

**L**atasha Garrett is currently a graduate student in chemistry at the University of Tennessee at Knoxville. She is pursuing research with Dr. George Schweitzer with a focus on inorganic chemistry and lanthanide chemistry. Ms. Garrett graduated from the University of Tennessee at Knoxville with a B.S. in Chemistry and a minor in psychology in 2005. At Oak Ridge National Laboratory, she participated in the SULI program for two appointments. She worked in the chemical sciences division under the supervision of Dr. Hugh O'Neill. Her research project was to study the surface chemistry of encapsulating proteins in silica gels.

**H**ugh O'Neill is a staff scientist at Oak Ridge National Laboratory (ORNL), Tennessee. He received his Ph.D. in 1998 from University College Dublin, Ireland. He was a post-doctoral researcher at ORNL and a research assistant professor at University of Tennessee, Knoxville prior to joining the Chemical Sciences Division at ORNL. His research interests are focused on both solar hydrogen and fermentative hydrogen renewable energy production. He is also interested in the investigation of the structure, function, and interactions of bio-molecules at the biotic/abiotic interface.

## THE EFFECTS OF SURFACE CHEMISTRY ON THE PROPERTIES OF PROTEINS CONFINED IN NANO-POROUS MATERIALS

LATASHA M. GARRETT AND HUGH O'NEILL

### ABSTRACT

The entrapment of proteins using the sol-gel route provides a means to retain its native properties and artificially reproduce the molecular crowding and confinement experienced by proteins in the cell allowing investigation of the physico-chemical and structural properties of biomolecules at the biotic/abiotic interface. The biomolecules are spatially separated and 'caged' in the gel structure but solutes can freely permeate the matrix. Thus, properties such as the folding of ensembles of individual molecules can be examined in the absence of aggregation effects that can occur in solution studies. Green fluorescent protein from *Aequorea coerulea* was used as a model protein to examine the unfolding/re-folding properties of protein in silica gels. The recombinant protein was isolated and purified from *Escherichia coli* extracts by cell lysis, three-phase partitioning, dialysis, and anion exchange chromatography. The purity of the protein was greater than 90% as judged by SDS PAGE gel analysis. Sol-gels were synthesized using tetramethylorthosilicate (TMOS) in combination with, methyltrimethoxyorthosilane (MTMOS), ethyltrimethoxyorthosilane (ETMOS), 3-aminopropyltriethoxysilane (APTES), and 3-glycidoxypropyltrimethoxysilane (GPTMS). The acid induced denaturation and renaturation of GFP was analyzed by UV-visible, fluorescence, and circular dichroism (CD) spectroscopies. No renaturation was observed in gels that were made with TMOS only, and in the presence of APTES, MTMOS, and ETMOS. However, in gels that were made with GPTMS, the CD and UV-visible spectra indicated that the protein had refolded. The fluorescence emission spectrum indicated that approximately 20% of fluorescence had returned. This study highlights the importance of the surface chemistry of the silica gels for the refolding properties of the entrapped GFP. Future studies will investigate the effect of surface chemistry on the thermal and solvent stability of the entrapped protein.

### INTRODUCTION

Cells can be described as crowded environments because they are composed of many different types of macromolecules, none of which are present at high concentration, but which collectively occupy a large fraction of the total volume of the fluid [1]. The complex mixture of nucleic acid, polysaccharide, protein and lipid comprises approximately 30–40% of the cell [2]. In this scenario, it is expected that non-specific interactions are individually weak but cumulatively quite strong. An important subset of these are excluded volume interactions that arise from steric repulsion between different macromolecules. They are always present and increase both the free energy of a solution and the chemical potential of each type of solute. Therefore, many proteins fold into their

native states in an environment that geometrically restricts their conformational space. This can dramatically affect protein stability, dynamics and function because of volume constraints, viscosity, and solvent and solute activity [1]. Conversely, the majority of protein characterization studies are carried out in dilute solution to mitigate against aggregation effects that can occur at higher concentrations. This approach fails to take into account physiological conditions where the excluded volume can influence the structure, function, and interactions of proteins.

Although sol-gel chemistry has been widely used for immobilization and stabilization of a range of different biomolecules [3] a recent and intriguing development is using sol-gel entrapment to mimic the crowded cellular environment [4]. The entrapment of proteins using the sol-gel route provides a means to retain their native

properties and artificially reproduce the molecular crowding and confinement experienced by proteins in the cell. The biomolecules are spatially separated and caged in interconnected nanopores such that solutes can freely permeate the matrix. The optically transparent matrix can be analyzed by a variety of spectroscopic techniques used to monitor the structure of macromolecules in dilute solutions. An important development in this area was the demonstration that circular dichroism spectropolarimetry could be used to directly measure the structural properties of proteins entrapped in sol-gels [4].

