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Communications

Enzymatic Activity of Glucose Oxidase Encapsulated in Transparent Glass by the Sol–Gel Method

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The sol–gel process is a technique that can be used to prepare optically transparent oxide glasses by hydrolysis and polycondensation of alkoxides.[2] Little or no heating is required, and consequently gels may be doped with molecules whose poor thermal stability precludes their incorporation in traditional inorganic hosts.[3,4] The sol–gel method also has the advantage that it may be used to prepare transparent monoliths with dimensions on the order of centimeters, as well as uniform films and fibers. Previous studies focused on the incorporation of organic molecules to probe spectroscopically the sol–gel process as well as to develop new optical properties. To extend our studies to include enzymes and other proteins as dopants in optically transparent sol–gel glasses, we modified the conventional procedures for preparation of sol–gel glasses, successfully applied our modified procedure to the encapsulation of several colored metalloproteins, and observed full retention of their absorption spectra.[5] We found that the large proteins were securely trapped but small substrates could diffuse readily into and out of the sol–gel matrix.[5] Prior to our studies, Avnir and co-workers reported that trapping of the enzyme alkaline phosphatase in sol–gel glass produced a bioactive opaque powder with improved thermal stability relative to the free enzyme.[4] In this communication, we report the synthesis of transparent silicate glass monoliths of the dimensions 10 mm × 5 mm × 2 mm containing the enzymes glucose oxidase and peroxidase. In addition to measuring the changes in the absorption spectra of the products of the enzymatic reaction in the glass, we follow the formation of the products in solution and demonstrate that these new enzyme-containing sol–gel glasses are enzymatically active and can be used as the active medium in optically based glucose sensors.

It is important to stress that the method of protein immobilization described here differs drastically from methods in which proteins have been immobilized by adsorption on glass surfaces or impregnation of porous glass powders.[6] Entrapment of the protein molecules in the sol–gel matrix apparently occurs because the silicate polymerizes around the biomolecule and physically traps it in the growing oxide network. The retention of the characteristic absorption spectra of several metalloproteins encapsulated in this matrix supports the conclusion that the proteins are entrapped.[5]

Two enzymatic reactions are involved in the system described here. First, glucose oxidase catalyzes the oxidation of β-D-glucose by dioxygen to give D-gluconic acid and hydrogen peroxide:

$$\text{glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \quad (1)$$

(1) (a) Department of Chemistry and Biochemistry. (b) Department of Materials Science and Engineering.

(2) Brinker, C. J.; Scherer, G. Sol–Gel Science: The Physics and Chemistry of Sol–Gel Processing; Academic Press: San Diego, 1989.

(3) (a) McKiernan, J. M.; Pouxviel, J. C.; Dunn, B.; Zink, J. I. *J. Phys. Chem.* **1989**, *93*, 2129. (b) Pouxviel, J. C.; Dunn, B.; Zink, J. I. *J. Phys. Chem.* **1989**, *93*, 2134. (c) McKiernan, J. M.; Yamanaka, S. A.; Dunn, B.; Zink, J. I. *J. Phys. Chem.* **1990**, *94*, 5652. (d) Knobbe, E. T.; Fuqua, P. D.; Nishida, F.; Dunn, B. *Appl Opt.* **1990**, *29*, 2729. (e) Dunn, B., Zink, J. I. *J. Mater. Chem.* **1991**, *1*, 903. (f) McKiernan, J. M.; Yamanaka, S. A.; Knobbe, E.; Pouxviel, J. C.; Parveneh, S.; Dunn, B.; Zink, J. I. *J. Inorg. Organomet. Polym.* **1991**, *1*, 87.

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

(5) Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Science* **1992**, *255*, 1113.

(6) (a) Norde, W. *Adv. Colloid Interface Sci.* **1986**, *25*, 267. (b) Brash, J. L., Horbett, T. A., Eds. *Proteins at Interfaces: Physicochemical and Biochemical Studies*; American Chemical Society: Washington, DC, 1987. (c) Mosbach, K., Ed. *Methods in Enzymology: Immobilized Enzymes and Cells, Part B* Academic Press: New York, 1987; Vol. 135.

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Peroxidase then catalyzes the reactions of dye precursors with hydrogen peroxide to produce colored dyes. This procedure is based on commonly employed methods for quantitation of glucose in solution.[7,8] Two different dye precursors were used. With 4-aminoantipyrine and *p*-hydroxybenzene sulfonate as the precursors, a quinoneimine dye with an absorption maximum at 510 nm was formed. *o*-Dianisidine was oxidized to produce a dye with an absorption maximum at 500 nm. The rate of formation of the colored dyes is indicative of the degree of activity of the enzymes and can be monitored by optical spectroscopy.

