A Novel Protocol To Entrap Active Urease in a Tetraethoxysilane-Derived Sol–Gel Thin-Film Architecture

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Enzyme and antibody entrapment by sol–gel processing has received increased attention in recent years.[1–8] A sol–gel matrix offers several advantages for biomolecule entrapment and the development of new chemical biosensors: inherent low-temperature processing conditions, simplicity, tunable porosity, chemical inertness, and negligible swelling of the final glass matrix.[9–11] To date, silicon alkoxide precursors have been most extensively studied because they are inexpensive and exhibit relatively slow overall reaction kinetics. Thus, one can readily prepare silica sol–gels that are doped with a wide variety of reagents (e.g., chemical recognition elements) *and* tune the characteristics of the final glass matrix by adjusting the processing conditions (pH, precursor ratios, etc.).[1–16]

In this paper, we report a new sol–gel:enzyme:sol–gel sandwich architecture based on the enzyme urease (hexamer, M_W = 590 000) encapsulated between two sol–gel-derived thin films. The new film architecture is used as a sensing element for the quantification of urea. The activity, detection limits, linear dynamic range, preparation repeatability and performance, storage and operation stability, and response time of this new sensor platform are characterized. The response of the new sensing element is followed by using the well-known complexation of Nessler's reagent ($K_2Hg^{II}I_4$) with ammonia produced by the enzymatic hydrolysis of urea.[17] The key reactions are the following:

$$NH_2CONH_2\ (\text{urea}) + H_2O \rightarrow 2NH_3 + CO_2 \quad (\text{catalyzed by urease})$$

$$2NH_3 + 2Hg^{II}I_4^{2-} \rightarrow NH_2Hg_2I_3\ (\lambda_{max} = 405\ \text{nm}) + NH_4^+ + 5I^-$$

Urease catalyzes the hydrolysis of urea to produce ammonia which in turn reacts with Nessler's reagent to form a colored product. By following the absorbance of the colored product at 405 nm, urea can be quantified[18] and the analytical performance of the new sandwich architecture is determined.

A stock sol–gel solution was prepared by mixing 4.5 mL of TEOS (tetraethoxysilane), 1.4 mL of H_20, and 100 μL of 0.1 M HCl in a glass vial. Mixing was terminated after a clear solution was formed (3 h). The sol–gel: urease:sol–gel sandwich films were prepared on glass slides. Prior to film preparation, the glass slides were soaked in concentrated HNO_3 for 2 h and then rinsed with copious amounts of distilled–deionized water. The slides were wetted with *n*-propyl alcohol prior to initial film casting. The thin films were then formed by spin coating (3000 rpm for 30 s) onto the substrate using 200 μL of the appropriate sol–gel solution. The lower film was produced from a 1:2 dilution of the sol–gel stock solution with methanol. After drying under ambient conditions for 6 h, the film was cured for 10 min at 200 °C. After removal from the oven, the film was kept in air at room temperature for 30 min. The final film thickness was 0.30 μm (determined using a surfometer, Model SF 200; Planner Industrial). Twenty-five microliters of a 10 mg/mL urease (Sigma; 69 000 μM units/g of solid) solution was then spread onto the sol–gel-coated substrate. The urease-coated film was then kept in the refrigerator at 4 °C. After 24 h, a diluted stock sol–gel solution (1:4, sol-gel stock:water) was spun cast on top of the sol–gel:urease surface to form a 0.10-μm-thick upper film to complete the sol–gel:urease:sol–gel sandwich film. All films were stored dry at 4 °C.

After preparing the sol–gel:urease:sol–gel films, we began our characterization by testing for enzymatic activity. Toward this end, films were placed in a test tube that contained 10 mL of Tris buffer (0.01 M, pH = 7.0) and incubated at 30 °C for 15 min. Urea (UltraSigma, Sigma) was then added to the test tube. After

