Entrapment of Avidin in Sol-Gel Derived Silica Glasses

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Abstract: *Avidin has been entrapped within silica matrices under ambient processing conditions. Properties of the entrapped avidin including stability and its binding with biotin have been investigated by measuring changes in fluorescence. The relative rates of binding to biotin in a range of organically modified silicate were used to compare diffusion properties within gels and their suitability for entrapment of biosensor reagents. Results showed that none of the sol-gel processing conditions used in this study denatured the biomolecules, with the silicate-entrapped generally avidin having better stability towards denaturing conditions than in solution. The avidin retains its binding ability even as the gels aged. Addition of specific organically modified silanes to sol-gel derived materials resulted in a slight improvement in stability and greater accessibility of avidin to external reagents.*

Keywords: avidin, biotin, entrapment, organically modified silicate, sol-gel

1. INTRODUCTION

Over the past decade, several groups have reported the development and characterization of proteins entrapped into inorganic silica sol-gel matrices.[1–3] Research in this field indicates that upon entrapment, the proteins generally retained their spectral properties and biological activity. Upon entrapment, the protein may find a more stable environment as the polymeric framework grows around the biomolecule creating a cage and thus protecting the protein from aggregation and unfolding.[4] The reaction chemistry of sol-gel entrapped biomolecular systems has been shown to be analogous to that in aqueous solution except for the observed rates of chemical reaction which are generally slower due to diffusion limitations in a porous silica matrix.[5]

The successful entrapment of biomolecules suggests a wide range of novel materials and applications. Sol-gel glasses doped with biomolecules have been used as optical and electrochemical probes for a number of analytes.

Avidin, a basic tetrameric glycoprotein, is an interesting protein being used in many areas of application for its unusually strong interaction with biotin.[6,7] Each avidin monomer can bind up to four molecules of biotin with exceptionally high affinity. The high binding activity between avidin and biotin is the basis of their use as molecular tools in biotechnological, diagnostic and therapeutic applications.[6–8] Besides biotin, this protein can also interact more weakly with other ligands, including fluorescein and 1-anilino-8-naphthalene sulfonic acid (ANS). The reactions of avidin with these ligands have been well studied and established in aqueous solution.[9–11] Immobilization of avidin in fatty acid (arachidic acid) films has also been studied. The immobilization and reaction of immobilized avidin with ligands in inorganic silica matrices, however, has not yet been reported.

This paper presents the entrapment of avidin in a silica glass network prepared by sol-gel processing of tetramethyl orthosilicate (TMOS) and a mixture of TMOS with organosiloxanes. Issues regarding how this protein is affected by entrapment in the porous inorganic matrix and the effect on the matrix of entrapping the protein are described.

2. EXPERIMENTAL

2.1 Materials

Tetramethyl orthosilicate (TMOS), methyltrimethoxysilane (MTMOS), polydimethylsiloxane (PDMS), d-biotin and ANS were obtained from Sigma Aldrich and used as received. All water was twice distilled and deionized to a specific resistance of at least 18 MΩ cm using a milli-Q water purification system. All other chemicals were analytical grade and used without purification.

2.2 Preparation of Precursor Solutions

An appropriate amount of organosilane (MTMOS or PDMS) was first added to TMOS to provide organosilane:TMOS ratios ranging up to 10 mol % for MTMOS and up to 5 wt. % for PDMS. A total of 1 ml of the silane solution was mixed with 0.24 ml of water and 10 μl of 0.15 N HCl. The mixture was sonicated for 30 min at ambient temperature until a clear, colorless and monophasic solution was obtained. The solution was then cooled and stored at –20^{o}C before use.

2.3 Preparation of Monoliths

A volume of 0.75 ml of the prehydrolyzed silane solution was rapidly mixed with 1.25 ml of phosphate buffer solution (100 mM, pH 7.02 with 100 mM NaCl, with or without 0.246 μM avidin). Monoliths were also prepared with ANS pre-bound to avidin by mixing a 0.75 ml of the prehydrolyzed silane solution and 375 μl of 0.820 μM avidin in phosphate buffer solution with 875 μl of phosphate buffer solution containing 1.405 μM ANS solution. The mixture was immediately placed into a disposable cuvette which was then sealed with parafilm and placed in an upright position until gelation occurred. Following gelation, the cuvettes were immediately filled with phosphate buffer solution and allowed to stand overnight at 4°C. The monoliths were then rinsed and were allowed to aged at 4°C.