Three types of samples were prepared. The first, used to monitor color changes in the glass, contained glucose oxidase, peroxidase, 4-aminoantipyrine, and *p*-hydroxybenzene sulfonate. The second and third, used to quantify the enzymatic activity as well as demonstrate the reactions, did not contain the dyes. For each type of glass, the silica sol was prepared by sonication of tetramethoxysilane (TMOS-Aldrich, 15.22 g), deionized water (3.38 g), and 0.04 N aqueous hydrochloric acid (0.22 g) in an ice-cooled ultrasonic bath for approximately 20 min.[9] The sol was then buffered to create more favorable pH conditions for the enzymes. For the gels containing the glucose oxidase, peroxidase and dye components, 4.05 mL of the sol was mixed with 4.95 mL of 0.01 M sodium phosphate buffer pH 6. Enzyme stock solutions were prepared by dissolving 0.96 mg of horseradish peroxidase Type II (Sigma) and 1.46 mg of glucose oxidase from *Aspergillus niger* Type X-S (Sigma), each individually in 1 mL of buffer. Then, 138 μL of peroxidase and 21 μL of glucose oxidase stock solutions were mixed with 2.84 mL of buffer yielding 3 mL of enzyme mixture to be used for the encapsulation. To the buffered sol, 3 mL of enzyme mixture and 8.32 mL of dye precursor solution (Aldrich, 0.5 mM of 4-aminoantipyrine and 2 mM of *p*-hydroxybenzene sulfonate in buffer) were added immediately. All of the additions were carried out on ice. The doped sol was quickly transferred to polystyrene cuvettes and sealed with Parafilm. All samples were stored at 4 °C for aging and drying.

The second type of sample, containing the glucose oxidase and peroxidase without the dye precursors, was prepared and handled in a similar manner. For these samples, 4.05 mL of TMOS sol was mixed with 4.95 mL of buffer. To this solution, 3 mL of the enzyme mixture was added. All steps were carried out on ice. The resulting sol was transferred to cuvettes and handled as with the glucose oxidase/peroxidase/dye precursor gels. The third type of sample, containing only glucose oxidase, was also prepared as described above.

The activity of the encapsulated enzymes was demonstrated by the formation of color in the glass. When solid monoliths containing both of the enzymes 4-aminoantipyrine and *p*-hydroxybenzene sulfonate were placed in a solution containing β-D-glucose, the glass turned red and the absorption maximum was observed at 520 nm as shown in Figure 1. To confirm that the enzymatic activity was distributed throughout the gel and that the color change was not due to enzyme adsorbed onto the surface of the gel, the gel was cleaved. All sections of the gel including the interior exhibited the same degree of coloration indicating that the activity was distributed throughout the

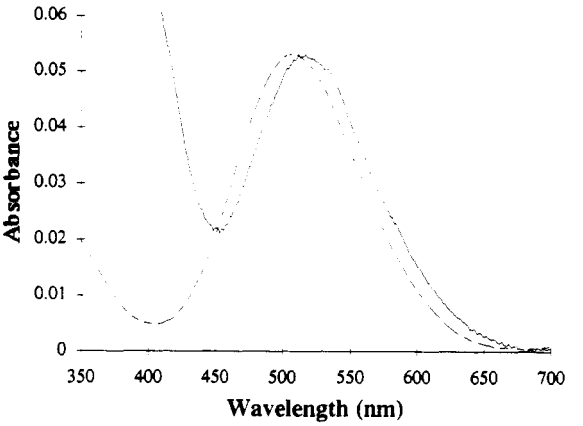

Figure 1. Solid line: absorption spectrum of a 5 mm × 5 mm × 2 mm solid monolith containing glucose oxidase, peroxidase, 4-aminoantipyrine, and *p*-hydroxybenzene sulfonate after exposure to an aqueous glucose solution. See text for experimental details. Dashed line: Solution absorption spectrum of the products of the enzymatic reaction. For comparison, the peak height was normalized to that of the glass spectrum.

Table I. Determination of k_{cat} and K_m for Glucose Oxidase with β-D-Glucose

	lit.[10,11]	solution[a]	aged gel[a]
K_m, M	0.026	0.028 ± 0.003	≥0.05 ± 0.05
k_{cat}, s^{-1}	293	251 ± 25	250 ■ 80

[a] Conditions: 0.01 M potassium acetate, pH 5.6, at 25 °C. The analysis was carried out as described in refs 10 and 11.

gel. Immobilization of the enzymes was tested by washing the gel with buffer and adding β-D-glucose and the dye precursor to the wash. No color change was observed indicating that the enzymes were not leached from the gel.

