

## Encapsulation of Proteins in Bulk and Thin Film Sol-Gel Matrices

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**Abstract.** This paper considers the nature of the interactions between the sol-gel derived inorganic matrix and a specific biomolecule, cytochrome c. Optical absorption and impedance spectroscopies are used to characterize the influence of synthesis conditions on the protein's stability and conformation within the silica matrix. In some instances, encapsulation within the sol-gel matrix provides stabilization. For example, protein denaturation is reversible and aggregation is prevented. Moreover, the drying process does not negatively affect the protein; it is possible to regenerate the aged gel state by rehydration. The flexibility of the sol-gel process enables high quality cytochrome c-doped SiO<sub>2</sub> thin films to be prepared. These films possess the characteristic reactivity and chemical function of cytochrome c in solution.

**Keywords:** protein encapsulation, absorption spectroscopy, thin films, cytochrome c

### 1. Introduction

The encapsulation of enzymes and other proteins into inorganic host materials using sol-gel processing has attracted considerable attention over the past few years [1, 2]. This research has demonstrated that the biomolecules immobilized in the sol-gel derived matrix retain their functional characteristics to a large extent. These new composite materials are of interest for their applications as optically based biosensors. The porosity of sol-gel glasses allows small analyte molecules to diffuse into the matrix while the large protein macromolecules remain physically trapped in the pores. The transparency of the matrix enables one to use optical spectroscopy methods to characterize the reactions that occur in the pores of the glass.

Sol-gel materials are ideal candidates as hosts for biomolecular dopants because they are synthesized at low temperatures under fairly mild reaction conditions [3]. The variation of synthesis conditions has enabled

researchers to tailor the sol-gel chemistry so that the encapsulation of a variety of proteins, enzymes and other biological molecules has been reported. Among the various biomolecular dopants studied to date are alkaline phosphatase, glucose oxidase, cytochrome c, trypsin, urease, Cu-Zn superoxide dismutase as well as yeast cells [1, 2]. These and other studies have clearly established that biological molecules encapsulated in inorganic matrices retain their characteristic chemical and biochemical functionality as, for example, ligand binding, oxidation/reduction, fermentation and enzymatic activity.

The present paper considers the nature of the interactions between the inorganic matrix and the dopant biomolecule, an area of study which has received very little attention. In this work we use cytochrome c (cyt c), an electron transfer protein as a model protein in which to investigate the structural and conformational effects which result from its encapsulation in the silica gel matrix. One topic addressed in this paper is

the influence of the gel network on the structure and microenvironment of encapsulated biomolecules. A related area we describe is the influence of sol-gel synthesis conditions on the stability and chemical function of cyt c. These results are then used to develop synthesis conditions appropriate for sol-gel thin films with encapsulated biomolecules.

## 2. Experimental

Sol-gel derived silica gels using tetramethoxysilane (or TMOS) were used exclusively for the experiments reported in this paper. The range of compositions investigated went well beyond the buffered TMOS sol used in prior work [4]. Instead, a wide range of ternary compositions comprised of TMOS sol, methanol and buffer were prepared. In this approach a sonicated TMOS sol (acid catalyzed with 1 : 2 molar ratio TMOS: water) was the silica precursor. Methanol was then added to the sol, followed by buffer to adjust sol pH. Cytochrome c (dissolved in buffer) was added to the sol at a concentration of  $2.74 \times 10^{-5} M$ . The samples contained at least 40 vol% TMOS sol to ensure fairly rapid gelation. The MeOH and buffer concentrations both ranged from 0 to 60 vol%. The final sols were cast into polystyrene cuvettes. Gelation times varied from 30 seconds to 45 minutes depending upon composition. The samples were then aged in sealed cuvettes for at least two weeks prior to characterization. In addition, a series of methanol/buffer solutions were prepared so that the properties of cytochrome c in reference solutions could be compared with the properties of the protein in the sol-gel matrix.

The preparation of thin films followed a similar procedure. The composition used for the thin films was the ratio 40 : 50 : 10 (vol%) of MeOH, TMOS sol and buffer. The latter was 0.1M acetate buffer (pH 4.25). A higher concentration ( $2 \times 10^{-3} M$ ) of cyt c was utilized in the thin film studies in order to compensate for the shorter path length and enable optical absorption measurements to be used. The films were prepared by a dip coating technique [3] where glass substrates or silicon wafers were withdrawn from a low viscosity sol containing the protein dopant.

