Sol−Gel-Encapsulated Alcohol Dehydrogenase as a Versatile, Environmentally Stabilized Sensor for Alcohols and Aldehydes

Amy K. Williams and Joseph T. Hupp*

Contribution from the Department of Chemistry, Northwestern University, Evanston IL, 60208

Received October 31, 1997

Abstract: Silicate-encapsulated yeast alcohol dehydrogenase (ADH) can be employed as a sensor for short-chained alcohols in standard aqueous, harsh nonaqueous, and gas-phase environments. Specifically, the implementation of sensing schemes based on encapsulated ADH/NAD^+ or ADH/NADH, and utilization of changes in fluorescence from the soluble, reduced cofactor nicotinamide adenine dinucleotide (NADH) upon exposure to alcohols or aldehydes, allows for semiquantitative determination of both substrates. Additionally, by using fluorescence from NADH, we find that cycling of the enzymatic probe can be accomplished via successive exposure to alcohol and aldehyde substrates, thus converting the system into a multiple-use sensor. Finally, we find that the gel matrix provides sufficient enzyme stabilization to permit the assemblies to be used analytically in hostile and inherently denaturing sample environments, including vapor-phase and nonpolar liquid (e.g., hexane) environments.

Introduction

Sol−gel-based encapsulation of delicate molecules and biomolecules, such as enzymes and coenzymes, in transparent, porous silicate matrixes is now proven technology.[1] Notably, the comparatively mild room-temperature processes of hydrolysis and condensation of semimetal alkoxides[2] produce liquid sols that can be buffered to pH values compatible with retention of enzyme activity. Equally important for retention of activity has been the discovery of gelation protocols that avoid the use of exogenous alcohol, a synthetic component often capable of inducing enzyme denaturation.[1c,3] The design of sensors or probes[4] that use the natural discrimination and selectivity of enzymes for substrates, together with the purported increased stability provided by the silicate matrix, is an obvious extension of this marriage between materials and biomolecules.

Numerous studies of enzyme encapsulation now indicate that while enzymes can be firmly trapped within a gel or glass matrix, pores remain that permit enzyme access by substrates. (A recent report shows, however, that not all substrates are equally well incorporated into the negatively charged silicate matrix.[5]) In addition, the clear siloxane gels allow facile spectroscopic observation of electronic transitions associated with products, enzymes, or other reaction components.[6,7] Unfortunately, it has also been shown that some enzymes lose their activity upon encapsulation in the matrix.[8]

Oxidoreductases which utilize a soluble nicotinamide adenine dinucleotide cation (NAD^+) as a cofactor represent an interesting class of enzymes that have yet to be exploited extensively in solid-state sensors.[9] While NAD^+ is largely silent spectroscopically, the reduced form of the cofactor (NADH) is an excellent light absorber at 340 nm and an efficient light emitter at 450 nm. Indeed, exposure of the encapsulated enzyme glucose-6-phosphate dehydrogenase (which utilizes an NADH derivative as its cofactor) to its substrate has been shown to yield measurable increases in cofactor fluorescence.[10] Our initial efforts have focused on extending this technology to encapsulation of alcohol dehydrogenase (ADH) with subsequent sensing of short-chained alcohols and aldehydes based on fluorescence of the cofactor (NADH) as shown in Scheme 1.

It should be noted that free yeast ADH has been used previously in a *solution* probe scheme for alcohol that takes advantage of NADH fluorescence.[11] Also known are solution assays based on ADH and NADH in which aldehyde is evaluated via decreases in cofactor fluorescence.[12] In part, on the basis of this work, we believed that the highly active yeast

(1) (a) Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, M. *Chem. Mater*. **1994**, *6*, 1605−1614. (b) Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Science* **1992**, *255*, 1113. (c) Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Anal. Chem*. **1994**, *66* (22), 1120A−1126A.

(2) Brinker, C. J.; Scherer, G. W. *Sol−Gel Science*; Academic Press: New York, 1990.

(3) Avnir, D.; Braun, S.; Ottolenghi, M. In *Supramolecular Architecture*; Bein, T., Ed.; American Chemical Society: Washington, DC, 1992; pp 384−404.

(4) For a general review of sensors and probes, see: Wolfbeis, O. S. *Fiber Optic and Chemical Sensors and Biosensors*; CRC Press: Boca Raton, FL, 1991; Vols. I−II.

(5) Shen, C.; Kostic, N. M. *J. Am. Chem. Soc*. **1997**, *119*, 1304−1312.