Once it was established that the enzymes were active in the sol–gel samples, the next step was to quantify the activity. In the initial experiments, crushed sol–gel samples containing glucose oxidase and peroxidase were added to a solution containing 32 mL of dye precursor solution and 6.15 mL of a 10% glucose solution. The glasses were crushed to increase the surface area. The studies were carried out with glasses at various stages of aging and drying. Triton X-100 (8 mL, Sigma) was added to decrease the adsorption of the dye on the glass surfaces. The reaction rates were measured by monitoring the increase in the absorbance of quinoneimine. Changes in the absorbance of the solution exposed to the glass-encapsulated enzyme and to reference solutions of the enzyme are shown in Figure 2. With the approximation that the slope, given by a linear regression, represents the overall apparent rate constant, the rates in the aged samples were approximately 20% of that of the reference solution. Dried samples exhibited values of about 10%.

To quantify further the enzyme activity, glucose oxidase was immoblized separately and the turnover number (k_{cat}) and the apparent dissociation constant, (K_m) with β-D-glucose were determined. Table I summarizes the results of steady-state turnover experiments. Measurements were carried out using an oxygen meter as described previously using air-saturated solutions.[10-12] The k_{cat} for glucose oxidase immobilized in glass is quite similar to that found for the enzyme in solution. The apparent dissociation

(7) Trinder, P. *Ann. Clin. Biochem.* **1969**, *6*, 24.
(8) Worthington, C. S.; Teller, J. D. *Worthington Enzyme Manual*; Freehold, NJ, 1972; p 43.
(9) Esquivias, L.; Zarzycki, J. *Proceedings of the Third International Conference on Ultrastructure Processing*; Mackenzie, J. D., Ulrich, D. R., Eds.; John Wiley & Sons: New York, 1988; pp 255–270.
(10) Bright, H. J.; Gibson, Q. H. *J. Biol. Chem.* **1967**, *242*, 994.
(11) Rogers, M. J.; Brandt, K. G. *Biochemistry* **1971**, *10*, 4624.
(12) Gibson, Q. H.; Swoboda, B. E. P.; Massey, V. *J. Biol. Chem.* **1964**, *239*, 3927.

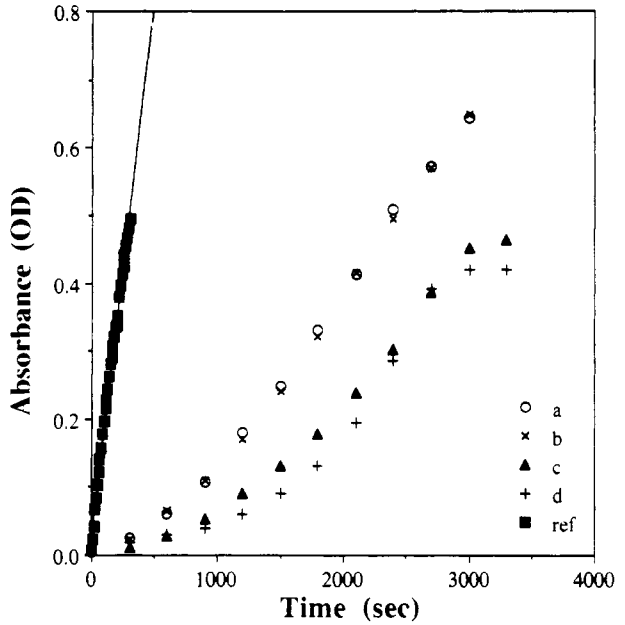

Figure 2. Plots of the absorbance as a function of the time in which glass samples containing glucose oxidase and peroxidase were exposed to a solution of glucose, 4-aminoantipyrine, and *p*-hydroxybenzene sulfonate. Sample (a) was aged 2 weeks, (b) was aged 3 weeks, (c) was aged 2 weeks and dried 1 week, and (d) was dried 2 weeks (no aging). The squares connected by the straight line are the results from the reference solution containing 3 mL of the enzyme mixture and 9.0 mL of sodium phosphate buffer pH 6.

constant for the enzyme, i.e., K_m, is 2-fold higher than that of the enzyme in solution indicating that binding of the substrate is weaker. The lower apparent activity found for the mixed enzyme system with dyes (see Figure 2) is not due to a slower turnover number for glucose oxidase. The difference in the relative rate may be influenced by the diffusion of both of the dye components and glucose into the gel structure in order to reach the enzymes, and the diffusion of the products back into solution to be measured.

In summary, optically transparent silica glass containing the active enzymes glucose oxidase and peroxidase was synthesized by the sol-gel method. The enzymes were immobilized, but small molecules such as glucose diffused readily through the porous glass. The enzymatic reactions that occur normally in solution were readily carried out in the pores of the glass matrix. When all of the enzymes and colorimetric precursors were encapsulated and the glass was exposed to glucose solutions, the colored products which were formed in the glass produced a colored glass which is suitable for use as the active element in a solid-state optically based detector. The turnover number for encapsulated glucose oxidase with β-D-glucose is the same in the glass and in solution. The results show not only that the enzymes retain similar activities when immobilized in an inorganic matrix but also that optically transparent biochemically active glasses can be synthesized.