Absorption spectroscopy was the principal means of characterizing the interactions between cyt c and the silica matrix. Cyt c is an electron transfer protein which functions by oxidation and reduction of iron ( $Fe^{3+} \rightleftharpoons Fe^{2+}$ ) present in the central heme group. The protein molecule has a diameter of approximately 32.4 Å and a molecular weight of  $\approx 12,000$  g/mole with

a net charge of +7.5 in its oxidized state. Cyt c has well defined optical characteristics due to the presence of the ferriheme active site. There are two absorption bands in the visible;  $\alpha$  at 521 nm and  $\beta$  at 550 nm and an intense UV absorption band, the Soret at 415 nm [5]. These absorption bands arise because of  $\pi \rightarrow \pi^*$  electronic transitions within the highly conjugated heme group. The absorption characteristics have been studied carefully and established in solution media. Denaturation, or unfolding of the protein, is evident by wavelength shifts in the peak absorption of the Soret band as well as by intensity changes and peak broadening [6]. Protein aggregation, an effect which can occur when some proteins are placed in a solution with a dielectric constant lower than water [7], exhibits little if any absorption because the protein settles to the bottom of the cuvette, out of the light path. The extensive literature available on the optical properties of cyt c are extremely useful for interpreting the effects observed upon encapsulation.

## 3. Results and Discussion

### 3.A. Effects of Drying

In the drying of gels, pore collapse is associated with the evaporation of the solvent phase. Thus, the question arises as to whether proteins are affected by solvent loss and/or physical confinement as the gel-xerogel transformation occurs. The absorption spectra of cyt c in the solution phase and in aged silica gel sample are quite similar (Fig. 1). The heme signature absorptions due to the Soret and the Q bands are preserved upon encapsulation of the protein in the gel, although slight changes in frequencies are evident. The solution spectrum of the protein shows the Soret band centered at 406 nm, while in the aged gel sample there is a slight blue shift from 406 nm to 404 nm. Upon ambient drying, the blue shift in the Soret band continues as the gel-xerogel transformation occurs. The Soret band maximum for cyt c doped in dried xerogel samples is blue-shifted to 395 nm. This decrease of 9 nm can be attributed to drying effects and must arise from either pore collapse and/or from loss of the solvent phase. In order to determine the cause of the blue shift, absorption spectra were obtained on rehydrated xerogel samples. Absorption spectra of xerogels immersed in 0.1M acetate buffer (pH 4.5) exhibit a distinct red-shift in the Soret band maximum to 404 nm (Fig. 1). This value is exactly the same as that observed for aged gel samples. It can, therefore, be concluded that the changes in absorption spectra accompanying the drying process are not

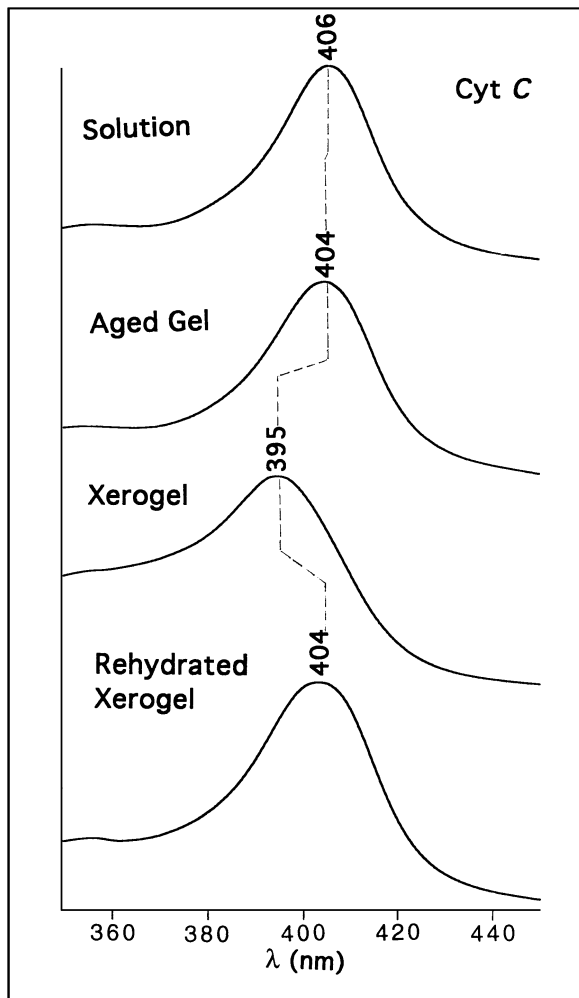


Figure 1. Optical absorption spectra for cytochrome c in solution, aged gel, xerogel and a rehydrated xerogel.