The unfolding equilibrium and kinetics of a mutated green fluorescent protein (GFPmut2) from *Aequorea victoria* in silica gels were investigated to gain insight into the effects of caging and crowding on protein structure and large scale dynamics [5]. The guanidium-HCl induced unfolding of GFP was a biphasic process in the sol-gel environment as opposed to monophasic in solution. This suggested the presence of a significant fraction of encapsulated molecules with a different conformation to the dominant species in solution. A further extension this work was to investigate unfolding and refolding of single GFP molecules entrapped in wet silica gels using two-photon fluorescence spectroscopy [6]. Several periodic oscillations among the chemical substates of the protein fluorophore immediately before unfolding could be resolved. This study highlights the effectiveness of protein encapsulation in silica gels to characterize the dynamic properties of individual molecules. The water-rich matrices offer a good simulation of the natural cellular environment: the proteins under investigation are spatially separated preventing interprotein interactions.

In the present study, a green fluorescent protein (GFP) from *Aequorea coerulea* was used to investigate the effect of the surface chemistry of sol-gel on the properties of the encapsulated protein [7]. The wild-type *A. coerulea* GFP homologue shares 92% sequence identity with *A. victoria* GFP, the most widely studied variant of this protein. The fluorophore forming residues, Ser<sup>65</sup>, Tyr<sup>66</sup>, and Gly<sup>67</sup> are conserved as are the evolutionary invariant Arg<sup>96</sup>, Glu<sup>222</sup>, His<sup>148</sup>, Phe<sup>165</sup>, Ile<sup>167</sup>, and Thr<sup>203</sup>, all of which are very spatially very close to the chromophore. The *A. coerulea* mutated variant used in this study underwent five amino acid substitutions, V11I, F64L, K101E, T206A, and E222G, in comparison to its wild-type non-fluorescent parent protein. The E222G mutation is the key event that confers the fluorescent properties on the protein. The other mutations are suggested to improve the folding properties of the protein. It has an excitation maximum at 480nm and emission at 505nm.

The effect of surface chemistry on the refolding of acid-denatured GFP was investigated in sol-gels whose surface chemistry was altered by introduction of charged polar, uncharged polar and hydrophobic functional groups. Various spectrophotometric techniques were employed to determine how the microenvironment of the sol-gel pores affected the folding properties of the protein.

## MATERIALS AND METHODS

### ***Over-Expression of GFP in Escherichia coli***

All recombinant DNA techniques and *E. coli* maintenance and propagation procedures were carried out as previously described [9]. *E. coli* JM109 cells were transformed with pAcGFP1 (BD Biosciences, USA) that encodes the gene for *A. coerulea* GFP. The transformed *E. coli* was grown in Luria Bertani medium at 37°C supplemented with ampicillin at a final concentration of 50µg/ml. For production of recombinant GFP, protein expression was induced at an optical density of 0.5 recorded at 600nm by addition of isopropyl-B-d-thiogalactopyranoside (IPTG) at a final concentration of 1.0mM. Growth was continued at 37°C for approximately 18h.

### ***Protein extraction and purification***

The bacterial cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The cells were resuspended in sonication buffer (20mM Tris HCl, 10mM NaCl, 5mM EDTA, pH 7.5) that contained 1.0 mg/ml lysozyme. Sonication was performed using a Branson Sonicator equipped with a microtip for a total of 6 min with 20 sec sonication intervals followed by 1 min of cooling on ice/NaCl. The broken cells were centrifuged at 15,000 x g for 15 min and the pellet was resuspended and sonicated for a further 2 min with 30 sec bursts. The supernatants, containing the GFP were then pooled.

The combined crude extracts were subjected to the three-phase partitioning technique as previously described [9]. The protein preparation was further purified by anion-exchange chromatography (AEC) on Hi-Trap Q Sepharose (Amersham Biosciences). The equilibration buffer was 50mM Tris-HCl pH 8.0 (buffer A) and the limit buffer was 50mM Tris-HCl pH 8.0, 1M NaCl (buffer B). The protein was dialyzed into 50mM Tris-HCl pH 7.5 and filter-sterilized before applying it to the column. The column was washed with 6 volumes of buffer A to remove unbound protein, followed by application of a gradient from 0 to 100% of buffer B (15 column volumes) to elute GFP. The absorbance of the eluant was monitored at 490nm and 280nm.