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(1) Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Science* **1992**, *25*, 1113.
(2) Tatsu, Y.; Yamashita, K.; Yamaguchi, M.; Yamamura, S.; Yamamoto, H.; Yoshikawa, S. *Chem. Lett.* **1992**, 1615.
(3) Yamanaka, S. A.; Nishida, F.; Ellerby, L. M.; Nishida, C. R.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Chem. Mater.* **1992**, *4*, 495.
(4) Wang, R.; Narang, U.; Bright, F. V.; Prasad, P. N. *Anal. Chem.* **1993**, *65*, 2671.
(5) Kurokawa, Y.; Sano, T.; Ohta, H.; Nakagawa, Y. *Biotechnol. Bioeng.* **1993**, *42*, 394.
(6) Kurokawa, Y.; Ohta, H.; Okubo, M.; Takahashi, M. *Carbohydr. Polym.* **1994**, *23*, 1.
(7) Aharonson, N.; Alstein, M.; Avidan, G.; Avnir, G.; Bronshtein, A.; Lewis, A.; Lieberman, K.; Ottolenghi, M.; Polevaya, Y.; Rottman, C.; Samuel, J.; Shalom, S.; Strinkovski, A.; Turniansky, A. In *Better Ceramics Through Chemistry*; Sanchez, C., Mecartney, M. L., Brinker, C. J., Cheetham, A., Eds.; Materials Res. Soc. Symp. Proc.; MRS: Pittsburgh, 1994, Vol. 346, p 1.
(8) Shtelzer, S.; Braun, S. *Biotechnol. Appl. Biochem.*, in press.
(9) Zang, Y.; Prasad, P. N.; Burzynski, R. In *Chemical Processing of Advanced Materials*; Hench, L. L., West, J. K., Eds.; Wiley: New York, 1992.
(10) *Chemical Processing of Advanced Materials*; Hench, L. L., West, J. K., Eds.; Wiley: New York, 1992.
(11) Narang, U.; Wang, R.; Bright, F. V.; Prasad, P. N. *J. Phys. Chem.* **1994**, *98*, 17.
(12) Narang, U.; Dunbar, R. A.; Bright, F. V.; Prasad, P. N. *Appl. Spectrosc.* **1993**, *47*, 1700.
(13) Narang, U.; Bright, F. V.; Prasad, P. N. *Appl. Spectrosc.* **1993**, *47*, 229.
(14) Narang, U.; Prasad, P. N.; Bright, F. V.; Ramanathan, K.; Kumar, N. D.; Malhotra, B. D.; Kamalasanan, M. N.; Chandra, S. *Anal. Chem.*, in press.
(15) MacCraith, B. D.; McDonagh, C. M.; O'Keefe, G.; Keyes, E. T.; Vos, J. G.; O'Kelly, B.; McGlip, J. F. *Analyst* **1993**, *118*, 385.
(16) Klein, R.; Voges, E. *Sensors Actuators B* **1993**, *11*, 221.
(17) Pierce, C. W.; Haenisch, E. L.; Sawyer, D. T. *Quantitative Analysis*; John Wiley & Sons: New York, 1958, p 410.
(18) Wolf, P. L.; Williams, D.; Tsudaka, T.; Acosta, L. *Methods and Techniques in Clinical Chemistry*; Wolf, P. L., Ed.; Wiley-Interscience: New York, 1972, p 42.

0897-4756/94/2806-1596$04.50/0

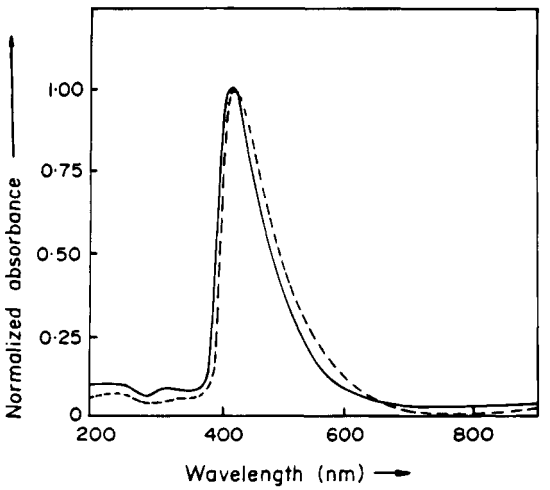

Figure 1. Normalized UV–vis absorbance spectra resulting from the reaction of the new sol–gel:urease:sol–gel sandwich film (—) and native urease (- - -) to Nessler's reagent and 10 mM urea.

15 min, the film was removed and 1 mL of Nessler's reagent (Qualigens, Glaxo India Ltd.) was added to the test tube and the total volume of liquid in the test tube increased to 20 mL with distilled–deionized water. This solution was subsequently diluted 1:10 with water and the absorbance read at 405 nm. Blanks were run under identical conditions using sandwich films fabricated with bovine serum albumin in place of urease.

Figure 1 shows typical normalized absorbance spectra that result after 10 mM urea is exposed to a 7-day-old sol–gel:urease:sol–gel film (—) or native urease (- - -) and treated subsequently with Nessler's reagent. The results illustrate two key points. First, urease is clearly active within the sol–gel sandwich film architecture. Second, there is little spectral dissimilarity, suggesting no unusual behavior of the enzyme in the sol–gel matrix.

We tested for urease leaching by storing a sol–gel: urease:sol–gel film in Tris buffer for 3 days and assaying (urea and Nessler's reagent added) the solution for urease. No detectable urease was found in the buffer. This suggests that the sol–gel sandwich scheme is a good, stable matrix in which to entrap enzymes like urease. Of course, it is also possible that any urease that may have leached from the film is inactive.