2.4 Fluorescence Measurements

Fluorescence spectra were measured on a LS 50 B Perkin Elmer Luminescence Spectrometer at room temperature. For samples containing avidin, samples were excited at 280 nm and emission was measured from 305 nm to 420 nm in 1 nm increments at a rate of 100 nm min^{-1} using 6-nm slits in both excitation and emission path. To observe samples containing the complex avidin-ANS, spectra were measured from 400 nm to 600 nm with an excitation of 380 nm using 6-nm slits.

2.5 Biotin Binding Studies

The rate of interaction between avidin and biotin was examined for samples containing avidin which were aged for 21 days. The rehydrated monolith was placed into a cuvette containing 1 ml of 1.25 μM biotin in phosphate buffer solution at pH 7.02. Fluorescence emission spectra were measured every 30 min during equilibration.

The biotin binding affinity of entrapped proteins was examined for samples containing avidin which were aged for 21 days. The avidin samples were equilibrated in 1 ml of phosphate buffer solution pH 7.02 containing 0, 0.153, 0.307, 0.461, 0.614, 0.921, and 1.228 μM biotin solution for 10 h. Fluorescence was measured at the end of the equilibration period. Fluorescence emission spectra were measured for the protein at various levels of biotin with excitation at 280 nm and emission were measured from 305 nm to 420 nm. For comparison, these experiments were done for avidin in buffer solution.

2.6 Displacement Studies

Samples containing complex avidin-ANS were used to examine ANS displacement. Samples were equilibrated in 1 ml of phosphate buffer solution pH 7.02 containing 0, 0.153, 0.307, 0.614, 0.921, and 1.228 μM biotin solution for 10 h. Fluorescence emission spectra were measured at the end of equilibration period with excitation at 380 nm. Fluorescence spectra were measured from 400 nm to 600 nm.

2.7 Thermal Stability Studies

The thermal stability was examined for free and entrapped avidin and avidin-biotin. For solution-based studies, a volume of 1.5 ml of protein in phosphate buffer solution was used. For monolith-based studies, the rehydrated monolith was placed into a cuvette containing 1.5 ml of phosphate buffer solution. In both cases, the proteins were denatured by placing the cuvettes into a water bath. The temperature was raised in 5^{o}C increments starting at 20^{o}C to 95^{o}C. The samples were allowed to equilibrate for 60 min at each temperature. A fluorescence spectrum was measured at each point for the sample and blank at an identical temperature.

3. RESULTS AND DISCUSSION

3.1 Preparation of Protein-Containing Sol-Gel

Transparent, monolithic protein-containing silica glasses have been prepared utilizing modified literature methods[1–3] suitable for biomolecule entrapments under biocompatible conditions. The preparation of avidin containing sol-gel materials was accomplished simply by adding the protein into the TMOS derived sol before gelation occurred, followed by aging and drying. In this study, 0.075 μM avidin was used for 1 ml TMOS sol. A high buffer concentration was used to reduce the gelation time in order to minimize the time the protein spent in the methanol-containing silane solution. In this synthesis, after all the components are mixed together in the sol state and before gelling, the sol was a homogeneous, viscous fluid. The gelation time was shortened significantly by the addition of the buffered protein solution which raised the pH of the mixture. Immediately after gelation, the monoliths were rinsed so that the residual methanol was removed from the monolith and the fluorescent impurities were removed. The monoliths were then allowed to aged.

The as-synthesized sol-gels did not show any change in the protein activity (as determined by biotin binding) even after the gels had aged for several months, indicating good stability of the materials and completion of the sol-gel reactions.

To compare the activity of the protein in different matrices, organic modification of the silica matrix has been studied through the co-condensation of TMOS and organosiloxanes. It was previously observed that with higher ratios of alkyl-substituted silanes (MTMOS or PDMS), the resulting materials were translucent. Results showed that transparent hybrid silica materials can be obtained with MTMOS less than 30 mol % or PDMS less than 20 wt. %. In this work, a relatively low portion of MTMOS (5 and 10 mol %) and PDMS (2.5 and 5 wt. %) were co-condensed with TMOS in order to obtain optically transparent host matrices for avidin entrapment. All the resulting samples were homogeneous and transparent, indicating the absence of macroscopic phase separation.