### ***Sol-gel synthesis***

For sol-solutions made with tetramethylorthosilicate (TMOS), methyltrimethoxysilane (MTMOS), aminopropyltriethoxysilane (APTES) or 3-glycidoxypropyltrimethoxysilane (GPTMS) as precursors, the sol gel precursor (0.01 mol) was mixed with 0.338g H<sub>2</sub>O and 0.02g 40mM HCl, and then sonicated (Laboratory Supply Sonicator, Model G112SPIT) at 4°C until a homogeneous solution was formed. Sol-solutions made with ethyltrimethoxysilane (ETMOS) were formed as described above except that the mixture was stirred at 4°C for several hours. Entrapment of GFP was achieved by initiation of the condensation reaction by mixing the protein solution, dissolved in 10mM Na phosphate, pH 7.0, with the hydrolyzed sol solution in a ratio of 2:3. The mol ratio of TMOS

hydrolyzed sol to ORMSIL hydrolyzed sol is shown in Fig 2B. After condensation occurred, the gels were allowed to age for at least 24h while immersed in 50mM Na phosphate, pH 7.0 before carrying out experiments. There were two types of gels made: gels (60–80 $\mu$ L) that were formed on quartz slides (9  $\times$  25mm) to form a thick film that was  $\sim$ 0.5mm and gels that were formed in plastic cassettes that were 8cm  $\times$  8cm  $\times$  0.1cm. The protein unfolding/refolding reactions were performed at 25°C.

### Analysis Methods

Circular dichroism spectra were recorded on a Jasco 810 CD spectropolarimeter from 190–600nm at 25°C. UV-visible spectra were recorded between 190–800nm using a Cary 50 spectrophotometer. Protein content was estimated using the modified Lowry assay method (Pierce Biochemicals) with Bovine Serum Albumin as the standard protein. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and native PAGE were carried out as previously described [10].

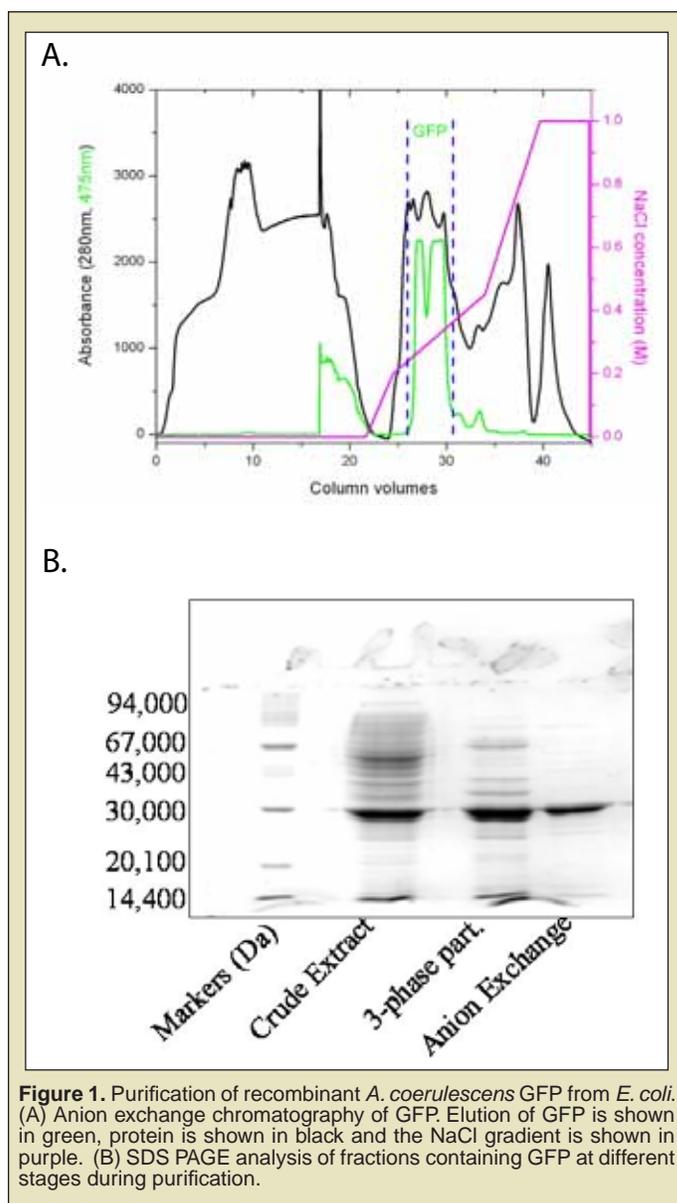
## RESULTS AND DISCUSSION

### Purification of GFP

Over-expression of GFP in *E. coli* was carried out as described in Materials and Methods. The first step in the purification procedure was by three-phase partitioning [9]. Briefly, ammonium sulfate was added to the crude extract to a final concentration of 20% followed by addition of an equal volume of tert-butanol. After an incubation period the reaction was centrifuged to separate three phases. The aqueous phase containing the GFP fraction was removed and the ammonium sulfate concentration was adjusted to 60% and 2 volumes of tert butanol were then added. The incubation and centrifugation steps were repeated and this time GFP was present as a precipitate at the interface of the aqueous and organic phases. The protein was dissolved in 0.5ml of 10mM Tris-HCl pH 7.5.

