Synthesis of Protein-Doped Sol–Gel SiO_2 Thin Films: Evidence for Rotational Mobility of Encapsulated Cytochrome *c*

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Introduction

The design of efficient optically based biosensors places a premium on synthesis of transparent thin films. Sol–gel encapsulated biomolecules are attractive candidates as biosensors,[1] but most of the research on these materials thus far has involved either bulk monoliths or powders.[2] The development of sol–gel techniques for processing optical-quality thin films of silica glass is well established for a variety of applications.[3] Thin-film-based materials inherently give a better dimensional control of final product material, require smaller amounts of dopant molecules, have faster response times with external reagents, have better compatibility with optics and electronics, show potential for miniaturization, and provide the possibility of fabricating multilayer configurations. Standard synthesis conditions required for sol–gel processing of thin films necessitate the use of substantial alcohol content and low pH, both of which cause aggregation and denaturation of fragile biomolecules.[4]

The fundamental question of how the chemical conditions and microstructural considerations imposed by thin-film synthesis may affect the stability and function of the encapsulated biomolecule remains to be resolved. In this communication, we report the first successful encapsulation of the redox-active protein cytochrome *c* in sol–gel-derived thin films of silica. Synthesis conditions for preparing homogeneously-doped, optical-quality thin films are identified and optical absorption measurements of these films indicate that the encapsulated protein retains its characteristic chemical properties in the inorganic matrix. In addition, dielectric relaxation measurements provide the first conclusive evidence that the biomolecule retains its rotational freedom upon encapsulation.

Synthesis of Protein-Doped Thin Films

The recently devised sol–gel route for encapsulation of biomolecules involves tetramethoxysilane (TMOS) precursor mixed-in with suitable buffers which raise the pH of the medium to near biological values.[1a] Using this and other procedures, a large number of biomolecules have been encapsulated in monolithic silica glasses.[5] These synthesis methods, however, are incompatible with the common sol–gel film deposition approaches where thin films are formed from a low-viscosity sol. If protein-doped thin films are to be synthesized, longer gelation times are necessary.[6] A lower water to TMOS ratio, high methanol concentration, and low pH are conducive for forming stable sols with considerably longer gelation periods.[7] Unfortunately, increased concentrations of alcohol and lower pH are detrimental to protein stability.[8] In our synthesis method, conditions of optimal pH coupled with moderate concentrations of TMOS and methanol were used to obtain longer gelation times without compromising the stability of the protein.

To identify the set of parameters necessary to obtain high-quality thin films, different preparations with varying fractions of TMOS sol, methanol, and buffer (0.1 M acetate, pH 4.25) were tested. Figure 1 displays the experimentally determined stability region for cytochrome *c* with respect to these variables. Stability of the protein was estimated from the absorption spectrum; the intensity of the Soret transition at ~400 nm is directly related to the structure of the protein. Aggregation effects lead to a decrease in intensity. Superimposed on this stability region is the film formation region for films incorporating cytochrome *c*. Outside of this region, film quality was extremely poor when using high concentrations of protein (>2 mM). One composition which combined long gelation time and protein stability (vide infra) and produced high-quality optically transparent thin films was the ratio 40:50:10 (volume percentages) of MeOH, TMOS sol, and buffer,

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(5) The proteins and enzymes stabilized so far in monolithic glasses include: horse heart cytochrome *c*, horse heart myoglobin, hemoglobin, Cu–Zn superoxide dismutase, horseradish peroxidase, beef liver catalase, glucose oxidase, oxalate oxidase, alcohol dehydrogenase, ferritin, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, trypsin, aspartase, L-amino acid oxidase, carbonic anhydrase, chitinase, cytochrome *c* peroxidase, β-glucosidase, monoamine oxidase, nitrate reductase, parathion hydrolase, alkaline phosphatase, urease, bacteriorhodopsin, and green plant photosystem II.