Because the sol-gel method produces silica glass that is transparent at wavelengths as low as 250 nm, the concentration of the entrapped protein was accurately determined on the basis of the absorbance at 282 nm. Based on the UV absorption spectrum, the concentration of the protein in monolith was 0.218 mg ml^{-1} and on the basis of the amount of protein used in the encapsulation experiments, the concentration of the protein was 0.220 mg ml^{-1}. The similarity of these values indicates that frequent rinsing of the monoliths during the aging of silica does not leach significant amounts of entrapped protein.

3.2 Characteristics of Avidin Encapsulated Sol-Gel Monolith

3.2.1 FTIR studies of entrapped protein

An important aspect of entrapment of protein molecules in host structures is to ascertain whether biological activity of the protein is retained. The host should not distort the secondary structure of the proteins after entrapment. The secondary structure of protein is conveniently studied using FTIR measurements. The amide I and amide II bands which occur at 1620–1680 cm^{-1} and 1500–1580 cm^{-1}, respectively, are known to be indicators of the environment in which the proteins are entrapped. FTIR spectra of pure avidin, TMOS gel, avidin entrapped TMOS and avidin-biotin entrapped TMOS, respectively are shown in Figure 1. The spectrum of pure TMOS gel clearly reveals the typical bands ascribed to the network structure of SiO_2 gels:[12,13] Si-O-Si asymmetric band stretching at 1088 and 794 cm^{-1}, Si-O-Si bending at 456 cm^{-1}, SiO-H stretching at 3440, Si-OH or Si-O stretching at 950 and 560 cm^{-1}. The peaks associated with amide I and amide II were observed in the spectrum of pure avidin. The FTIR spectrum in Figure 1(c) illustrates the TMOS monolith after avidin entrapment, where all

amide stretching vibration bands are present with a slight shift, indicating that the avidin molecules are entrapped in the TMOS gel without significant perturbation to their secondary structure. It is observed that upon binding with biotin as shown in Figure 1(d), the amide I band increases in intensity. The results are in agreement with previous studies on the interaction of avidin-biotin in solution reported by Torregiani et al.[14]

3.2.2 SEM of entrapped protein

The SEM micrographs presented in Figure 2 reveal the morphology and distribution of avidin in TMOS gel. The avidin is seen to be distributed throughout the gel, though there also seems to be some aggregation.

3.2.3 Fluorescence spectra of entrapped protein

In this study, the intrinsic and extrinsic fluorescence spectra of avidin were used to monitor the behavior of the protein after entrapment in sol-gel glasses. The fluorescence spectra of the silica entrapped protein gels were followed through the aging process. Figure 3 shows the fluorescence spectra of free and entrapped avidin. It can be seen that the fluorescence spectra of wet-aged gels were almost identical to that of the corresponding buffer solution having the same avidin concentration with only a slight increases in the full width at half maximum (fwhm) for the entrapped avidin. The emission maximum of free and entrapped avidin occurred at 341 nm. There was no change in the fluorescence spectra of avidin after aging.

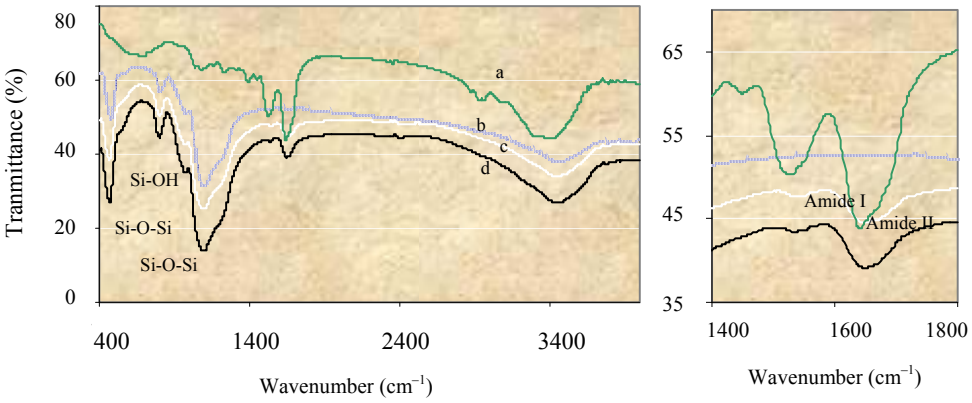

Figure 1: Infrared spectra of a) avidin; b) TMOS monolith; c) avidin in TMOS monolith; and d) avidin-biotin in TMOS monolith (amide region expanded, shown on right).