Jain et al. [9] reported the purification of GFP to homogeneity using this approach. In our hands we were only able to achieve approximately 80% purification by this method, as judged by SDS PAGE analysis. One difference was that in the present study *E. coli* JM109 replaced *E. coli* DH5 $\alpha$  which is known to produce less carbohydrate and extracellular protein than the JM109 strain. The presence of extracellular carbohydrate and protein have been shown to effect the purity of biomacromolecules purified from *E. coli* by organic extraction [8]. The other major difference was that the ratio of cell mass to solvent was increased 10-fold in this study compared to the published procedure which may impact the isolation procedure. A thick interfacial phase was observed during the first round of three phase partitioning and to reduce the amount of GFP trapped in that phase, DNAase was added for 1 hour before starting the extraction. The addition of DNAase did not improve in the yield of GFP and hence this step could be removed in the future.

GFP purified by three-phase extraction was subjected to anion exchange chromatography on a HiTrapQ column. The chromatogram is shown in Figure 1a. GFP eluted from the column between 25-35% Buffer B as indicated by the 475nm absorbance

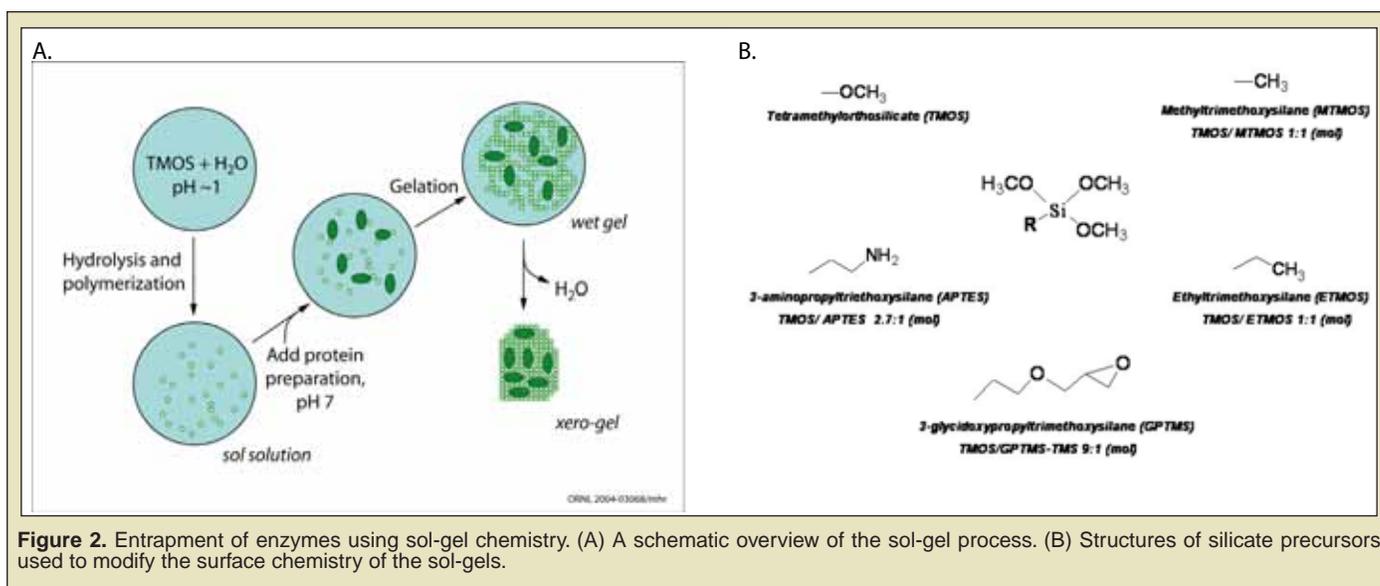


**Figure 1.** Purification of recombinant *A. coeruleus* GFP from *E. coli*. (A) Anion exchange chromatography of GFP. Elution of GFP is shown in green, protein is shown in black and the NaCl gradient is shown in purple. (B) SDS PAGE analysis of fractions containing GFP at different stages during purification.

profile. There were two peaks corresponding to GFP activity. These fractions were analyzed using spectrophotometry and fluorescence emission spectroscopy. The second peak contained the purest GFP. The SDS-PAGE analysis (Figure 1b) showed a single band at 27kDa after anion exchange chromatography which agreed with the known molecular mass of GFP. It was estimated that the purity of GFP was greater than 90%.