(6) Braun, S.; Shtelzer, S.; Rapporport, S.; Avnir, D.; Ottolenghi, M. *J. Non-Cryst. Solids* **1992**, *147/148*, 739−743.

(7) See, for example: (a)Yamanaka, S. A.; Nishida, F.; Ellerby, L. M.; Nishida, C. R.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Chem. Mater*. **1992**, *4*, 495−497. (b) Blyth, D. J.; Aylott, J. W.; Richardson, D. J.; Russel, D. A. *Analyst* **1995**, *120*, 2725−2730. (c) Edmiston, P. L.; Wambolt, C. L.; Smith, M. K.; Saavedra, S. S. *J. Colloid Interface Sci*. **1994**, *163*, 395−406. (d) Iosefzon-Kuyavskaya, B.; Gigozin, I.; Ottolenghi, M.; Avnir, D.; Lev, O. *J. Non-Cryst. Solids* **1992**, *147/148*, 808−812.

(8) Shtelzer, S.; Rapporport, S.; Avnir, D.; Ottolenghi, M.; Braun, S. *Biotech. App. Biochem*. **1992**, *15*, 227−235.

(9) We note, however, that detection of vapor-phase ethanol has recently been reported by Lan, Dave, Dunn, Valentine, and Zink (*Mater. Res. Soc. Symp. Proc.* **1995**, *371*, 267−76) based on catalytic current assessment within a sol−gel-encapsulated ADH/ferricyanide assembly. Other approaches involve immobilization *on* membranes with subsequent detection either through potentiometric or optical methods. (See, for example: Wangsa, J.; Arnold, M. *Anal. Chem*. **1988**, *60*, 1080−1082.).

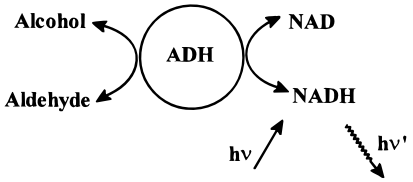
(10) Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Am. Chem. Soc*. **1995**, *117*, 9095−9096.

(11) Walters, B. S.; Nielsen, T. J.; Arnold, M. A. *Talanta* **1988**, *35*, 151−155.

(12) Bernt, E.; Bergmeyer, H. U. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 3, p 1506.

S0002-7863(97)03772-4 CCC: $15.00
Published on Web 04/18/1998

Scheme 1



ADH enzyme could be developed into a versatile solid-state probe (i.e., noncontaminating probe) for either alcohols or aldehydes by taking advantage of sol−gel encapsulation. We were also interested in assessing the ability of the gel matrix to stabilize the probe in environments that would ordinarily be viewed as hostile to enzymes and in evaluating the analytical utility of the proposed probe scheme in these environments. Real world applications of alcohol detection involving "gasohol" vapor, liquor samples, and the breath of a person who had consumed alcoholic drinks were targeted. Finally, we additionally hoped to utilize the inherent dual response capability (i.e., alcohol + aldehyde) of the yeast ADH enzyme/cofactor combination to convert irreversible probes into reversible (reusable) chemical sensors. As shown below, gel based encapsulation of yeast ADH has indeed proven possible, occurring with retention of observable enzymatic activity. Furthermore, the activity has been successfully exploited in harsh nonaqueous and gas-phase environments.

Experimental Section

Chemicals. Tetramethyl orthosilicate (TMOS) was purchased from Aldrich. Yeast alcohol dehydrogenase (ADH) EC 1.1.1.1 with activity 430 U/mg, nicotinamide adenine dinucleotide (NAD^+), and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Sigma Chemical Company. All chemicals were used as received and all water used was purified by using a Millipore purification system.

Enzyme Encapsulation: ADH with NADH. A typical encapsulation procedure involved sonication of TMOS (5.12 g, 0.0336 mol) with water (1.20 mL, 0.066 mol) and three drops of HCl solution (0.05 M) at 0 °C for 20 min to hydrolyze the monomer and generate a sol comprised of orthosilicic acid.[1b] Phosphate buffer (1.0 mL, 0.01 M, pH = 8.2) was added to the sol (1.0 mL) which was then shaken vigorously. The enzyme solution (0.20 mL) was then quickly added to the buffered sol and shaken gently. (High buffer concentrations, >50mM, lead to rapid gelation, leaving insufficient time for doping of the desired molecules into the liquid sol.) The enzyme solution usually was comprised of ADH (15 mg/mL) and NADH (7.0×10^{-2} M) in water. Aliquots of the enzyme-doped sol (0.25 mL) were pipetted into 5×10 mm polystyrene cuvettes, covered with Parafilm, and stored at 4 °C within a refrigerator until use. All manipulations of the sol were carried out in ice to slow gelation and minimize denaturation of the enzyme. After 24 h of gelling, the Parafilm was removed and the monoliths were further aged for a 5-day period at 4 °C. Monoliths were aged at reduced temperatures in order to slow the rate of evaporation and limit cracking within the gels. At this point the rectangular monolith dimensions were ca. $5 \times 3 \times 8$ mm corresponding to ca. 50% loss in volume. Phosphate buffer (0.25 mL, 0.1 M, pH = 8.2) was added to each monolith at least 24 h prior to a fluorescence measurement. The monoliths were further washed with buffer and dried with a tissue before being placed into a fresh cuvette and used for an experiment.

