Optical Biosensing of Gaseous Nitric Oxide Using Spin-Coated Sol–Gel Thin Films

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Through room-temperature manufacturing conditions and their physical nature, bulk sol–gel monoliths have been shown to provide suitable host matrices for biological materials retaining their function and activity upon encapsulation. Since the sol–gel entrapment of the enzyme alkaline phosphatase[1] in 1990, there have been a number of reports, including review articles, regarding the development of protocols for the encapsulation of a variety of biological molecules in sol–gel materials for the application of optical biosensing.[2–9]

The majority of the reported sol–gel encapsulated biosensors were prepared as bulk monoliths or powders using a two-stage sol–gel formulation process.[2] The two-step process overcomes the low pH and high organic solvent content associated with the "conventional" sol–gel method,[10] which could denature the biological recognition molecule. The main disadvantage of bulk sol–gel monolith systems for biosensing applications is the diffusion-limited speed of response for an equilibrium signal, taking ca. 15 min for a 1.5 mm thick sol–gel.[7] This problem can be addressed by either increasing the pore size of the sol–gel using a higher water-to-silicate ratio (high r value) or by reducing the thickness of the sol–gel thereby reducing the time of diffusion. The former solution results in fragile sol–gels with the possibility of biomolecular leaching if the pore size becomes too large,[11] while the latter approach involves the production of sol–gel thin films for which the processing conditions are not conducive to biomolecular encapsulation. Specifically these conditions are high methanol content to reduce viscosity of the sol and improve substrate wetting, low pH to provide longer gelation times,[12] and relatively high annealing temperatures, ca. 70 °C[13] all of which are liable to cause denaturation of the biomolecule. There have, however, been three recent reports on the manufacture of thin sol–gel films for biosensing, viz. dip-coating[12] and the aerosol-generated[14] formation of sol–gel thin films for the development of optical biosensors and the use of screen printing technology for the development of an electrochemical sensor.[15]

A particular problem area for the development of optical biosensors based on sol–gel technology is that of gaseous sensing. As the gas flows over the surface of the biosensing medium, it causes drying of the sol–gel surface through evaporation. In this communication we report on our investigations into the formulation of cytochrome *c* immobilized in thin sol–gel films, through the development of the spin-coating technique, for the optical biosensing of gaseous nitric oxide.

A silica sol comprising tetramethyl orthosilicate (TMOS, 1.5 mL), deionized water (1 mL), methanol (1.5 mL), and HCl (0.05 M, 0.1 mL) was sonicated on ice for 30 min and stored in a freezer for 36–72 h to allow complete hydrolysis to occur. The sonicated sol (0.9 mL) was then added to an aqueous solution of 10 mM horse-heart cytochrome *c* (Sigma). To prevent gelation the protein doped sol–gel mixture was stored on ice prior to film manufacture and the thin films were cast within 30 min of making the protein sol–gel solution. The glass substrates (microscope slides) were wiped clean and then washed with methanol. The substrate was then placed on a spin-coating instrument (Headway Instruments Inc.) which used a vacuum to hold the substrate in position. A few drops of methanol were pipeted onto the glass surface and the substrate was then spun at 1750 rpm for 30 s to ensure a clean surface. The spin-coating instrument was stopped, and 100 μL of the protein doped sol–gel solution was pipeted onto the substrate which was then spun at 1750 rpm for 30 s. This method produced optically transparent, crack free sol–gel films, with an initial thickness of approximately 5 μm (determined using $\epsilon_{\mathrm{Soret}}$ of the cytochrome *c*), which were dried in a covered Petri dish under ambient conditions. Experimental measurements using the spin-coated protein encapsulated sol–gel films were made between 1 and 8 h after the films were prepared.

The encapsulation of the cytochrome *c* in the spin-coated sol–gel thin film did not show any deleterious effects on the structure or redox properties of the protein molecule, as established by UV–visible spectroscopy. When the protein-immobilized sol–gel thin film was placed in an aqueous solution and reduced with excess sodium dithionite, the oxidized and reduced spectra of the immobilized cytochrome *c* were identical to those obtained from the protein in solution and those previously reported for cytochrome *c* encapsulated in sol–