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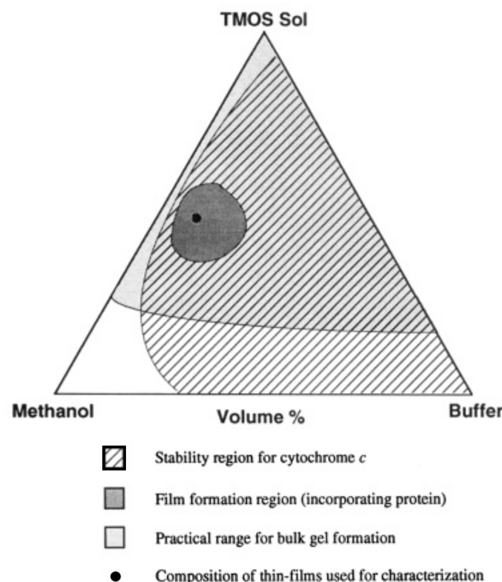

Figure 1. Ternary phase diagram illustrating the composition used for fabricating sol–gel thin films for encapsulation of cytochrome *c*. The bulk gel formation region is more extensive than indicated, but experiments are limited due to excess solvent concentrations which dramatically reduce gelation kinetics.

respectively, as indicated in Figure 1. No aggregation or denaturation of the protein was observed for this sol composition. This composition was used for all the thin films reported in this study. Dip coating techniques were used exclusively to produce films.[3a]

In addition to establishing the concentrations of methanol and pH required for thin-film processing, it was also necessary to identify the self-aggregation thresholds for the dopant protein at different buffered conditions. The limits of self-aggregation for equine ferricytochrome *c* were studied in different buffers. The protein showed a tendency to self-aggregate but remained stable to at least 60% vol/vol ratios of methanol–buffer mixtures.[4] Also, the stability of the protein was found to be a function of the pH.

Thin films containing cytochrome *c* were fabricated by substituting a solution of dissolved protein in place of the buffer. Typically a homogeneous sol was prepared by sonicating 15.27 g of TMOS, 3.38 g of water, and 0.22 mL of 0.04 M HCl for 10 min in a sonication bath containing a mixture of ice and water. The freshly prepared sol, methanol, and a buffered solution of protein were kept in an ice bath before mixing. The mixture from which the thin films were deposited was then obtained by combining 2.0 mL of the sol with 1.6 mL of MeOH, and 0.4 mL of cytochrome *c* solution containing 0.25 mg/mL of the protein dissolved in 0.3 mL of 0.1 M acetate buffer (pH 4.25) and 0.1 mL of 1 M HCl. Prior to film deposition, glass microscope slides (3 cm × 1 cm × 1 mm) were cleaned first with No-Chromix (Aldrich, Milwaukee) and then with deionized water. These substrates were connected to a weighted float in a water reservoir whose drainage rate was controlled by a flow valve. The films were prepared by a dip coating technique where glass substrates were withdrawn from a low-viscosity sol containing the protein dopant. Excellent quality protein-doped thin films that were homogeneous, optically transparent, crack-free, and adherent to the substrate were produced

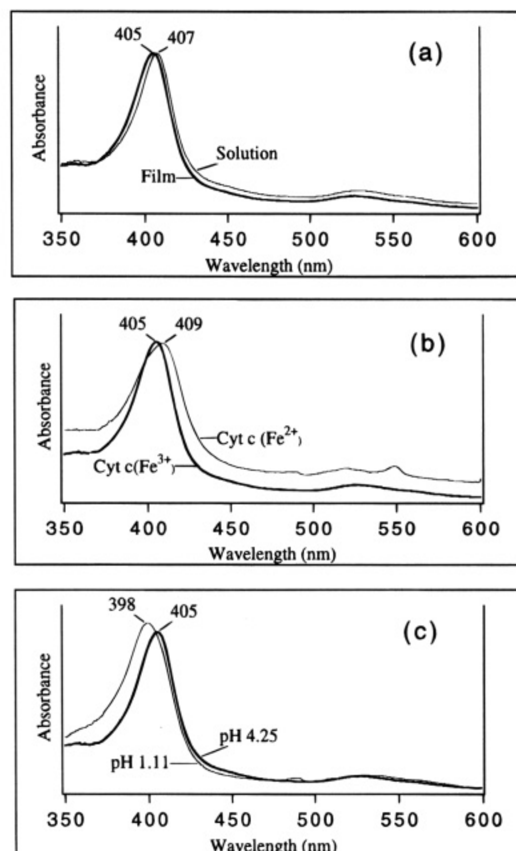

Figure 2. Optical absorption spectra of cytochrome *c*. (a) Comparison of solution (0.1 M acetate buffer, pH 4.25) and as-prepared thin films impregnated with protein. (b) Comparison of oxidized and reduced forms of protein in thin films. (c) Spectra of thin films exposed to pH 4.25 and 1.11 external buffers.