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Registry No. Glucose oxidase, 9001-37-0; peroxidase, 9003-99-0; β-D-glucose, 492-61-5.

Preparation of Titanium Diboride from Titanium Alkoxides and Boron Powder

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The high chemical and thermal stability of TiB_2, along with its low electrical resistivity and high thermal conductivity, has made it a very interesting engineering ceramic material.[1] An important element controlling the commercial success of TiB_2 as an engineered ceramic material is the availability of pure, submicron TiB_2 powders at low processing temperatures. The methods most often used for preparing TiB_2 powders include the direct reaction between Ti and B[2] and the reduction of TiO_2 and B_2O_3 (or B_4C) by carbon.[3,4] These methods generally require high temperatures (around 2000 °C) and yield TiB_2 powders with low purity and large particle sizes (5–30 μm). High-purity TiB_2 powders have been prepared by a gas-phase reaction[5] or from organometallic precursors.[6,7]

This communication reports our results on the preparation of highly crystalline TiB_2 powders with small particle sizes (0.3–0.4 μm), by pyrolyzing mixtures of boron and a polymeric precursor. It is well-known that thermal decomposition of furfuryl alcohol (FuOH) and hydrolysis of $Ti(OBu)_4$ produces carbon[8] and TiO_2,[9] respectively. Therefore, heating a mixture of boron, titanium butoxide, and furfuryl alcohol should result in the generation of carbon and titanium oxide. As the temperature is increased, the carbon causes the reduction of TiO_2, forming TiB_2 as the final product as indicated by reaction 1.

$$2C + 2B + TiO_2 = TiB_2 + 2CO \quad (1)$$

The amorphous boron powder used in the present study had an average size of 0.22 μm, based on a centrifugal particle size analysis and SEM micrograph (Figure 1a). The elemental analysis of the powder indicated that it contained 94.06% B; oxygen was assumed to be the major impurity due to surface oxidation. The precursor with a nominal composition of $2.1B/[(FuO)_{0.63}(BuO)_{0.37}TiO_{1.5}]$ was

(1) (a) Kim, J. J.; McMurtry, C. H. *Ceram. Eng. Sci. Proc.* **1985**, *6*, 1313. (b) Greenwood, N. N. In *Comprehensive Inorganic Chemistry*; Bailar, J. C., Jr., Emeleus, H. J., Nyholm, R., Trotman-Dickenson, A. F., Eds.; Pergamon Press: Oxford, 1973; Vol. 1, p 697. (c) Funk, R.; Lux, B.; Schachner, H.; Tannenberger, H. Fr. Patent 2,144,823, 1973; *Chem. Abstr.* **1973**, *79*, 107206. (d) Murata, Y.; Miccioli, B. R. *Am. Ceram. Soc. Bull.* **1971**, *50*, 182. (e) Janney, M. A., *Am. Ceram. Soc. Bull.* **1987**, *66*, 322. (f) McMurtry, C. H.; Boecker, W. D. G.; Seshadri, S. G.; Zanghi, J. S.; Garnier, J. E. *Am. Ceram. Soc. Bull.* **1987**, *66*, 325. (g) Watanabe, T., *J. Ceram. Soc. Jpn.* **1991**, *99*, 146.

(2) Palty, A. E.; Margolin, H.; Nielsen, J. P. *Trans. Am. Soc. Met.* **1954**, *46*, 312.

(3) Thompson, R. In *Progress in Boron Chemistry*; Pergamon: New York, 1975; p 195.

(4) Greenwood, N. N.; Parish, R. V.; Thornton, P. *Q. Rev.* **1966**, *20*, 444.

(5) Baumgartner, H. R.; Steiger, R. A. *J. Am. Ceram. Soc.* **1984**, *67*, 207.

(6) Gallagher, M. K.; Rhine, W. E.; Bowen, H. K. *Ultra-Structure Processing of Advanced Ceramics*; Mackenzie, J. D., Ulrich, D. R., Eds.; John Wiley & Sons Inc.: New York, 1988; p 901.

(7) Su, K.; Sneddon, L. G. *Chem. Mater.* **1991**, *3*, 10.

(8) Mark, H. F., Bikales, N. M., Overberger, C. G., Menges, G., Eds. *Encyclopedia of Polymer Science and Engineering*; John Wiley & Sons Inc.; New York, 1987; Vol. 7.

(9) Bradley, D. C.; Mehrotra, R. C.; Gaup, D. P. *Metal Alkoxides*: Academic Press: New York, 1978.

0897-4756/92/2804-0497$03.00/0