### Entrapment of GFP in sol-gels

The procedure for entrapment of GFP in sol-gels was carried out using a previously described procedure [11] as described in Materials and Methods. An overview of the process is described in Figure 2A. Wet gels that were aged for 24–48 hours were used in this study. Two different approaches for casting the sol-gels were tested. The first approach was to layer the sol solution on a clean quartz slide and following gelation to immerse it in 10mM Na phosphate buffer pH 7.0. The second approach involved casting the gels in plastic gel



**Figure 2.** Entrapment of enzymes using sol-gel chemistry. (A) A schematic overview of the sol-gel process. (B) Structures of silicate precursors used to modify the surface chemistry of the sol-gels.

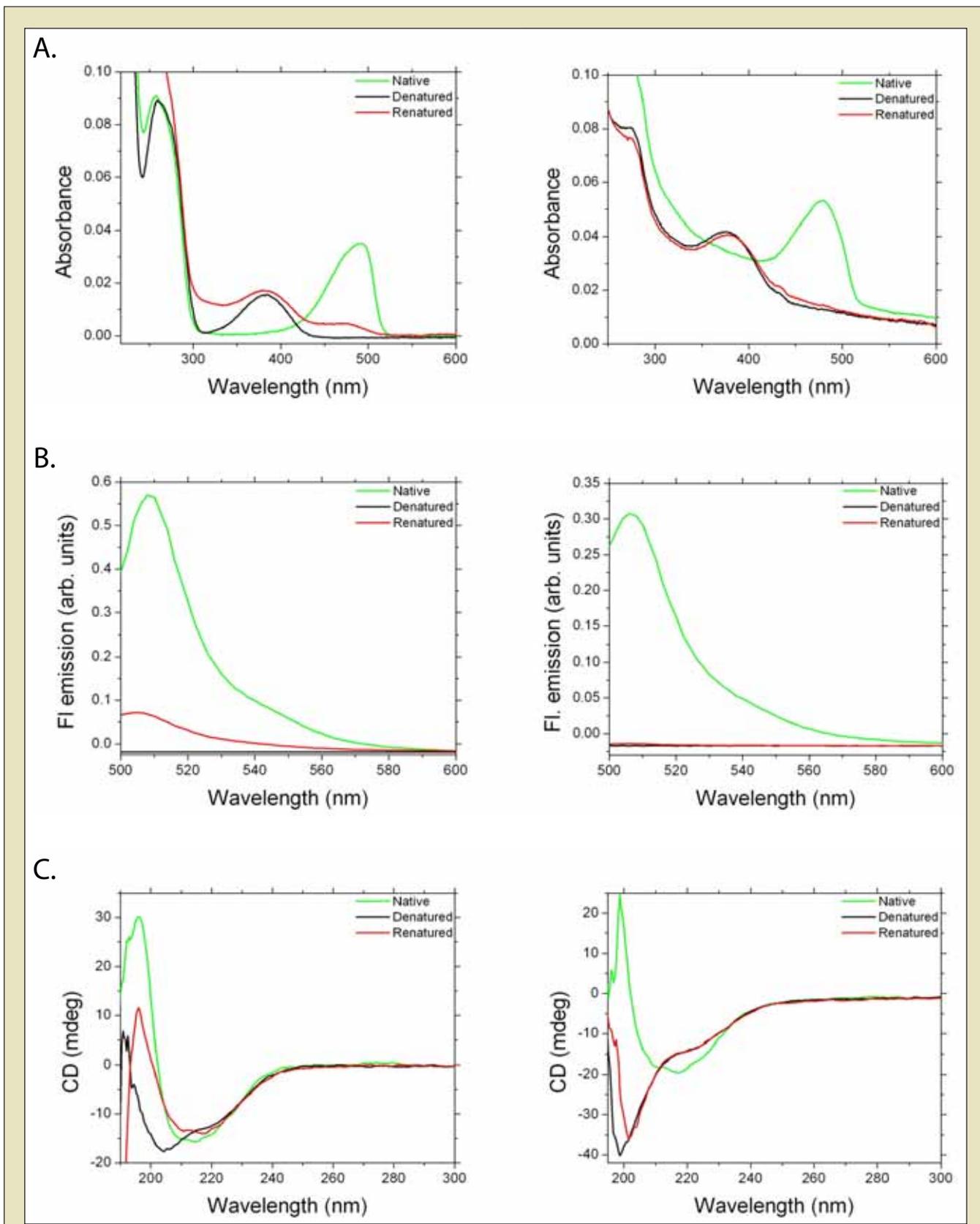
cassettes usually employed for PAGE applications. After gelation, the gels were overlaid with 10mM Na phosphate buffer pH 7 and aged at 4°C. The latter approach had the advantage that the gels were of uniform thickness (1mm) and not as prone to cracking due to changes in surface tension during the gelation process.