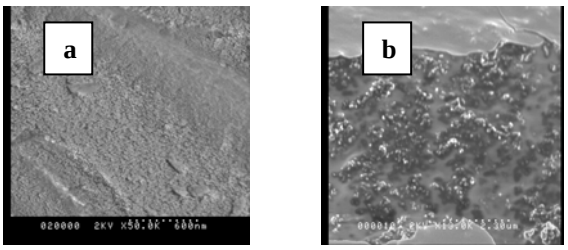

Figure 2: Typical SEM structure of a) TMOS and b) TMOS-avidin gel (wt. % avidin = 10). (Note the different scales).

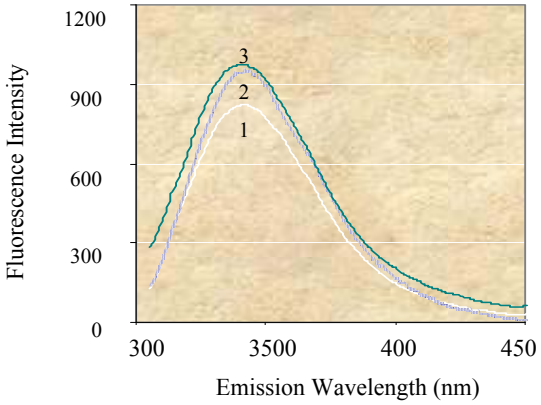

Figure 3: Fluorescence spectra of avidin 1) in buffer solution pH 7.04; 2) in wet-aged monolith; and 3) in dry-aged monolith (21 days).

The similarity in the emission spectral characteristics for avidin entrapped in gels to the solution phase indicates that the local environments surrounding the tryptophan residues in avidin solution were not significantly altered when the protein was entrapped. This also means that the native conformation of this protein was not significantly altered by sol-gel glass entrapment. This behavior is consistent with the hypothesis that the protein designs a specific pore when the silica network was formed during the sol-gel process and the silica cage around the protein. The presence of the protein prevents its surrounding pore from collapsing during aging and drying. However, the fluorescence spectra are broad and more subtle changes cannot be detected by this method.

Leaching studies of entrapped protein by monitoring the loss of avidin during aging showed that no significant leaching occurred over time or during repeated washes. It suggests that most of the protein molecules were sterically confined in smaller pores. This is in accordance with previous data[15] that the average pore diameter of the glass used is typically ~14 Å, even though the

protein [with the dimensions of (56 Å x 50 Å x 40 Å)] is larger and may then cause its own pore templating.[16]

Figure 4 shows the fluorescence spectra of avidin that were entrapped in hybrid silica (TMOS-MTMOS and TMOS-PDMS) sol-gels. The emission spectra of the entrapped avidin samples are again very similar to the spectrum of avidin in solution, with only small broadening of the peaks as the organosilane content increased. The results suggest that avidin appears to be entrapped with retention of its native conformation and that the sol-gel processing conditions used in these studies did not damage the protein though this will need to be confirmed by other techniques. The wavelength of maximum emission was not dependent on the concentration of organically modified silane, suggesting that the internal solvent composition dominated the emission behavior.

In order to compare ligand binding of avidin in solid matrix to those in buffer solution, these proteins were reacted with biotin in buffer solution. The fluorescence spectra for avidin in solution, and in 21 days aged silica monolith are shown in Figure 5. Equilibration of the entrapped samples into buffer solution containing biotin decreased the intensity of avidin fluorescence and shifted the emission wavelength from 342 nm to 329 nm. The results showed that the protein in silica glass was able to bind to biotin and that binding induced conformational changes with similar fluorescence properties to those in aqueous buffer. This indicates that a substantial proportion of the biotin binding sites in the avidin inside the pores of silica matrix retained their characteristic ligand binding ability.