associated with dimensional changes occurring from pore shrinkage. Pore shrinkage of the silica gel upon drying is an irreversible process. In contrast, the present results indicate that shifts in the absorption spectra are reversible. If the blue-shift observed from dehydration was caused by pore collapse due to drying, the original spectrum of the aged gel would not be recovered upon rehydration. Therefore, we conclude that the absorption spectra changes are due to changes in the microenvironment of the trapped protein caused by loss of the solvent phase upon drying and not by changes in pore size.

The spectroscopic reason for the blue shift in the absorption spectrum can be interpreted in terms of increased overlap of the heme  $\pi$  orbitals with  $d_{\pi}$  orbitals of the central Fe(III) ion. The process of encapsulation

within the electrostatically charged pores leads to increased electronic conjugation and a relatively more planar conformation of the heme group. The physical cause of this effect is not clear. Pressure-induced effects causing nonplanar heme conformations can be ruled out [8].

### 3.B. Methanol and pH Effects

The prospects of using sol-gel encapsulated biomolecules for a new generation of biosensors will be greatly advanced if thin films of these materials can be prepared. Thin films are desirable for optical or electrochemical based sensors because of faster response times, lower dopant levels and compatibility with microelectronics and signal processing methods. The potential problem is the effect of the chemical environment on the protein. Films are typically prepared using a sol diluted with alcohol in order to decrease viscosity, enhance sol stability and improve substrate wetting [3]. The excess alcohol, however, can cause protein aggregation in the sol which inhibits protein function. Another important consideration is the desirability of low pH to stabilize the sol and provide long gelation times. Low pH, however, is also detrimental to protein stability.

To identify the set of processing parameters appropriate for producing films with encapsulated proteins, the influence of sol-gel synthesis conditions on the stability and chemical function of cyt c was investigated. In general, the stability of cyt c in the sol-gel environment is largely determined by the stability of the protein in MeOH/buffer solutions. Cyt c was found to be stable against aggregation to at least 60 vol% methanol, regardless of buffer type or pH over the range 2.9–7.0 [9]. Some buffers, such as acetate and monobasic phosphate, stabilized the protein against aggregation to greater than 90 vol% methanol. With regard to denaturation, cyt c partially denatured in solutions with less than 60 vol% MeOH, with the degree of denaturation increasing as the amount of methanol increased. The spectroscopic behavior for the denaturation of cyt c is well established and is characterized by changes in the absorption intensity, wavelength shifts and broadening of the Soret band [6]. Figure 2 summarizes the stability of cyt c in the MeOH/acetate buffer/TMOS sol ternary system. There is a substantial stability region for cyt c from which it is possible to identify compositions which are chemically compatible with the protein and also feasible for the preparation of thin films.

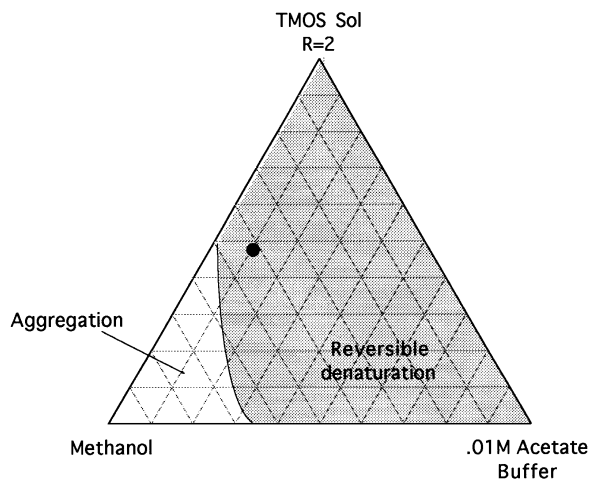


Figure 2. Ternary diagram indicating the stability region for cytochrome c in sol-gel materials prepared from TMOS sol, methanol and 0.1M acetate buffer. The composition used for thin films is shown by the filled circle.

