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STRATEGIES FOR ENCAPSULATING BIOMOLECULES IN SOL–GEL MATRICES†

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Abstract—The encapsulation of biological molecules in sol–gel matrices has received considerable attention because of the prospect of creating novel materials which exhibit the characteristic chemical and biochemical functionalities of enzymes and other proteins. This paper explores the nature of the interactions between a sol–gel derived silica matrix and the electron transfer protein, cytochrome *c*. These interactions were found to be of central importance in determining the synthesis conditions for these materials, in creating the pores which confine the protein and in stabilizing the protein so that it retains its structure and chemical function. © *1998 Acta Metallurgica Inc.*

1. INTRODUCTION

The sol–gel process is a chemical synthesis technique for preparing gels, glasses and ceramic powders [1–3]. The method has received considerable attention because it leads to a number of unique features for oxide materials including the ability to form inorganic glasses at far lower temperatures than is possible by using conventional melting, to produce highly reactive, fine powders with molecular-level mixing of constituents and to synthesize compositions which are difficult to obtain by conventional means. In addition, the sol–gel method is a solution-based process, which is adaptable to producing materials as films and fibers as well as in bulk geometries. The growth and significance of the sol–gel field are such that there are international conferences, symposia and a journal devoted to sol–gel related topics [4].

The synthesis of materials by the sol–gel process generally involves the use of metal alkoxides. $M(OR)_n$ (where M is Al, Si, Ti, ... and R is an organic group), which undergo hydrolysis and condensation polymerization reactions to give gels. The formation of silica glass, for example, is achieved by hydrolysis of $Si(OCH_3)_4$ (tetramethyl orthosilicate or TMOS) followed by condensation to yield a polymeric oxo-bridged SiO_2 network. Hydrolysis converts the Si—OCH_3 bonds to Si—OH bonds which condense together to form the oxo-bridged Si—O—Si structure. These reactions occur in a localized region leading to the formation of sol particles. As polycondensation continues, the degree of cross-linking between particles increases and sol viscosity increases. This viscous material solidifies and leads to the formation of a porous gel.

The gel formed in this fashion is a two-phase system comprised of an inorganic porous solid and the trapped solvent phase. Even after gelation, the structure and properties of the gel continue to change as polycondensation reactions occur with Si—OH fragments. During this aging process, the formation of new Si—O—Si bonds result in contraction of the gel network. There is some shrinkage of the gel, the pore size decreases and some of the solvent phase is expelled from the pores. When the gel is dried, evaporation of the pore liquid takes place and substantial shrinkage occurs, leading to pore collapse. The fully dried gels, termed xerogels, are approximately 1/8 the volume of the initial gel and show no further loss of solvent phase. These materials are optically transparent solids which are chemically, thermally and dimensionally stable [1–3].

One of the novel features of the sol–gel method is that it enables one to synthesize inorganic glasses at room temperature without melting. A number of groups have used this characteristic to synthesize new nanocomposite materials by encapsulating different organic, organometallic and even biological molecules in the sol–gel derived inorganic matrix [5–7]. The key consideration here is that the low temperature sol–gel approach circumvents the inability of these molecules to withstand the high temperatures required in the processing of oxides. The use of the sol–gel process to incorporate a variety of organic and organometallic guest molecules in an inorganic sol–gel host is well recognized as being applicable to a wide range of organic mol-

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ecules. The work in this area has generally proceeded along two directions; (1) to use the guest molecules as luminescence probes of sol–gel chemistry and structure; and (2) to develop new optical materials by taking advantage of the spectroscopic or photochemical properties of the deliberately chosen guest molecule. The latter has led to new materials which exhibit specific optical properties such as laser action, nonlinear optical effects, photochromism, chemiluminescence and detection of chemical reagents.

The present paper concerns a more recent development in this field, that of encapsulating biological molecules into inorganic host materials using sol–gel processing [8, 9]. It is now well established that a wide variety of enzymes and other proteins retain their characteristic reactivities and chemical function when they are confined within the pores of the sol–gel derived matrix. The porosity of sol–gel glasses allows small molecules and ions to diffuse into the matrix while the large protein macromolecules remain physically trapped in the pores. This characteristic in combination with the transparency of the matrix has led to the prospect of developing an entirely new class of optically-based chemical and biomedical sensors which possess the specificity and sensitivity of enzymes and proteins. Among the various biomolecular dopants studied to date are alkaline phosphatase, glucose oxidase, urease, Cu–Zn superoxide dismutase, horseradish peroxidase, lactate dehydrogenase, alcohol dehydrogenase, myoglobin, hemoglobin, cytochrome *c*, trypsin, as well as yeast cells and photo-reactive biosystems like bacteriorhodopsin (bR) [8, 9]. 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.