Enzyme Encapsulation: ADH with NAD^+. This enzyme/cofactor encapsulation procedure was analogous to the procedure for ADH with NADH. Here, however, it was necessary to dilute enzyme solutions to 2 mg ADH/mL and $[NAD^+] = 7.5 \times 10^{-3}$ M in order to avoid excessive cloudiness in the resulting gel.[13]

Fluorescence Measurements. All fluorescence measurements were made with a Perkin-Elmer MPF 44A spectrofluorimeter that had been computerized by using LabView software. A typical static fluorescence experiment involved adding 0.25-mL aliquots of cold aldehyde containing solutions to a monolith in a covered polystyrene cuvette. (Low temperatures were employed to diminish aldehyde volatility.) The cuvette windows were purged with nitrogen to reduce water condensation caused by adding cold substrate solutions. Emission at 450 nm was collected at 90° to excitation at 350 nm. Static fluorescence was collected over time and stored in an ASCII format. A new monolith was used for each aldehyde sample. The relative velocity of the enzymatic reaction at each aldehyde concentration was determined by a linear fit to the initial fluorescence decrease or to the decrease that followed an induction period. A similar protocol was used with alcohol substrates except that measurements were made at ambient temperature (ca. 23 °C) and fluorescence increases were monitored.

Reversible alcohol−aldehyde experiments involved adding aldehyde solution and monitoring a decrease in signal. The monolith was then removed from the cuvette, washed with cold buffer, and placed in a fresh cuvette. Alcohol solutions were then added to the monolith.

Results and Discussion

Encapsulation and Qualitative Kinetic Response. While silica sol−gel encapsulation of enzymes and retention of activity have previously been demonstrated, successful encapsulation of the ADH/NAD^+ pair was not a foregone conclusion. Although copious amounts of exogenous alcohol were not needed to form a homogeneous sol, the TMOS monomer used during sol formation yields up to four methanol molecules per silicon atom.[14] Methanol, in turn, can react with the dehydrogenase prior to intentional exposure to substrate. From Scheme 1, a fluorescence signal would then be expected even in the absence of added analyte. Our initial studies indeed did occasionally yield enzyme and cofactor containing monoliths that exhibited substantial fluorescence prior to exposure of substrate. In the initial studies, we used a tri(hydroxymethyl)-aminomethane (TRIS) based buffer that has been shown to trap the formed aldehyde,[11] which, in turn, could drive the formation of NADH (the fluorescent form of the cofactor). We found that the interference effect could be largely eliminated, however, by using a phosphate-based buffer and initiating encapsulation under less basic conditions where ADH activity for alcohol reduction was suppressed. Postsynthetic buffering to ca. pH 8.8 then served the dual purpose of washing away residual methanol and restoring enzyme activity. As shown in Figure 1, monoliths prepared in this way exhibited readily measurable increases in fluorescence upon exposure to aqueous alcohol solutions, with little fluorescence prior to exposure. Similar responses were obtained with *n*-propanol, consistent with the somewhat broad selectivity of ADH for short-chained alcohols.

Semiquantitative Kinetic Responses: Ethanol. The responses in Figure 1 suggested that a useful correlation between fluorescence signal intensities and substrate concentrations might exist. We chose to model the response in standard Michaelis−Menten fashion where the rate of fluorescence signal increase (ν) is expected to be governed by the apparent substrate/catalyst Michaelis constant (K_s), the cofactor/catalyst Michaelis constant (K_c), the concentration of cofactor [C], and the concentration

(13) Exposure of the cloudy gels to substrate resulted in no change in fluorescence. We are currently investigating whether the cloudiness is due to aggregation thus indicating an upper limit on achievable enzyme concentration.

(14) Note that post gelation washing of the monoliths obviously does not preclude formation of NADH at an earlier stage, for example, during doping of the sol. Recall that initial sol formation entails TMOS hydrolysis and methanol formation.