We next investigated the response of the sol–gel: urease:sol–gel films to varying concentrations of urea. Figure 2 presents a typical calibration curve for urea. The linear range covers the physiological concentration of urea found in blood[19] and detection limits were on the order of 0.5 mM.

One of the key features of any viable biosensor is a rapid response to analyte. Sol–gel-derived chemical sensors have been previously fabricated for quantification of O_2[15] and NH_3[16] and exhibit response times on the order of 5–10 s. However, these sensor schemes operated in the *gas phase* only and one[16] required processing at 150 °C. Many enzymes like urease cannot survive such harsh processing conditions.

Any real biosensor is required to respond rapidly in aqueous media. To date, one of the key problems

(19) Fischbach, F. T. *A Manual of Laboratory & Diagnostic Tests*; J. B. Lippincott Co.: New York, 1992, p 312.

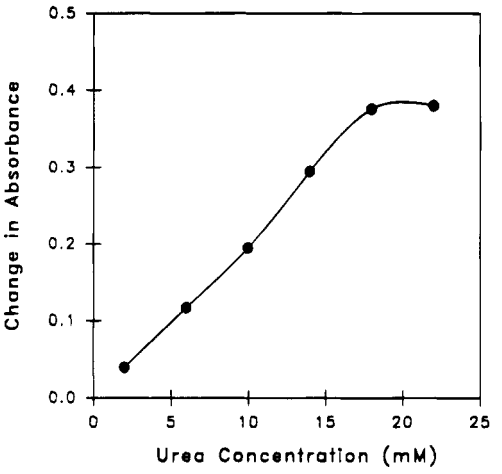

Figure 2. Photometric calibration (λ = 405 nm) curve using a sol–gel:urease:sol–gel film (aged for 7 days) on exposure to varying concentrations of urea and identical amounts of Nessler's reagent.

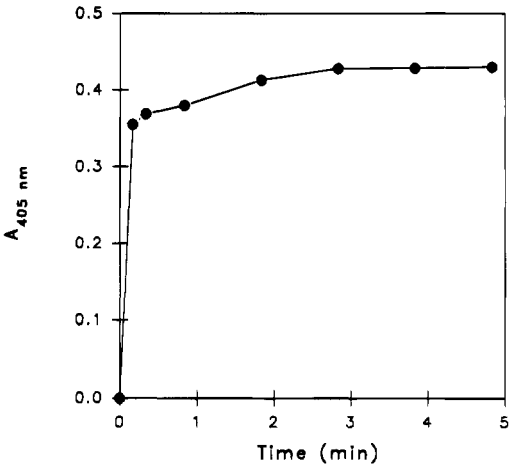

Figure 3. Absorbance (405 nm) vs time profile for a sol–gel: urease:sol–gel sandwich film on exposure to Nessler's reagent and 20 mM urea.

associated with the use of sol–gel-derived chemical sensors in *liquids* is the slow response time.[3,4,20,21] For example, the response time of sol–gel monoliths containing glucose oxidase, peroxidase, and an oxidizable dye to β-D-glucose was approximately 1 h.[3] Lev et al.[20] reported that 1,10-phenanthroline, encapsulated within sol–gel-derived disks, responded to Fe^{2+} in 1–24 h. Kurokawa et al.[5,6] demonstrated urease activity in ZrO_2– and TiO_2–cellulose composites, but (in the only system reported) response times were more than 30 min.

In the present work, we attempt to alleviate this shortcoming by using a thin-film sandwich architecture that provides one simultaneously with high levels of active enzyme and rapid diffusion. The analytical response vs time profile of our urease sandwich film to an aqueous urea sample is shown in Figure 3. The response is 85% of the maximum value in approximately 10 s. These results suggest fast diffusion of urea into and ammonia out of the sol–gel sandwich architecture.

(20) Lev, O.; Kuyavskaya, B. I.; Gigozin, I.; Ottolenghi, M.; Avnir, D. *Fresenius J. Anal. Chem.* **1992**, *343*, 370.

(21) Braun, S.; Shtelzer, S.; Rappoport, S.; Avnir, D.; Ottolenghi, M. *J. Non-Cryst. Solids* **1992**, *147*, 739.

(22) *Biosensors: Fundamentals, Technologies, and Applications;* Scheller, F., Schmid, R. D., Eds.; VCH: Weinheim, Germany, 1992, pp 159–174.