There are two additional interesting results from the stability experiments which show the effect of protein encapsulation. One is the observation that denaturation is reversible when cyt c is encapsulated in the sol-gel matrix. When samples containing partially denatured cyt c were soaked in pure buffer, the absorption spectra indicated that the cyt c reverted to its native form (Fig. 3). The Soret band maximum was at 405 nm and the FWHM of the peak was 22.8 nm. Upon soaking these same gels in MeOH, however, the protein

partially denatured once again; the Soret band shifted to 402 nm and the peak broadened to 23.1 nm. The reversibility of the denaturation is shown in Fig. 3. The second significant feature indicated in these experiments is that protein aggregation did not occur, even if the gels were soaked in pure MeOH, a condition which produces protein aggregation in solution. Thus, these stability experiments indicate that encapsulation in the sol-gel matrix partially constrains the mobility of the protein so that aggregation is prevented but the restricted mobility does not interfere with its chemical function.

### 3.C. Preparation and Properties of Thin Films

Compositions corresponding to the ratio 40:50:10 (MeOH:TMOS sol:buffer) were found to produce optical quality thin films (Fig. 2). The protein was incorporated by substituting a solution of the dissolved protein in place of the buffer. The dip-coated, protein-doped thin films, 200 to 250 nm thick, were homogeneous, optically transparent, crack-free and adherent to the substrate.

The cyt c encapsulated in the sol-gel SiO<sub>2</sub> thin films retained its characteristic reactivity and chemical function [10]. Its redox behavior is completely reversible as air-oxidation and dithionite reduction steps could be carried out successively without deterioration of its spectral properties. Another characteristic of cytochrome c, reversible proton induced conformational

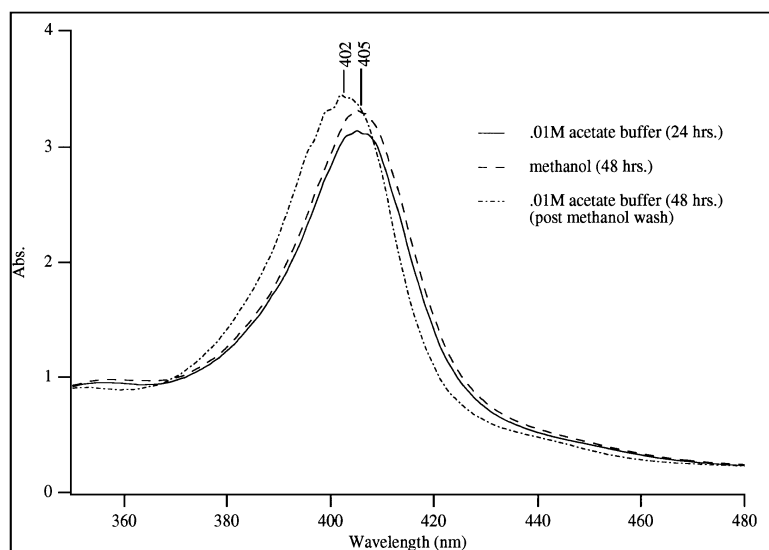


Figure 3. Absorption spectra for cytochrome c encapsulated in SiO<sub>2</sub> gels demonstrating the reversibility of denaturation upon immersion in pure methanol and acetate buffer solutions.