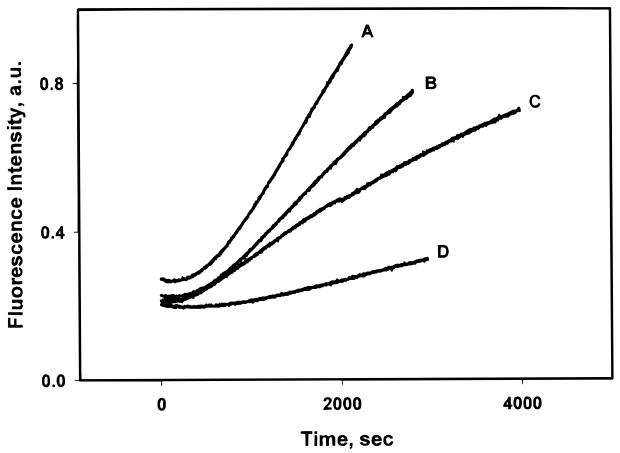


Figure 1. Fluorescence temporal response from ADH and NAD^+ containing monoliths following liquid-phase exposure to (A) 1.0 M ethanol, (B) 0.50 M ethanol, (C) 0.20 M ethanol, and (D) 0.10 M ethanol in 0.1 M phosphate buffer at pH = 8.8.

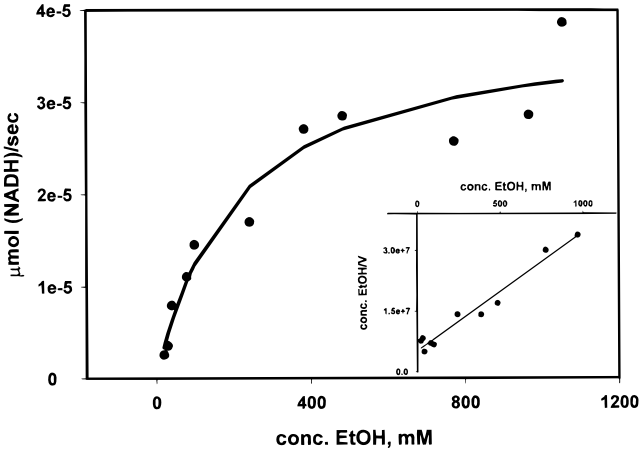


Figure 2. Plot of rate of fluorescence increase, expressed as μmols of NADH generated per second, versus ethanol concentration. Best fit curve obtained by fitting to eq 2 using kinetic parameter estimates from the Hanes plot. All samples were buffered at pH = 8.8. Inset: Hanes plot of concentrated EtOH/(rate of fluorescence increase, (moles of NADH per sec)) versus concentrated EtOH. The slope ($1/V$) and y-intercept (K_s/V) were equated with an apparent V_{max} (μmol min^{-1}) and K_s (mM), respectively.

of substrate [S]:[15]

$$v = \frac{V[C][S]}{(K_s + [S])(K_c + [C])} \quad (1)$$

In the equation, V is the hypothetical maximal velocity (μmol min^{-1}) at infinitely high concentrations of coenzyme and substrate. By assuming a sufficiently large concentration in cofactor, the expression can be simplified to

$$v = V' - K_s\left(\frac{v}{[C]}\right) \quad (2)$$

where V' is the apparent limiting velocity and K_s is the apparent Michaelis constant for the substrate at large [S] for a given [C]. Given the potential for product inhibition due to the chemical reversibility of Scheme 1, *initial* reaction velocities (slopes from plots of fluorescence vs time) were deemed the most pertinent parameters for model utilization.