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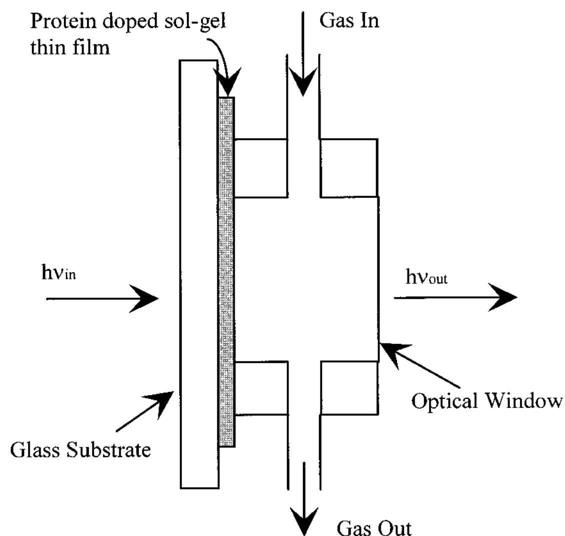

Figure 1. Schematic representation of the spin-coated sol–gel thin film gas flow-through cell.

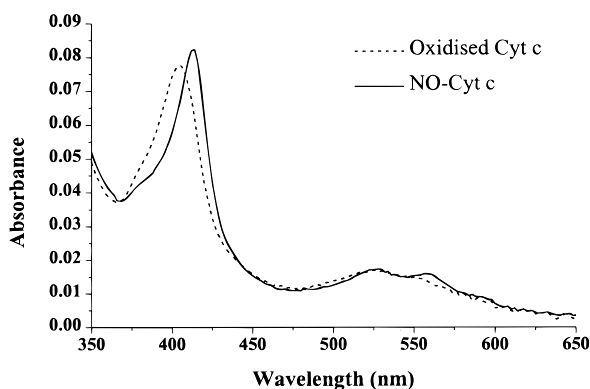

Figure 2. Absorption spectra of oxidized and NO bound cytochrome *c* encapsulated in a sol–gel thin film.

gel monoliths[2,7] and dip-coated thin films.[12] However, the speed of equilibrium response to changing redox conditions for the sol–gel thin film was much faster than that reported for the bulk monolith,[7] 6 and 15 min respectively, results similar to those obtained by Dave et al. for a dip-coated sol–gel film.[12]

In addition to maintaining redox activity, the immobilized cytochrome *c* was able to bind NO gas. NO binding was established by using a gas flow-through cell which was clamped onto the sol–gel-coated glass substrate using a rubber O-ring to ensure a gastight seal (Figure 1). By use of a gas blender (Series 850, Signal Instrument Co. Ltd.), the concentration and flow rate of the NO gas passing through the cell were controlled (within the limits of 0.1–100 ppm). Measurements were made using NO balanced with nitrogen and subsequently diluted with oxygen-free nitrogen gas (BOC Gases). Oxygen-free nitrogen was passed through the gas cell for ~15 min to remove any oxygen from the system (to prevent NO from oxidizing to NO_2) and to obtain an equilibrium response from the sol–gel thin film. After this initial period there were no changes in the absorption intensity of the cytochrome *c* due to solvent evaporation effects. The binding of gaseous NO to the Fe^{3+} center of the cytochrome *c* resulted in a shift in the Soret (γ) absorption band of the protein from 406 to 414 nm (Figure 2). This shift is in agreement with previous reports of NO binding to Fe^{3+}–cytochrome *c*

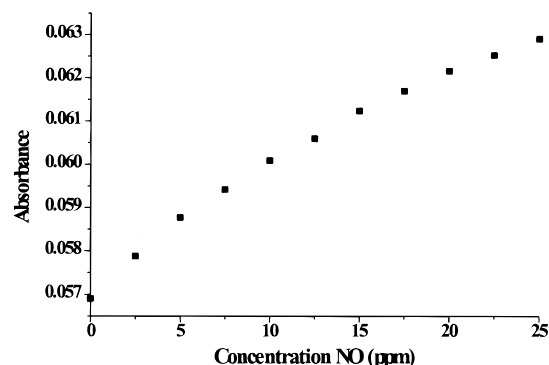

Figure 3. Calibration curve for the reaction of NO with the cytochrome *c* encapsulated in a sol–gel thin film.