by this method. Thickness of these films varied between 2000 and 2500 Å, as determined by surface profilometry (Alpha-Step 200; Tencor Instruments, Mountain View, CA). The films were dried in ambient for approximately 30 min before the experimental measurements were initiated.

Optical Properties

Optical absorption characteristics of the heme Soret band of the ferricytochrome *c* are able to establish the structural integrity of the trapped protein.[9] The absorption spectrum for a reference solution of the protein dissolved in pH 4.25 acetate buffer (0.1 M) has its Soret maximum at 407 nm, whereas the absorption maximum of the as-prepared film is centered at 405 nm (Figure 2a). Immersing the thin-films in either a 0.1 M acetate buffer (pH 4.25) or 0.1 M phosphate buffer (pH 7.0) does not alter the optical absorption characteristics of the films. Immersing the films in a lower pH (3.0) phosphate buffer (0.1 M) does not shift the absorption maximum either, but at pH 2.02 a small blue shift (~2 nm) is observed, which becomes very pronounced at pH 1.11. As can be seen in Figure 2c, a blue shift of 7 nm occurs in the films exposed to pH 1.11 external solution. A further lowering of the pH results in a decreased intensity of the Soret band, indicating partial damage to the trapped protein. The solution chemistry of

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ferricytochrome c is characterized by proton-induced conformational changes.[10] Different conformational isomers of the protein exist at pH ~4 and at pH ~2 with a transition point centered around pH ~3. Our results show that similar behavior is retained in sol–gels, although the midpoint pH of the transition is lowered. The low and high pH forms of the trapped protein can be obtained reversibly as long as the films are not exposed to pH lower than 1.

The protein-doped thin films exhibited the characteristic redox properties of ferricytochrome c. Immersing the as-prepared films in a solution of sodium dithionite resulted in an increased intensity peak in the ~550 nm region, which is indicative of the reduced form of the protein. The reduction of the encapsulated protein was also accompanied by a 4 nm red shift of the Soret transition to 409 nm and a concomitant change in intensity (Figure 2b). Air oxidation of the reduced form produces the oxidized species and the original spectrum could be reobtained. It is important to emphasize that the redox and pH-induced structural changes and accompanying optical absorption changes occurring in these films were totally reversible. We carried out 10 cycling experiments and found that the redox (or the pH dependent) forms of the protein could be successively interconverted without any significant deterioration in the optical properties of the films. These results are in general agreement with previously reported redox and pH dependent spectral changes observed in aqueous media and in monolithic sol–gels.[2a] It is important to mention that the protein-doped thin films responded much more rapidly to the external reagents. Remarkably, total reduction of the protein in the thin films was observed after only a few minutes of immersion with sodium dithionite as compared to monolithic glass samples (1 cm × 1 cm × 2 cm) where about an hour is needed for a similar change to take place.[2a]

Dielectric Relaxation Measurements

The dipolar relaxation measurements provide insight concerning the nature of the interaction between molecules and their environment.[11] The rotational activation energy associated with an orienting dipolar molecule when placed in an ac field is directly related to the characteristics of the immediate microenvironment. The cytochrome c molecule has an estimated dipole moment of ~300 D.[12] The differences in dipolar relaxation energies in the sol–gel matrix as compared to aqueous media should provide useful information about the extent to which the molecule experiences an altered environment upon encapsulation in silica gel. A plot of the imaginary component of the impedance versus log frequency exhibits a maximum corresponding to the dipolar transition. In general, proteins show a dipolar transition centered at ~10^6 Hz.[11] Using a set of interdigitated gold electrodes on a silicon substrate, complex impedance measurements were performed on the films