Figure 2 shows, consistent with a Michaelis−Menten description, an asymptotic increase in reaction velocity with increasing substrate (ethanol) concentration. From the reciprocal of the x-intercept from a Hanes plot (see inset and eq 2),[16] the effective value for K_s is 0.18 M which can be compared with a reported value of 1.3×10^{-2} M for the same system in a phosphate-buffered solution environment at pH = 7.15.[17] Note, however, that the effective K_s value for the encapsulated enzyme/substrate pair will be convoluted with a coefficient for partitioning of substrate into the gel phase from the solution phase. In principle, from the reciprocal of the y-intercept from the Hanes plot in Figure 2, k_{cat} ($=V_{max}/(\mu$mols of enzyme active sites)) for ethanol dehydrogenation can also be estimated. The estimate, however, requires absolute reaction velocities; obtaining these, in turn, requires either accurate information about absolute fluorescence intensities and emission quantum yields or accurate intensity calibrations against fluorophore samples of known concentration in equivalent environments.[18] On the basis of the latter approach, k_{cat} is roughly 3 min^{-1}, assuming an active site loading of 1.2×10^{-3} μmol and further assuming that all encapsulated enzymes are accessible and that all four subunits of each encapsulated enzyme are active. For comparison, k_{cat} for the same enzyme/cofactor/substrate system in a glycine/pyrophosphate-buffered solution at pH = 9 is reportedly 14 000 min^{-1}.[19] Thus, the enzyme activity is tremendously diminished in the gel environment—a somewhat surprising finding in view of the retention of ca. 50% of solution activity in other enzyme systems.[8,10] We speculatively attribute the diminution here to enzyme aggregation during encapsulation, although additional effects including denaturation or gel trapping of the enzyme in a less-active conformation cannot be discounted.[20]

Semiquantitative Kinetic Responses: Propionaldehyde. Given that the yeast organism generally uses ADH to convert acetaldehyde into ethanol, rather than vice versa, we reasoned that Scheme 1 could be run in reverse with encapsulated ADH now comprising the catalytic component of an aldehyde sensor. An attenuation of fluorescence accompanies exposure of ADH/NADH containing monoliths to propionaldehyde (see the Supporting Information). A more-concentrated aldehyde solution yields a larger decrease in fluorescence than a less-concentrated solution, at least over the short term. Eventually, fluorescence from monoliths exposed to the aldehyde solution of lower concentration diminish to the same level as observed for those exposed to the more concentrated solution. A plot of the rate of fluorescence diminution (initial rate) against the propionaldehyde concentration yielded a clear correlation between velocity or rate and substrate concentration (see the Supporting Information), indicating that the assembly indeed can function as a semiquantitative probe of condensed phase aldehyde concentration. A second Hanes plot (Supporting Information) yielded an effective K_s value of 9.1×10^{-4} M and a k_{cat} value of 10 min^{-1} based on an active site loading of 8.3×10^{-3} μmol. It should be noted that in addition to propionaldehyde, the encapsulated enzyme also turns over acetaldehyde; however, significant reproducibility problems

(15) (a) Hayes, J. E.; Velick, S. F. *J. Biol. Chem.* **1954**, *207*, 225. (b) Wratten, C. C.; Cleland, W. W. *Biochemistry* **1963**, *2*, 935−941.

(16) Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*; Portland Press Ltd.: London, 1995.

(17) Bergmeyer, H. U., Ed.; *Methods of Enzymatic Analysis*; Academic Press: New York, 1974; Vol. I.

(18) Eisenthal, R.; Danson, M. J. *Enzyme Assays: A Practical Approach*; Oxford University Press: New York, 1992.

(19) Calculated by using data from ref 17, page 178 with $V_{max} = 0.061$ U (for 0.16 μg of ADH).

(20) We are currently attempting to evaluate aggregation effects by spatially interrogating gels via confocal fluorescence microscopy.

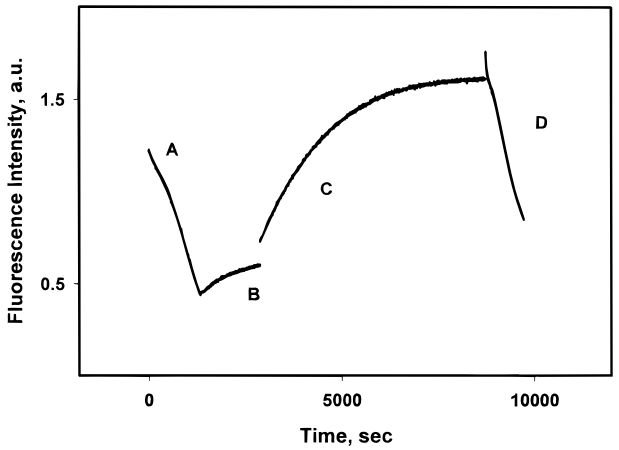


Figure 3. Monolithic enzymatic sensor response to aqueous solutions containing (A) 6×10^{-4} M propionaldehyde, (B) 0.36 M propanol, (C) 1.10 M propanol, and (D) 4.0 M propionaldehyde

were encountered with the latter. The problems appear to stem from an inability to maintain constant concentrations for the highly volatile acetaldehyde standards (acetaldehyde boiling point = 21 °C).