A series of gels were synthesized using TMOS singly and in combination with the ORMSILs (organically modified silicates) MTMOS, ETMOS, APTES and GPTMS. The structures of the precursors are shown in Figure 2B. The main criterion for the synthesis of the hybrid gels was to add the maximum amount of ORMSIL that would allow the entrapment of GFP in its native form and preserve the optical properties of the gels. The synthesis conditions are described in Materials and Methods. The absorbance, fluorescence emission spectra and CD spectra of the entrapped GFP in all gel types were identical to the solution spectra Figure 3A. No leakage of GFP was detected from the gels during the time frame the experiments were carried out.

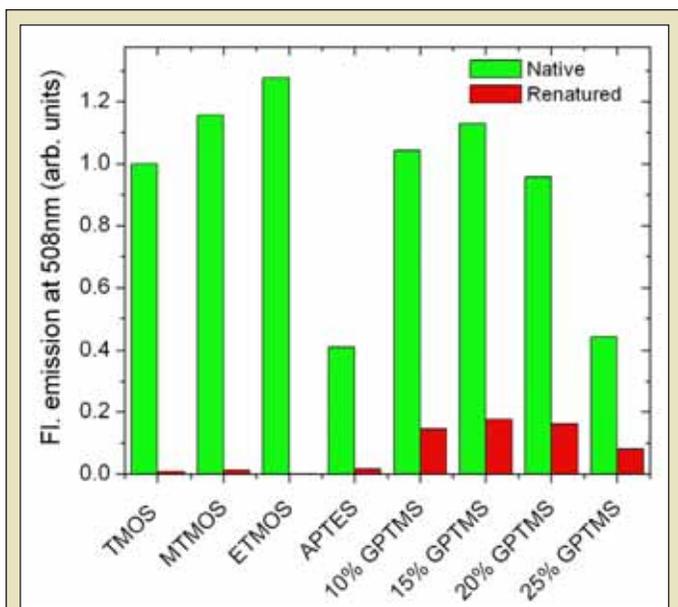
The aim of the project was to investigate how the microenvironment of the entrapped protein affected the refolding of GFP entrapped in the gels. After aging for 24h in 10mM Na-phosphate pH 7.0 the protein sol-gel was immersed in 10mM HCl for at least 15 min or until all fluorescence had disappeared. This took less than 5 min for all types of gel except the APTES gels which required longer incubation times (~30 min). The gels were then placed in 50mM Na-phosphate buffer, pH7.0. The visible absorption, fluorescence emission and circular dichroism spectra of the native, denatured and renatured protein were recorded. A comparison of the acid denaturation-renaturation of GFP in solution and entrapped in silica gels is shown in Figure 3. The chromophore peak at 490nm shifted to 295nm when the protein was exposed to acid due to the protonation of the tyrosyl OH group on the chromophore (Figure 3B) [12]. It is usually in the anionic form in its native fluorescent state. The loss in fluorescence was accompanied by a change in the CD spectrum also. Re-equilibration of the protein at pH 7.0 resulted in partial recovery of fluorescence for GFP in solution (Figure 3C). This was accompanied by a recovery in the native CD spectrum of the protein. Conversely, no recovery of

fluorescence was observed for the protein entrapped in the silica gel. A similar result was obtained for gels functionalized with methyl, ethyl and aminopropyl groups. There was no recovery of fluorescence or evidence for the recovery of the native structure of the protein. A summary of the fluorescence emission data is shown in Figure 4.

In contrast, gels made with GPTMS produced a microenvironment that was favorable for refolding GFP. Gels were made in which the mol ratio of TMOS/GPTMS was varied from 9 to 3. A TMOS/GPTMS mol ratio of 6.7 appeared optimal, with approximately 20% recovery of native fluorescence of GFP (Figure 4). Figure 5 shows the absorption, fluorescence emission and CD spectra of the GFP entrapped in a TMOS/GPTMS gel (6.7 mol ratio). The spectra of the denatured protein was identical to other sol-gel formulations. However, after replacement of the protein in 50mM Na-phosphate buffer pH 7.0 a recovery in fluorescence at 509nm was observed. In addition, the CD spectrum partially regained its native shape. A deconvolution algorithm [13] was used to quantitatively estimate the fraction of each type of secondary structure present in GFP in its three different states (Table 1). There was a decrease in the amount of unordered polypeptide and a concomitant increase in the amount of  $\beta$ -sheet and  $\alpha$ -helix present compared to the denatured protein, confirming that the protein refolded after replacement in phosphate buffer after denaturation.



**Figure 3.** The properties of GFP in solution and entrapped in sol-gels. Spectrophotometric (A), fluorescence emission (B) and circular dichroism spectra (C) were recorded. The left panels correspond to GFP in solution and the right panels are GFP in TMOS gels. Spectra were recorded for native GFP in 10mM Na-phosphate buffer pH 7.0 (green), GFP in 10mM HCl (black) and renatured GFP in 10mM Na-phosphate pH 7.0 (red).