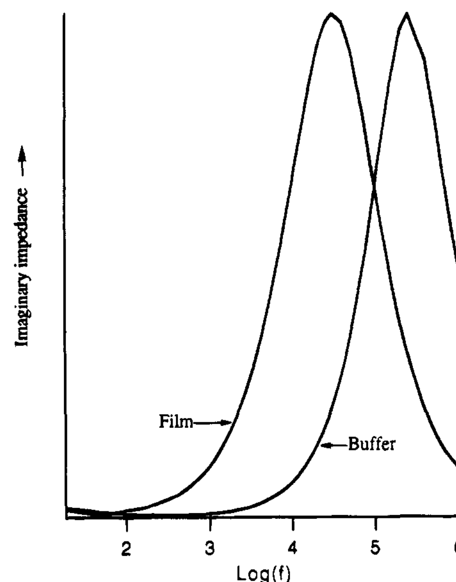

Figure 3. Plot of imaginary component of impedance vs log of frequency for ferricytochrome c in 0.1 M acetate buffer (pH 4.25) and for ferricytochrome c encapsulated in thin films that were immersed in 0.1 M acetate buffer (pH 4.25). For both the measurements the temperature was kept constant at 20 °C.

that were immersed in buffer. The buffer solution itself was placed in a thermostat, the temperature of which was set at 20 °C (±0.5 °C). Measurements were performed with a precision LCR meter (Hewlett-Packard Model HP4284A) over the frequency range 20 Hz to 1 MHz using a two- probe method. Undoped sol–gel films did not show any well-defined transition in this frequency range. As shown in Figure 3, ferricytochrome c in 0.01 M acetate buffer (pH 4.25) undergoes relaxation at ~$10^{5.5}$ Hz whereas sol–gel thin film encapsulated cytochrome c showed a similar transition centered at ~$10^{4.5}$ Hz. The activation energy (E) for dipolar relaxation process is $E = RT \ln(RT/2\pi hf)$ where R is the gas constant, h is the Planck's constant, f is the frequency, and T is the absolute temperature.[11] An additional activation barrier of ~1.1 kcal/mol in thin films can therefore be associated with matrix encapsulation. This increase suggests that interaction between the biomolecule and the matrix restricts the rotational movement of the protein to a slightly greater extent inside the sol–gel environment. The small 1.1 kcal/mol difference indicates minimal change in protein noncovalent interaction with the medium and that only a slightly perturbed microenvironment is experienced by the protein upon entrapment in the sol–gel glass film.

Conclusions

Modification of the buffered sol–gel synthesis method enables one to prepare protein-doped silica thin films by the dip coating method. Optical-quality thin films containing the redox protein cytochrome c show the characteristic optical properties of both the oxidized and the reduced forms of the protein. The redox behavior of the protein is totally reversible in the silica films, and successive air-oxidation and dithionite reductions steps could be carried out without a deterioration of the spectral properties. In addition, the structure of ferricytochrome c undergoes proton induced reversible structural changes that can be monitored by optical absorp-

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tion spectroscopy. Dipolar relaxation of the trapped proteins shows that the biomolecule is free to rotate in order to align its dipole with the applied electric field. The very small increase in dipole orientation barrier suggests an essentially unchanged microenvironment for the protein in sol–gel films.

A particularly noteworthy aspect of the chemistry of cytochrome *c* is the reversible structural changes and the changes in optical properties induced by external reagents such as protons or dithionite. Such a coupling of chemical, structural, and conformational changes with electronic and optical characteristics provides a facile pathway to control externally the physical properties of a material. The reversible changes observed in the optical properties of cytochrome *c* encapsulated thin films along a proton-dependent reaction coordinate furnish a simple molecular switching mechanism based on conformational isomers of the protein that may be of potential relevance to the development of sol–gel-based biomolecular optoelectronic materials. The faster optical response of the thin films to external reagents should be beneficial to fabricate efficient optically based biosensor devices, and prospects in this direction are currently being pursued.

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