Dual Responses. The ability of encapsulated ADH to function as a catalytic probe for both alcohols and aldehydes suggested to us that successive exposure to alcohol and aldehyde substrates (or vice versa) could be used to reset a given probe and thus convert it to a multiple-use sensor. As shown in Figure 3, the system can indeed be cycled via successive aldehyde and alcohol exposure, i.e., reversible conversion between dark (NAD^+) and luminescent (NADH) forms of the cofactor can be effected via the sequences in Scheme 1. For a given monolith, this procedure can be repeated several times; however, it should be noted that larger alcohol concentrations are needed at later times in order to elicit the same response. Although Figure 3 shows results only for propanol and propionaldehyde, we also examined ethanol + acetaldehyde and benzyl alcohol + benzaldehyde as substrates. All three pairs proved capable of inducing reversible changes in cofactor fluorescence intensity. Control experiments showed that the observed changes were not associated with leaching of the cofactor from the monoliths, but rather that fluorescence originated from within the monoliths.

Hostile Environments. One of the most frequently cited virtues of sol−gel encapsulation is apparent stabilization of enzyme systems[7,21] with respect to denaturation. We reasoned that if this stabilization were sufficiently great, enzyme activity might be retained even in comparatively hostile environments such as vapor-phase environments or completely nonpolar liquid environments. Exposure of an ADH and NAD^+ containing monolith to ethanol in liquid hexane yields a time-dependent increase in NADH fluorescence, as expected if Scheme 1 is still operative. In contrast, in the absence of a protective monolith, alcohol dehydrogenase is denatured almost immediately upon exposure to hexane solutions.

The ability of the encapsulated enzyme to retain activity in nonaqueous environments obviously expands greatly the number and range of possibilities for sensor applications. At the same time, it raises interesting fundamental questions about the underlying physical or chemical basis for catalyst stabilization. One possibility is that the semirigid gel matrix is effectively form fitting and physically constrains the enzyme from unfolding. Alternatively, the gel's hydrophilicity may be the primary stabilizing factor: A sufficiently hydrophilic environment would both retain water (for microsolvation of the enzyme) and exclude nonpolar (potentially denaturing) solvent components. Gel encapsulation and absorbance studies with the near-UV chromophore, *p*-nitroaniline (PNA), provide circumstantial support for the second explanation. When initially encapsulated, the moderately solvatochromic PNA displays an electronic absorption maximum at 380 nm that is moderately red shifted from that observed in ethanol (372 nm), but significantly red shifted with respect to its value in hexane or benzene solution (344 nm). With subsequent exposure of the dye-containing monolith to either hexane or benzene the dye retains its water-like absorption behavior.

As qualitative tests of the first hypothesis, we compared the thermal stabilities of the free (aqueous) and encapsulated forms of the enzyme, as indicated by the retention of catalytic activity. We found that encapsulation offered no stability advantage during cycling to 50 °C and only a slight advantage during brief cycling to 65 °C. It is known that ADH denaturation at 50 °C mainly involves peptide chain unfolding followed by disruption of covalent interactions.[22] The absence of significant thermal stabilization suggests that ADH retains considerable macromolecular mobility in the gel environment. In a second experiment, resistance to denaturation was probed by exposing the encapsulated enzyme and cofactor to a concentrated (5 M) aqueous urea solution for 24 h. From mammalian ADH studies, it is known that urea can diminish catalytic activity both by competitive binding at the enzyme's active sites and by stimulating disruption of the tertiary structure.[23] In any case, a significant, but not complete, loss of activity was observed for both the encapsulated system and a solution-phase analogue. We tentatively conclude, therefore, that the gel matrix provides little or no direct physical stabilization of the dehydrogenase with respect to denaturation, at least under the conditions employed here. Nevertheless, we caution against generalizing the conclusion, especially in view of previously published positive evidence for physical stabilization of other enzyme systems.[24] Presumably the efficacy of the gel matrix in inhibiting denaturation is a critical function of overall enzyme size, charge, shape, gel pore dimensions, and extent of gel cross linking. The latter, of course, could depend strongly on both the preparative protocol and the gel aging protocol.

Representative Applications. Given the evidence above for gel-based stabilization of ADH in at least some hostile chemical environments and the evidence for retention of Michaelis−Menten-type kinetic behavior in other environments, we additionally sought to evaluate the encapsulated enzyme's response to various "real" analytes. Among the analytes examined was a small collection of liquor samples. To avoid substrate absorbance or fluorescence interferences, only colorless samples of high transparency were examined. For all neat samples, a limiting fluorescence response was observed, consistent with saturation of the enzyme kinetics. Following a 250-fold dilution, however, a kinetic distinction based on alcohol content was achievable. Indeed, as shown in Figure 4, an approximately linear correlation between sensor response rate (fluorescence growth rate) and sample alcohol percentage was achievable. Given the probe sensitivity, vapor-phase sampling above a

(21) Braun, S.; Rappoport, S.; Zusman, R.; Avnir, D.; Ottolenghi, M. *Mater. Lett.* **1990**, *10*, 1.