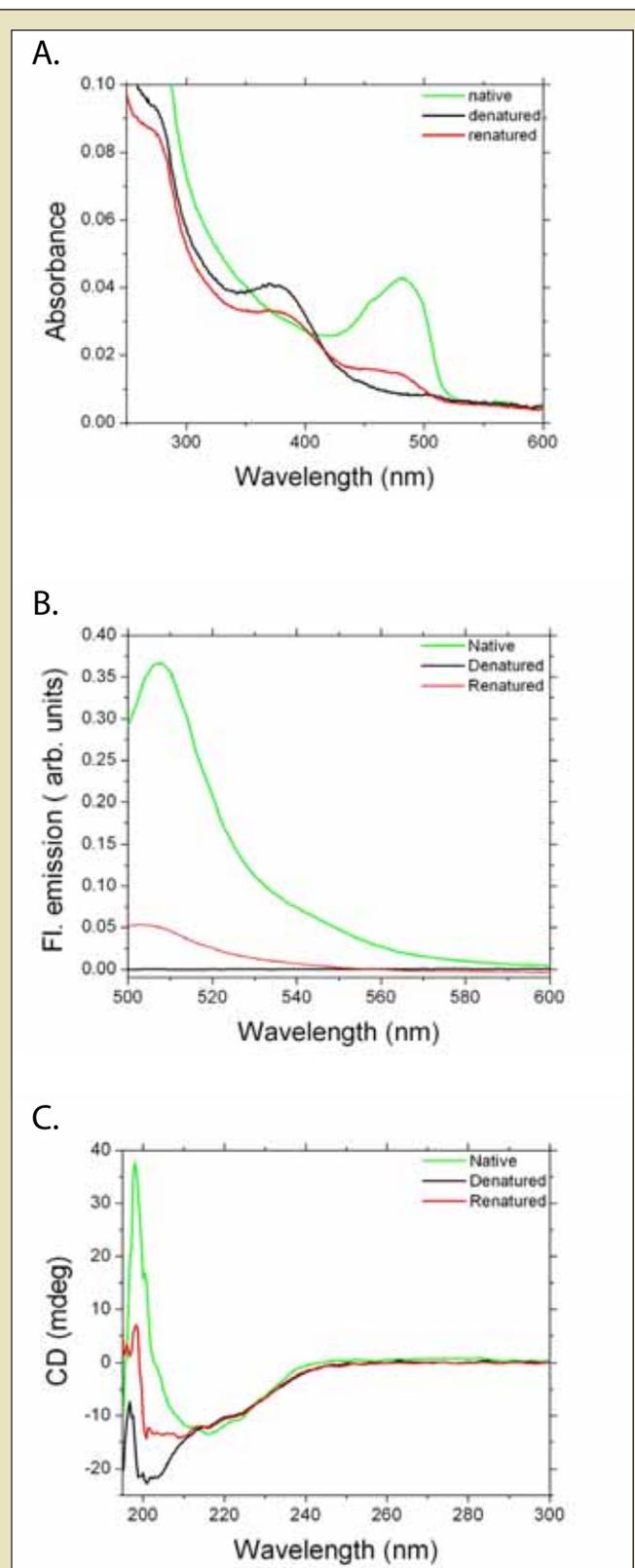


**Figure 4.** Comparison of fluorescence emission at 508nm of native GFP and the enzyme after renaturation in 10mM Na-phosphate buffer pH 7.0 in different formulations of sol-gels.

## DISCUSSION

The aim of the study was to investigate the unfolding properties of GFP entrapped in sol-gels. There were three important aspects to the design of the experimental set-up used. First, the entrapment process resulted in the protein molecules being caged in nanopores creating conditions similar to the crowded environment of the cell. Furthermore, the proteins were spatially separated in the gel matrix allowing ensembles of individual molecules be investigated without interfering effects such as protein-protein interactions. Finally, the surface properties of the pores were altered by incorporation of organic groups into the sol-gel backbone influencing excluded-volume interactions in the pores.

Three different types of environment were created to investigate the refolding properties of GFP in this study. A polar charged environment was formed by negatively charged native sol-gel matrix and by the introduction of amino ( $\text{NH}_3^+$ ) groups did not support the refolding of acid denatured GFP. Similarly, the hydrophobic groups such as methyl and ethyl groups also didn't support refolding of acid denatured GFP. GPTMS has a highly reactive epoxy functional group and under the conditions used for the hydrolysis of the sol precursors it is highly probable that the epoxy functional group was converted to a mixture of 1,2 diol and methoxy alcohol resulting in a hydrophilic uncharged polymer. These uncharged hydrophilic molecules formed by GPTMS precursor did support refolding of GFP. Inert polyethylene glycol like molecules have previously been shown to stabilize the compact native state relative to the less compact unfolded or partially unfolded state of polypeptides [14]. This study supports the view that under crowded conditions excluded volume interactions promoted by inert PEG-like molecules aid the folding properties of biomolecules.