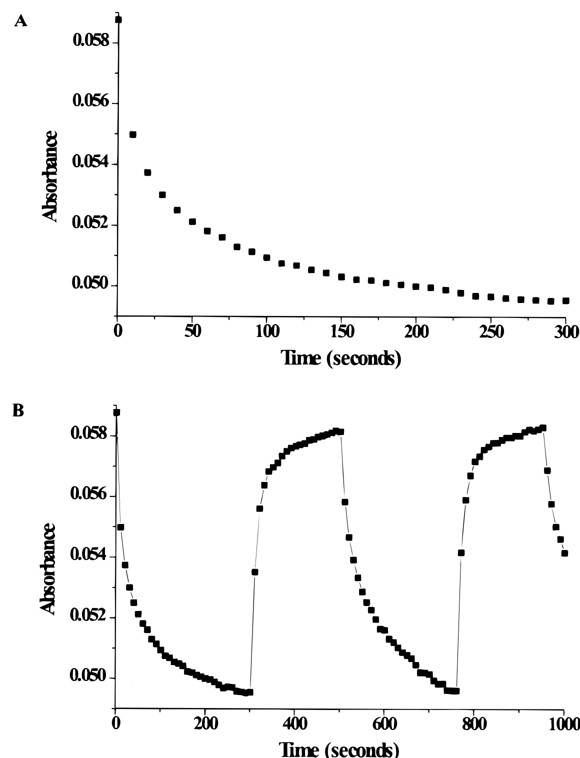

Figure 4. (a) Desorption of 20 ppm NO from the encapsulated cytochrome *c* during a 300 s nitrogen purge and (b) biosensor response to cycles of 20 ppm NO followed by nitrogen purge.

in solution[16] and in a bulk sol–gel monolith[7] and was used as the basis for the optical biosensing of the NO target species. By measurement of the intensity of the absorption signal at 414 nm as a function of changing concentration of gaseous NO passed over the protein-doped sol–gel thin film a calibration curve was constructed (Figure 3). The calibration curve suggests that the cytochrome *c* immobilized sol–gel thin films can be used to detect gaseous NO in the range 1–25 ppm, with a limit of detection of 1 ppm NO. To construct the calibration curve, absorption measurements were taken 200 s after the appropriate concentration of NO gas was passed over the protein film. This period of time enabled an equilibrium response to be measured. The desorption of the NO gas (20 ppm) from the cytochrome *c* biosensor during a 300 s purge of nitrogen gas is shown in Figure 4a. It is apparent that the cytochrome *c* reverts from the nitrosyl derivative to the nonbound

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form during this period as measured at 414 nm. Figure 4b shows the reversible sensor response of the spin-coated thin film of cytochrome *c* to cycles of 20 ppm NO and subsequent purges of nitrogen gas. A repeat cycle (×5) of 10 ppm NO followed by a flush of nitrogen gas was used to determine the reproducibility of the system. The absorption intensity at 414 nm was measured after the 200 s time period for each NO cycle. The gas cell was then flushed with the nitrogen gas for 300 s to return the biosensor response to its baseline value. This cycle established that the biosensing system had a reproducible response to 10 ppm NO with a relative standard deviation of less than 1%, for the five sequential cycles.

In developing a sensing system for NO using this spin-coating immobilization protocol, one is reliant on the specificity of the biomolecule to overcome background interferents. During the course of these investigations potential interferents were passed over the cytochrome *c* sol−gel thin film. It was observed that gaseous molecules such as oxygen, nitrogen, and carbon monoxide (all at 100% concentration) did not interfere with the binding of NO to the cytochrome *c*. However, NO_2 between 10 and 1000 ppm was found to bind to the heme center of cytochrome *c*, giving an absorption shift similar to NO. It is apparent therefore that the use of cytochrome *c* as a biological recognition molecule would be restricted to the development of an NO_x gas sensor.

With the importance of NO as a target analyte it remains our goal to develop a biosensing system which is specific for this molecule. We have extracted and purified another heme protein, cytochrome *c*′, from the bacterium *Paracoccus denitrificans*, which is known to possess high selectively for the binding of the NO molecule when the biomolecule is in the oxidized form. With the cytochrome *c*′ molecule the binding specificity toward NO over NO_2 stems from steric hindrance preventing the NO_2 molecule from gaining access to the heme binding site. We are currently characterizing this protein for the selective biosensing of NO.

The results reported in this present study show that spin-coated, protein encapsulated sol−gel thin films can be used for optical biosensing measurements of analytes in the gaseous phase. Indeed, it has been shown that quantitative data for the optical biosensing of gaseous NO in the 1−25 ppm range, with a reproducible and reversible response, can be readily obtained. While the particular biosensing system in this study was found to suffer from interference from NO_2, it is thought that the sol−gel thin film formation technique that has been developed using spin-coating methodologies should be compatible with other proteins, such as the heme protein cytochrome *c*′, which could be used to improve specificity.

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