This paper reviews two areas which are of central importance to these novel, biochemistry based solid-state materials. One topic concerns the synthesis strategies used in preparing these biomolecule-doped sol–gel materials. We have now established a protocol for obtaining sol–gel synthesis conditions which are biocompatible with the dopant. The second topic concerns the nature of the interactions between proteins and the sol–gel derived inorganic matrix. In this paper we describe the enhanced stability achieved when a biomolecule is encapsulated in the inorganic silica matrix.

2. STRATEGIES FOR SYNTHESIZING PROTEIN-DOPED SOL–GEL MATERIALS

The conventional sol–gel procedures are not generally suitable for encapsulation of proteins because high acidity and high concentrations of alcohol lead to denaturation of most proteins. We therefore modified the methods of Esquivias and Zarzycki [10]. The first and most important modification was the addition of buffer after HCl-catalyzed hydrolysis of the TMOS and before the protein to be encapsulated was added. We also found that there was no need to add alcohol to the silica sol provided sonication was used. By using this general approach, the sol–gel method has been found to be generally applicable to a wide range of biomolecules [11].

While the above approach is effective, it neither takes advantage of the flexible solution chemistry of the sol–gel process nor improves our understanding of the behavior of proteins in the sol–gel environment. For these reasons we recently carried out a more thorough investigation which considered the influence of sol–gel synthesis conditions on the stability, chemical function and reactivity of different proteins [12]. This work used the electron transfer protein cytochrome *c* (cyt-*c*) as a model protein in which to investigate the structural and conformational effects which result from its encapsulation in the silica gel matrix. The approach taken in this study was to compare the response of cyt-*c* dissolved in reference solutions with those responses observed for the protein when it was encapsulated in silica sol–gel matrices. This enabled us to use the extensive literature on this biomolecule to identify stable synthesis conditions and to characterize changes in protein behavior as synthesis conditions were extended in terms of pH, alcohol and silica sol content.

Cyt-*c* functions by oxidation and reduction of iron ($Fe^{3+} \Leftrightarrow Fe^{2+}$) present in the central heme group and has well defined optical characteristics. Therefore, absorption spectroscopy was the principal means of characterizing the interactions between cyt-*c* and the silica matrix. There are two absorption bands in the visible; α at 521 nm and β at 550 nm and an intense u.v. absorption band, the Soret at 415 nm [13]. These absorption bands arise because of $\pi \rightarrow \pi^*$ electronic transitions within the highly conjugated heme group. The absorption characteristics for both denaturation, or unfolding of the protein, and protein aggregation effects have been well studied for solution media [14, 15].

Reference solutions for cyt-*c* consisted of several different buffers (phosphate, succinate, acetate and oxalate) spanning the pH range 2.9–7.0 and a range of methanol (MeOH) concentrations. The addition of methanol to the aqueous buffer causes some denaturation of cyt-*c* to occur as evidenced by a shift in the Soret band maximum to shorter wavelengths and an increase in peak intensity. The protein, however, retains its chemical functionality. It is only when the methanol content reaches at least 60 vol% that protein aggregation occurs as the solution dielectric constant becomes too low to sustain uniform dissolution. These results are very significant for sol–gel synthesis because they indicate that cyt-*c* retains its stability and chemical function over

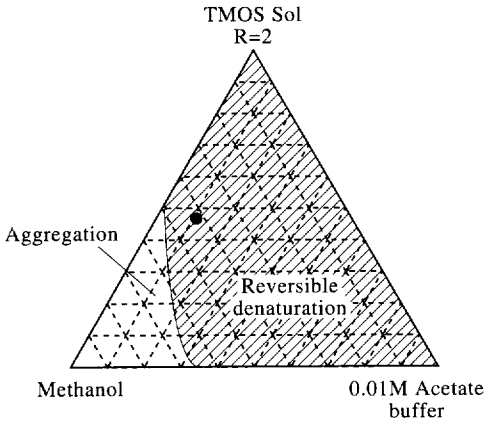

Fig. 1. Ternary diagram indicating the stability region for cytochrome *c* in sol-gel materials prepared from TMOS sol, methanol and 0.1 M acetate buffer. The composition used for thin films is shown by the filled circle [12].

pH values and methanol concentrations which are compatible with a wide range sol–gel synthesis conditions. Moreover, there are some buffer systems, such as acetate and monobasic phosphate, which stabilize the protein against aggregation to greater than 90 vol% methanol.