(22) Magonet, E.; Hayen, P.; Delforge, D.; Delaive, E.; Ramacle, J. *Biochem. J.* **1992**, *287*, 361−365.

(23) Gonnelli, M.; Strambini, G. B. *Biophys. Chem.* **1986**, *24*, 161−167.

(24) Braun, S.; Rappaport, S.; Shtelzer, S.; Zusman, R.; Druckmann, S.; Avnir, D.; Ottolenghi, M. In *Biotechnology: Bridging Research and Applications*; Kamely, D., et al., Eds.; Kluwer Academic Press: Dordrecht, The Netherlands, 1991; pp 205−219.

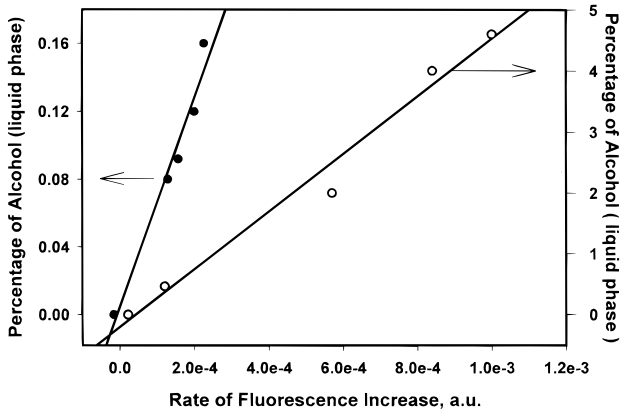


Figure 4. Rate of fluorescence increase versus percentage of alcohol in water-diluted beverage samples. Open circles: vapor-phase (head gas) sampling. Closed circles: liquid-phase sampling.

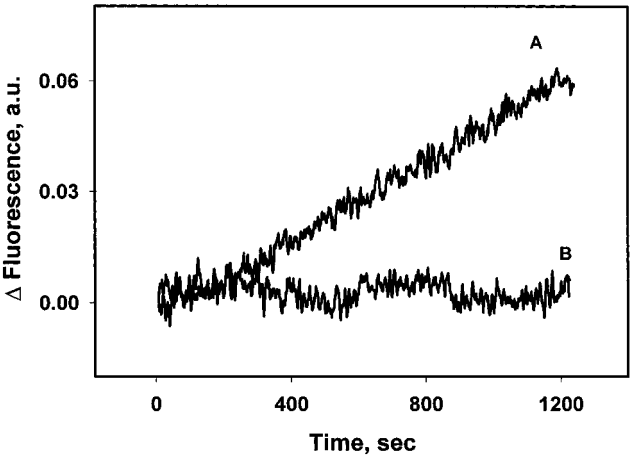


Figure 5. Evolution of fluorescence following gas-phase exposure to (A) breath of a person who had consumed three alcoholic drinks in 1 h and (B) breath of a person who had consumed no drinks.

solution reservoir was also attempted. Figure 4 (open circles) shows that an approximately linear correlation between response rate and alcohol content within the solution phase can also be obtained under these conditions. (Note that the gas-phase sampling approach largely negates the initial concern about interfering substrate absorbance or fluorescence and, thus, extends the realm of applicability of the sensor.) Taking the alcohol vapor experiment one step further, the breath of a person who had consumed three alcoholic drinks in 1 h was measured. The subject blew up a balloon that was placed over the cuvette. As seen in Figure 5, the enzyme-based ethanol sensor registered a readily detectable response.[25]

Finally, the alcohol sensor was also successfully employed in a vapor-phase gasoline testing experiment. An attempt at liquid-phase sample interrogation failed due to intense fluorescence or phosphorescence interferences from the samples themselves (presumably polycyclic aromatic components). As shown in Figure 6, the sensor easily distinguished gasoline from commercial gasohol (10% ethanol in gasoline) in a head gas sampling experiment. In addition, the sensor successfully

(25) The "Breathalyzer" experiment utilizes the vaporization of alcohol from the subject's lungs to the air. Assuming that three alcoholic drinks in an hour results in a blood alcohol level of ca. 0.1%, an approximate comparison of the "Breathalyzer" response (i.e., fluorescence growth rate) to the trend represented in the vapor-phase (head gas) experiment is possible. The combined kinetic responses are reasonably quantitatively self-consistent. The comparison additionally suggests that the lower limit of detection for the enzyme-based ethanol sensor, as currently configured, is ca. 0.02% alcohol present in the corresponding liquid phase.