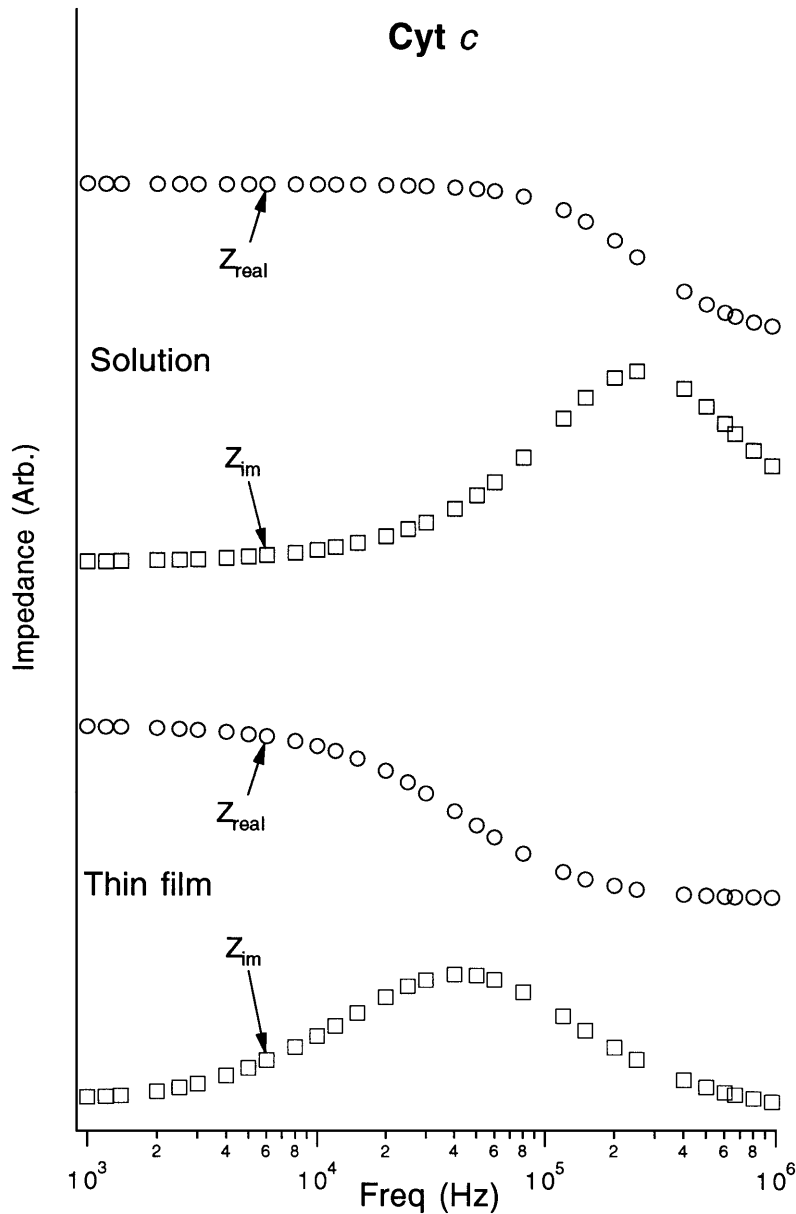


Figure 4. Frequency dependence of the impedance (real and imaginary) for cyt *c* in solution and in SiO<sub>2</sub> thin films.

changes [11], was also observed in the sol-gel films. A third property, dielectric relaxation, provides insight concerning the nature of the interaction between the biomolecule and its environment [12]. These measurements are based on having the biomolecule rotate so that its dipole aligns in the applied ac field. In the case of cyt *c*, the slight shift in the relaxation peak, from 10<sup>5.5</sup> Hz (solution) to 10<sup>4.5</sup> Hz (in the film), is an indication that interaction between the cyt *c* and the matrix

slightly restricts the rotational movement of the protein within the matrix (Fig. 4). The magnitude of the difference, however, is quite small (1.1 kcal/mol) suggesting that the protein experiences only a slightly perturbed microenvironment upon encapsulation in the sol-gel glass film [10]. An experimentally interesting consideration in these measurements is that the protein-doped sol-gel films are coated onto a set of interdigitated gold electrodes previously deposited on an oxidized

silicon substrate. The nature of this electrode pattern represents the type of configuration in which protein-doped films would be utilized in biosensor devices involving electrochemical detection. The ability to fabricate protein-doped thin films and obtain sufficient signal is a very positive development for this emerging field.

#### 4. Conclusions

The present paper has reported on the nature of the interactions between the inorganic matrix and a specific biomolecule, cytochrome c. A number of important results have been presented. It is possible to prepare cyt c doped sol-gel materials over a wide range of synthesis conditions, especially with regard to methanol content and pH. Encapsulation within the sol-gel matrix provides stabilization towards external denaturing agents. Once trapped within the network, denaturation of the biomolecule is reversible and the native form can be regenerated by soaking in buffer. In addition, protein aggregation is avoided because of the restriction in protein mobility. The flexible synthesis conditions have enabled excellent quality cyt c-doped SiO<sub>2</sub> thin films to be prepared. The resulting films exhibit the characteristic reactivity and chemical function of cyt c in solution, including reversible structural changes induced by external reagents such as protons or dithionite. An unexpected result in this study was the observation that the drying process does not affect the protein, that the aged gel state of the protein can be regenerated by rehydration. This suggests that pores containing the

protein behave differently than free pores which apparently collapse upon solvent removal.

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