The chemical stability conditions for cyt-*c* in sol–gel matrices closely follow those of the MeOH/buffer reference solutions. Figure 1 summarizes the stability of cyt-*c* encapsulated in sol–gel derived silica glasses. The silica precursor is a sol composed of TMOS:acidified water (pH 2.4) in a 1:2 molar ratio. The ternary diagram of MeOH/acetate buffer (pH 4.25; 0.01 M)/TMOS sol indicates the existence of two regions. As observed with the reference solutions, the protein tends to aggregate at high methanol concentrations and to partially unfold at low methanol concentrations. The stability range for synthesizing cyt-*c* encapsulated in sol–gel glass is quite extensive and is largely determined by the stability of the protein in MeOH/buffer solutions. Sodium acetate buffer stabilizes the protein against aggregation for MeOH:buffer ratios of 9:1. Although TMOS sols of this ratio were not prepared, gels with MeOH:buffer ratios of 6:1 were readily synthesized. Finally, it is important to note that while Fig. 1 presents data from one system, it is representative of the behavior observed for all the buffers studied.

An important advantage of having a wide stability region is that it is possible to select sol–gel compositions without compromising protein stability. This is especially important for preparing sol–gel materials as films where long gelation times are necessary for film processing purposes. In systems where protein stability is not a consideration, the general approach is to use a lower water:TMOS ratio, low pH and high methanol content [3]. The wide stability region for cyt-*c* in sol–gel derived silica enables one to obtain synthesis conditions which are feasible for film formation. Figure 1 indicates a composition region which provides optimum conditions for preparing optical quality films of cyt-*c* encapsulated in silica glass [16].

3. PROTEIN–MATRIX INTERACTIONS

One of the effects of the interactions between the biomolecule and the sol–gel silica matrix concerns the formation of pores which contain the protein. Our results indicate that as the gel is dried and pore collapse occurs, the pores containing the protein shrink differently from the empty pores. Optical absorption spectroscopy has been used to detail this effect (Fig. 2). The absorption spectra of cyt-*c* in the solution phase and in the aged silica gel sample are quite similar. The heme signature absorptions due to the Soret, α and β 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 to 404 nm. Upon ambient drying to 20% of their original weight, xerogel materials are obtained. The blue shift in the Soret band continues as the gel–xerogel transformation occurs. The Soret band maximum for cyt-*c* doped in the dried silica xerogel samples is

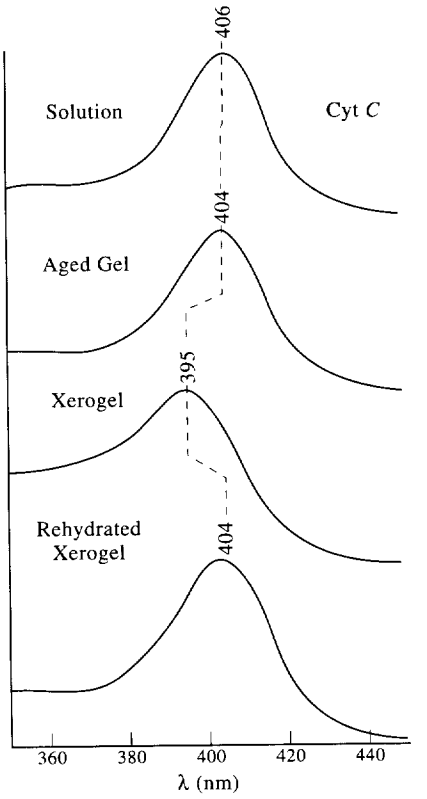

Fig. 2. Optical absorption spectra for cytochrome *c* in solution, aged gel, xerogel and a rehydrated xerogel.

blue-shifted to 395 nm. This decrease of 9 nm can be attributed to drying effects. These effects must be due to either pore collapse or loss of the liquid phase that solvates the protein in the pore. In order to determine the cause of the blue shift, absorption spectra were obtained on rehydrated xerogel samples. Absorption spectra of xerogels immersed in 0.1 M acetate buffer (pH 4.5) exhibit a distinct red-shift in the Soret band maximum to 404 nm (Fig. 2). This value is exactly the same as that observed for aged gel samples.