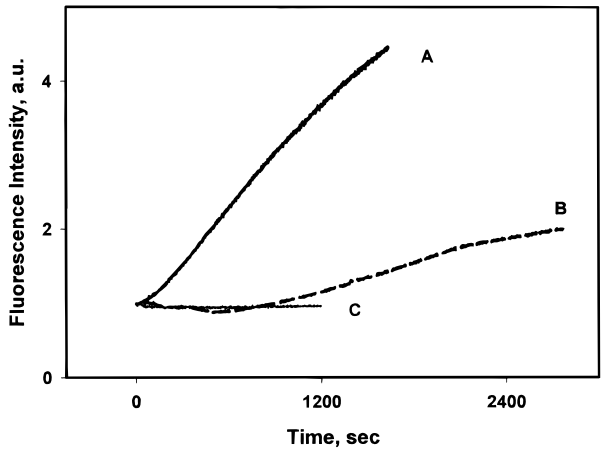


Figure 6. Evolution of fluorescence following gas-phase exposure to (A) 10% ethanol in liquid gasoline (commercial gasahol), (B) a 100-fold dilution of sample A in hexane, and (C) an alcohol-free sample of gasoline.

distinguished gasoline from a gasohol sample that had been diluted 100-fold with hexane (i.e., 0.1% ethanol solution). Nevertheless, the comparatively slow response of the sensor to the diluted gasohol vapor sample (see Figure 6) points to a potential practical limitation. Although the slow response largely reflects slow enzyme reaction kinetics (see above), slow diffusion of substrate species and/or cofactors through the comparatively thick gel monolith evidently also is important. In both liquid- and gas-phase alcohol detection experiments, significant induction periods preceded steep increases in fluorescence. Presumably the induction effects are associated with diffusion-limited transport of substrate molecules to active sites within the monolithic gel.[10] To accelerate the time response, we have begun exploring alternative thin film sensors (ca. 0.5 μm depth). At this point we have successfully encapsulated ADH and have observed a substrate response. We have encountered difficulties however in quantifying the response, apparently because of deleterious light scattering effects. Assuming that these difficulties can be overcome, we hope to report shortly on the details of the thin film studies.[26]

Conclusions

Yeast alcohol dehydrogenase and NADH or NAD^+ cofactors can be encapsulated in transparent silica monoliths via sol−gel techniques. Importantly, the techniques are sufficiently mild to permit retention of enzymatic activity although it was greatly diminished from solution values. The causes for the reduced activity are currently under investigation. Nevertheless, sufficient activity is retained to permit real-time observations of NADH fluorescence intensity changes and to permit use of the encapsulated assemblies as both selective chemical probes and sensors. Representative quantitative studies show, for example, that aqueous propionaldehyde concentrations can be evaluated readily over a 0.1−10 mM range and that aqueous ethanol concentrations can be evaluated over a 10−1000 mM range. Limitations of this monolith approach involve the length of time to measure a response accurately as well as the potential for eventual diffusion of the soluble cofactor ($NADH/NAD^+$) into the analyte solution. Sol−gel encapsulation additionally provides sufficient aqueous microsolvation to permit the enzyme

(26) Examples of successful film-based encapsulation include the following: (a) MacCraith, B. D.; McDonagh, C. M.; Keeffe, G. O.; McEvoy, A. K.; Butler, T.; Sheridan, F. R. *Sensors Actuators B* **1995,** *29*, 51−57. (b) Dave, B. C.; Soyez, H.; Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Chem. Mater*. **1995,** *7*, 1431−1434.

and cofactor to be used as vapor-phase alcohol and aldehyde concetration probes. Finally, the encapsulation methodology permits the enzymatic-catalysis/fluorescence-probe technique to be utilized in harsh sample environments, such as liquid hexane and gasoline, which would otherwise denature the dehydrogenase and render the probe inoperable.

Acknowledgment. We thank the Army Research Office (MURI program) for support of our research.

Supporting Information Available: Figures showing evolution of fluorescence for encapsulated ADH and NADH upon exposure to propionaldehyde solutions, correlation between rate of fluorescence decrease and propionaldehyde concentration, and Hanes plot of velocity data used to determine kinetic parameters (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA973772C