The doped sol was transferred to 4 mm × 10 mm polystyrene cuvettes, sealed with Parafilm, and placed in the refrigerator at 4 °C to age. When opened and allowed to dry, the samples shrink irreversibly to approximately one-eighth their volume.

The enzymatic activity of the G6PDH-doped aged gels and dried monoliths at 37 °C was monitored at 340 nm using a Cary 3 UV/vis spectrometer. Fluorescence measurements were taken on the G6PDH samples excited at 351 nm both before and after exposure to the substrate.

The rates of the G6PDH/$NADP^+$ reactions in the sol–gel as a function of substrate concentration are slower than those in solution. The plots of [$NADPH^+$] vs time for the sol–gel samples show an initial delay period of about 4 min where there is virtually no response (Figure 1). After the initial delay, the slopes gradually increase and the plots eventually begin to level off after a period of <1 h. The solution plots show no initial delay period and steeper slopes that level off within a few minutes. The slower response in sol–gels is probably due to the complexity of the diffusion in the gel matrix.

The activity in the gel was quantified by studying the kinetics of the reactions using Michaelis–Menten techniques. Using the slopes of the reaction rate plots for the sol–gel samples from 5 to 20 min, a Lineweaver–Burk plot was made (Figure 1). The slopes after 5 min were used in order to eliminate the initial period due to the diffusion of the substrate into the gel. The Lineweaver–Burk plots were linear for the substrate concentration range of 3.9×10^{-2}–3.3×10^{-3} M G6R, indicating good Michaelis–Menten behavior for the linear portion of the rates over this concentration range. The apparent K_m and k_{cat} values for the coupled enzyme system in both aged and dried gel samples are given in Table 1. All values were corrected for the changes in concentration that occur due to the shrinkage of the samples during drying. The calculated K_m was higher for the sol–gel samples than for the refrence solution, indicating that the binding of the substrate in the gel was weaker than in solution. Because the fraction of the enzyme that is active is not known, the values of k_{cat} were calculated on the

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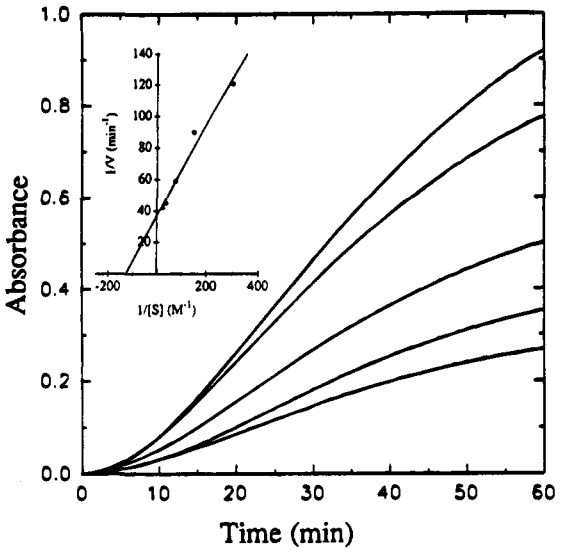

Figure 1. Plots of the absorbance at 340 nm of G6PDH/NADP$^+$-doped aged gel monoliths as a function of time. The substrate concentrations are (top to bottom) 39, 26, 13, 6, and 3 mM G6P. Inset: Lineweaver–Burk plot obtained from a 2 week aged gel sample.

Table 1. K_m (M) and Relative k_{cat} (min^{-1}) Values for the G6PDH/NADP$^+$ System in the Interior of Aged and Dried Sol–Gel Materials[a]

	K_m		k_{cat}	
processing time	aged gel	dried gel	aged gel	dried gel
2 weeks	78		0.27	
4 weeks	69	74	0.15	0.32
6 weeks	117	74	0.14	0.26
12 weeks	114	108	0.39	0.52

[a] In solution, K_m = 21.7 M and k_{cat} = 0.746 min^{-1}.

basis of the assumption that all of the enzymes encapsulated were active. The relative values of k_{cat} for the aged and dried gels were smaller than those obtained from solution, indicating that the turnover of G6P in the sol–gel encapsulated system was slower.

Fluorescence measurements provide a highly sensitive monitoring technique for optically based sensors. To date, only absorption has been used to monitor the enzymatic response to substrates in the sol–gel materials. The G6PDH/NADP$^+$ samples exhibit the characteristic fluorescence band of NADPH (Figure 2). The intensity of the luminescence at a given time after addition of the substrate was proportional to the substrate concentration.

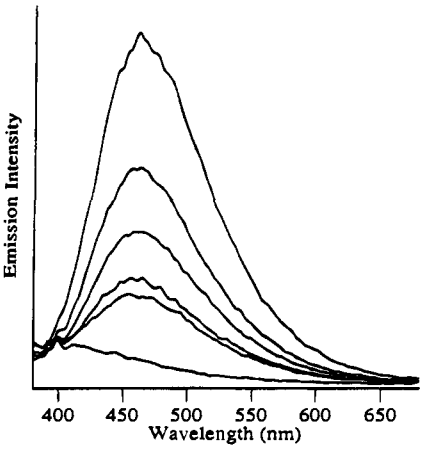

Figure 2. Fluorescence spectra excited at 351 nm of NADPH in G6PDH/NADP$^+$ aged gel monoliths after exposure to G6P. The substrate concentrations are (top to bottom) 39, 26, 13, 6.6, 3.3, and 0 mM G6P.

In summary, our studies show that pyridine nucleotide coenzymes can function in sol–gel materials. The enzymatic reactions which result in the reduction of the coenzymes occur fully within the interior of the transparent glass monoliths. The transparency of the sol–gel matrix allows these reactions to be monitored using both absorption and luminescence techniques. The reactivity of the G6PDH system in sol–gel materials demonstrates two important features. First, it uses the optical properties of a direct participant to monitor the enzymatic reactions. Second, it demonstrates the applicability of sol–gel materials with pyridine linked dehydrogenases. Our research shows that other dehydrogenase enzyme systems such as sorbitol dehydrogenase/NADH and lactate dehydrogenase/NADH are active in sol–gel glasses. The prospects for the development of optically based biosensors using sol–gel techniques are further expanding.

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