Our interpretation of these dehydration/rehydration experiments is that the changes in absorption spectra accompanying the drying process are not 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.

These results also suggest a somewhat more speculative conclusion; that pores containing the protein behave differently than the dopant-free pores [17]. The latter exhibit pore collapse when the pore liquid evaporates whereas, as Fig. 2 indicates, the cyt-*c* resides in a pore which conforms to its size (32.4 Å diameter). The curious feature here is that the gel network possesses a wide distribution of pore sizes, yet the proteins are selectively trapped in pores that conform to the size of the protein (or larger). Our interpretation of this selective behavior is that since the protein is added to the sol, there are protein–sol interactions which occur as the protein becomes encapsulated in the growing silica network. The large number of H-bonding groups on the surface of the biomolecule result in extensive interaction with the silicate polymer which, during the initial stages of network formation contain Si—O(H)—Si and Si—OH fragments. In this way, the dopant biomolecule serves as a nucleus which enables condensation polymerization to readily take place. Thus, the protein acts as a structural template around which the gel network can develop and form a porous inorganic polymer cage. When drying occurs, this pore shrinks but conforms to the dimensions of the dopant biomolecule while the protein undergoes slight conformation changes as it adapts to the new micro-environment furnished by the silicate network. Recent studies using resonance Raman spectroscopy provide additional details of this mechanism and further support the templating effect of the dopant biomolecules [18].

The encapsulation of the cyt-*c* in the silicate gel network also leads to stabilizing effects. First, protein denaturation due to MeOH is reversible 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) [12]. The Soret band maximum was located at 405 nm (FWHM of 22.8 nm). Upon soaking these same gels in MeOH, however, the protein partially denatured once again; the Soret band shifted to 402 (FWHM of 23.1 nm). Immersing the methanol-soaked samples in pure buffer led once again to the native cyt-*c*. The reversibility of the denaturation is shown in Fig. 3. These experiments establish that the encapsulation process stabilizes cyt-*c* in the folded state as compared to the unfolded (denatured form) in the sol–gel matrix. A second significant feature of these experiments is that protein aggregation did not occur, even when the gels were soaked in pure MeOH, a condition which produces protein aggregation in solution. These results suggest that confining the molecule within the silicate network constrains the mobility of the protein so that the aggregation is prevented yet the molecule remains sensitive to changes in the local environment.

4. CONCLUSIONS

The present paper has reported on the nature of the interactions between the sol–gel derived silica matrix and a specific biomolecule, cytochrome *c*. These interactions are of central importance in determining the synthesis conditions for these materials, in creating the pores which confine the protein, and in stabilizing the protein so that it retains its structure and chemical function. These results define a strategy for the synthesis of sol–gel matrices incorporating biomolecules. The pH/buffer/alcohol stability of the protein in solution pro-

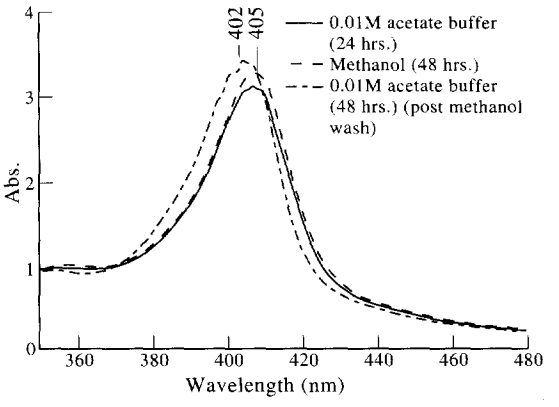

Fig. 3. Absorption spectra of the heme Soret band for cytochrome *c* encapsulated in TMOS derived silica gels demonstrating the reversibility of denaturation upon immersion in pure methanol and acetate buffer solutions [12].

vides an important guideline for successful sol–gel synthesis as well as for identifying optimum synthetic conditions for protein function. The experiments which demonstrate that solvent loss (and not pore collapse) occurs in the protein-doped xerogels have led us to suggest that protein–matrix interactions facilitate the formation of size-specific pores for the biomolecules. Finally, it was shown that encapsulation within the sol–gel matrix provides stabilization towards external reagents. Once trapped within the network, denaturation of the biomolecule is reversible and the native form can be regenerated. In addition, protein aggregation is avoided because of the restriction in protein mobility.

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