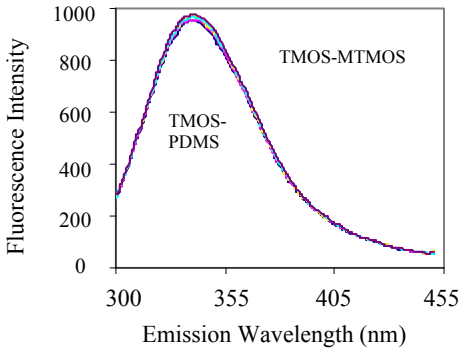

Figure 4: Fluorescence spectra of avidin entrapped into TMOS-MTMOS (10–30 mol %) and TMOS-PDMS (2.5–10 wt. %) sol-gel derived hybrid materials.

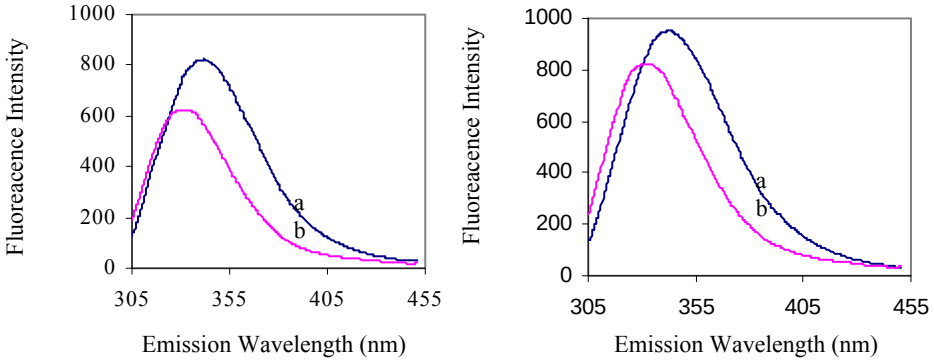

Figure 5: Fluorescence spectra for avidin (1) in buffer solution; and (2) in TMOS monolith. before and b) after binding with biotin.

The tryptophan residues in avidin are affected upon binding to biotin such that the energy and quantum yield of their fluorescence is altered.[10] As can be seen in Figure 5, biotin binding induces a blue shift in the emission maximum and quenches the avidin fluorescence both in solution and in silica monoliths. This proposed to be due to displacement of water from the avidin binding site with the ligand. This is in agreement with the report of Mei et al.[8] that in the absence of biotin, water contained in the avidin binding site may interact with tryptophan residues in their excited states, leading to a dipole relaxation towards red emitting species. This relaxation is associated with a greater mobility of the indolyl residues which may be responsible for the distribution of tryptophan fluorescence decay. The presence of biotin displaces all of the water molecules from the binding cavity inducing a blue shift of the protein fluorescence.

3.2.4 Concentration dependence of biotin binding

The binding of biotin was monitored spectroscopically by measuring the change in intensity for the tryptophan residues resulting from the binding of biotin. Figure 6 shows the change in intensity as a function of biotin concentration for avidin fluorescence. The binding curves showed that all samples retained at least 85% of their protein function as compared to the avidin in solution. This result suggests that TMOS had only a little effect on binding behavior. Entrapment of avidin into samples containing MTMOS or PDMS resulted in a slight improvement in the sensitivity of avidin to biotin as the MTMOS or PDMS content increased. It is presumed that the presence of organic groups in the silica matrix reduces the matrix-protein interactions increasing the interaction of protein with analyte.

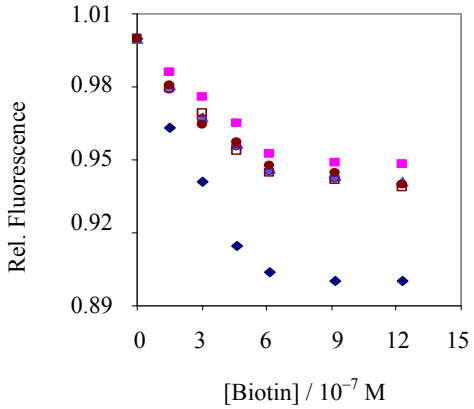

Figure 6: Fluorescence response of avidin as a function of biotin concentration. (■) TMOS; (▲) TMOS-MTMOS 5 mol %; (□) TMOS-MTMOS 10 mol %; (○) TMOS-PDMS 2.5 wt. %; (●) TMOS-PDMS 5 wt. %; (◆) solution. Concentration of avidin = 1.53 x 10^{-7} M, equilibration time = 10 h.