**Figure 5.** Properties of GFP entrapped in TMOS/GPTMS sol-gels. The spectrophotometric (A), fluorescence emission (B) and circular dichroism spectra (C) were recorded. Spectra were recorded for native GFP (green), GFP in 10mM HCl (black) and renatured GFP in 10mM Na-phosphate pH 7.0 (red).

| Structure | H(r)  | H(d)  | S(r)  | S(d)  | Trn   | Unrd  |
|-----------|-------|-------|-------|-------|-------|-------|
| Native    | 0.091 | 0.088 | 0.228 | 0.109 | 0.194 | 0.289 |
| Denatured | 0.030 | 0.045 | 0.137 | 0.075 | 0.147 | 0.565 |
| Renatured | 0.036 | 0.045 | 0.164 | 0.092 | 0.170 | 0.493 |

Abbreviations: H(r), regular  $\alpha$ -helix; H(d), distorted  $\alpha$ -helix; S(r), regular  $\beta$ -sheet; S(d), distorted  $\beta$ -sheet; Trn, turn; Unrd, unordered

**Table 1.** Fractions of each type of secondary structure in GFP entrapped in TMOS/GPTMS sol-gels.

## REFERENCES

- [1] Minton, A. P., Implications of macromolecular crowding for protein assembly. *Current Opinion in Structural Biology* **2000**, 10, (1), 34-39.
- [2] Zimmerman, S. B.; Trach, S. O., Estimation of Macromolecule Concentrations and Excluded Volume Effects for the Cytoplasm of Escherichia-Coli. *Journal of Molecular Biology* **1991**, 222, (3), 599-620.
- [3] Avnir, D.; Coradin, T.; Lev, O.; Livage, J., Recent bio-applications of sol-gel materials. *Journal of Materials Chemistry* **2006**, 16, (11), 1013-1030.
- [4] Eggers, D. K.; Valentine, J. S., Molecular confinement influences protein structure and enhances thermal protein stability. *Protein Science* **2001**, 10, (2), 250-261.
- [5] Campanini, B.; Bologna, S.; Cannone, F.; Chirico, G.; Mozzarelli, A.; Bettati, S., Unfolding of Green Fluorescent Protein mut2 in wet nanoporous silica gels. *Protein Science* **2005**, 14, (5), 1125-1133.
- [6] Cannone, F.; Bologna, S.; Campanini, B.; Diaspro, A.; Bettati, S.; Mozzarelli, A.; Chirico, G., Tracking unfolding and refolding of single GFPmut2 molecules. *Biophysical Journal* **2005**, 89, (3), 2033-2045.
- [7] Gurskaya, N. G.; Fradkov, A. F.; Pounkova, N. I.; Staroverov, D. B.; Bulina, M. E.; Yanushevich, Y. G.; Labas, Y. A.; Lukyanov, S.; Lukyanov, K. A., Colourless green fluorescent protein homologue from the non-fluorescent hydromedusa *Aequorea coerulea* and its fluorescent mutants. *Biochemical Journal* **2003**, 373, 403-408.
- [8] Sambrook, J., Fritsch, E.F. & Maniatis, T (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Press. Cold Spring Harbor..
- [9] Jain, S.; Singh, R.; Gupta, M. N., Purification of recombinant green fluorescent protein by three-phase partitioning. *Journal of Chromatography A* **2004**, 1035, (1), 83-86.
- [10] Laemmli, U. K., Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4. *Nature* **1970**, 227, (5259), 680-&.
- [11] Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I., Encapsulation of Proteins in Transparent Porous Silicate-Glasses Prepared by the Sol-Gel Method. *Science* **1992**, 255, (5048), 1113-1115.
- [12] Wiehler, J.; Jung, G.; Seebacher, C.; Zumbusch, Z.; Steipe, B., Mutagenic stabilization of the photocycle intermediate of green fluorescent protein (GFP). *ChemBiochem* **2003**, 4, (11), 1164-1171.
- [13] Sreerama, N.; Woody, R. W., Analysis of protein CD spectra: Comparison of CONTIN, SELCON3, and CDSSTR methods in CDPro software. *Biophysical Journal* **2000**, 78, (1), 334A-334A.
- [14] Tellam, R. L.; Sculley, M. J.; Nichol, L. W.; Wills, P. R., The Influence of Poly(Ethylene Glycol) 6000 on the Properties of Skeletal-Muscle Actin. *Biochemical Journal* **1983**, 213, (3), 651-659.