Table 1. Comparison of the New Sol–Gel:Urease:Sol–Gel Film Sensor Platform to Representative Urease-Based Biosensors or Urease Immobilization Schemes

composition of matrix/support	response time	detection limits	storage stability	run-to-run stability	activity vs native	ref
TiO_2–cellulose	>30 min	ND	few weeks	ND[e]	ND	5
TiO_2	ND	ND	few weeks	ND	ND	5
ZrO_2–cellulose	ND	ND	ND	100 ± 20%[a]	10%	6
antimony-based NH_3 electrode	30–45 s	0.1 mM	1–2 days[b]	ND	ND	23
avid–biotin–silica	70 s	0.1 mM	<1 week	ND	6%	24
collagen films	≥7 min	0.2 μM	ND	ND	ND	25
poly(vinyl alcohol)	2 min	0.1 mM	28 days[c]	1–5% RSD	ND	26
silanized silica	ND	ND	>168 h (25 °C)	ND	88%	27
			>1 h (100 °C)	ND	88%	27
			>1 h (100 °C)	ND	100%	28
sol–gel sandwich	10 s	0.5 mM	>6 weeks	3.9% RSD[d]	12%	this work

[a] Relative activity compared to initial preparation (20 replicates). [b] Due to enzyme leaching. [c] 14% decrease in response over this time period. [d] Response drops after the 5th replicate to 50% of original value. [e] ND: not determined or not reported.

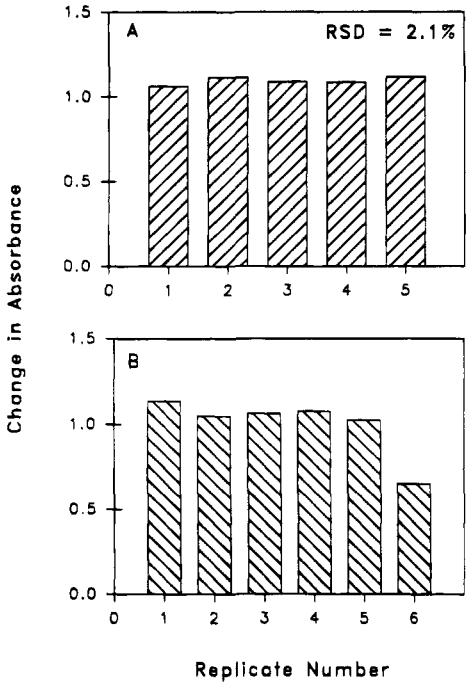

Figure 4. Reproducibility and stability of sol–gel:urease:sol–gel films. (A) Response of five different sol–gel:urease:sol–gel films (prepared under identical conditions) to Nessler's reagent and 6 mM urea. (B) Response of the same sol–gel: urease:sol–gel film to replicate samples containing 6 mM urea and Nessler's reagent.

Another issue associated with any chemical sensing platform is the reproducibility of the preparation scheme. Figure 4A shows the response (to 6 mM urea) of five replicate sol–gel:urease:sol–gel films. The recovered RSD is 2.1%. We also investigated the response of a single sol–gel:urease:sol–gel film to successive urea determinations (Figure 4B). The response remains moderately constant for the first five replicates (RSD = 3.9%) but decreases by approximately 50% after the sixth determination. We speculate that because each assay requires incubation of the film at 30 °C, the decrease in response is a result of temperature-induced loss in the urease activity. We have confirmed that temperature has an affect on the sensor performance and note that a film stored for 2 h at 30 °C in buffer is only 75 ± 3% as active as a similar film stored at 4 °C. The urease entrapped within our thin-film architecture remained active (>95% of original activity) for at least 6 weeks if stored at 4 °C.

Finally, there have been many urease-based urea sensors reported in the literature,[22] and it is reasonable to compare our scheme to previous designs. Table 1 present a summary of the performance characteristics of several representative sensor schemes[5,6,23–28] and the new sol–gel sandwich scheme. Although the current detection configuration is far from optimized, it is comparable to previous schemes in terms of detection limits. Activities are comparable to the majority of other urease sensor schemes. When compared to previous sensor platforms, the new sensor film exhibits a combination of more rapid response, high storage stability, good run-to-run stability, simplicity in fabrication, does not involve any chemical modification of the substrate or enzyme, and does not require secondary organic codopants.

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(23) Joseph, J. P. *Anal. Chim. Acta* **1985**, *169*, 249.
(24) Luo, S.; Walt, D. R. *Anal. Chem.* **1989**, *61*, 1069.
(25) Mikkelsen, S. R.; Rechnitz, G. A. *Anal. Chem.* **1989**, *61*, 1737.
(26) Narinesingh, D.; Mungal, R.; Ngo, T. T. *Anal. Chim. Acta* **1991**, *249*, 387.
(27) Kallury, K. M.; Lee, W. E.; Thompson, M. *Anal. Chem.* **1992**, *64*, 1062.
(28) Kallury, K. M.; Lee, W. E.; Thompson, M. *Anal. Chem.* **1993**, *65*, 2459.