3.2.5 Displacement studies

Avidin reactivity can also be measured using the fluorescence probe ANS since the presence of avidin results in increasing quantum yield of the fluorescence of the ANS. Biotin binding causes displacement of the weakly bound fluorophore with concomitant quenching of the fluorescence (Fig. 7). Therefore, the fluorometric monitoring of the displacement of ANS can be used as an alternative method of measuring the biotin-avidin interaction. Figure 8 presents data from displacement experiments with avidin-ANS entrapped in silicate organic-inorganic hybrid monoliths and subsequently equilibrated with biotin solution. As expected, the characteristic decrease in fluorescence intensity of ANS with increasing biotin concentration is observed both in solution and silica matrices. This provides evidence that the ANS had been released back into an aqueous environment. Since there are only small changes in the fluorescence, however, these data can not determine whether the probe returns to solution in the aqueous phase or is displaced to a position at which the water molecules can quench the probe fluorescence as if the probe were in solution.[13] As found in biotin binding studies, entrapment of avidin-ANS into samples containing MTMOS or PDMS also resulted in slight improvement in the sensitivity of the fluorescence response of ANS to biotin.

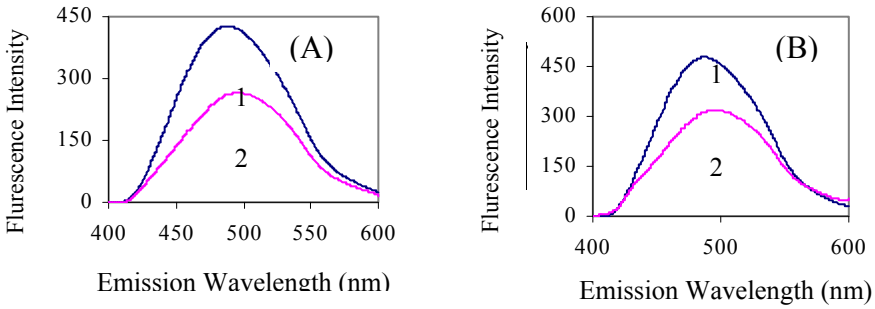

Figure 7: Fluorescence spectra for avidin-ANS (A) in buffer solution; and (B) in TMOS monolith. 1) before and 2) after binding with biotin.

3.2.6 Stability of entrapped protein

To examine the unfolding behavior of avidin, both free and entrapped proteins were subjected to thermal denaturation. Figure 9 depicts the plots of relative fluorescence intensity at different temperatures for avidin and its complex with biotin in solution and in sol-gel derived matrices. The intensity changes observed in the unfolding curves are due to thermally induced effects on the quantum yield of the tryptophan residues and are normally observed during thermal denaturation of proteins. It is shown in Figure 9 that avidin in solution denatures at a temperature of about 85°C. Avidin is more stable upon binding with biotin. Green[7] reported that the denaturation of avidin in solution in the absence and presence of biotin took place at 85°C and 132°C, respectively. To avoid cracking of the silica matrices, thermal stability experiments were done at a temperature of not more than 100°C. As Figure 9 shows, the unfolding temperature of the protein increased significantly upon entrapment. This suggests that the protein is conformationally restricted upon entrapment and this may lead to incomplete unfolding of the entrapped protein. Separated by silica matrices, the protein molecules cannot aggregate and precipitate. Both of these effects may be considered stabilization with respect to the state of the protein in solution.

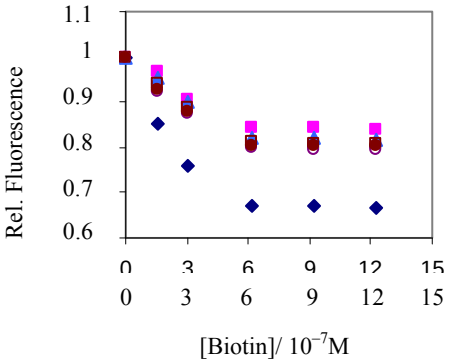

Figure 8: Fluorescence response of ANS as a function of biotin concentration. (■) TMOS; (▲) TMOS-MTMOS 5 mol %; (□) TMOS-MTMOS 10 mol %; (○) TMOS-PDMS 2.5 wt. %; (●) TMOS-PDMS 5 wt. % and; (◆) solution. Concentration of ANS = 6.18 x 10^{-7} M, equilibration time = 10 h.

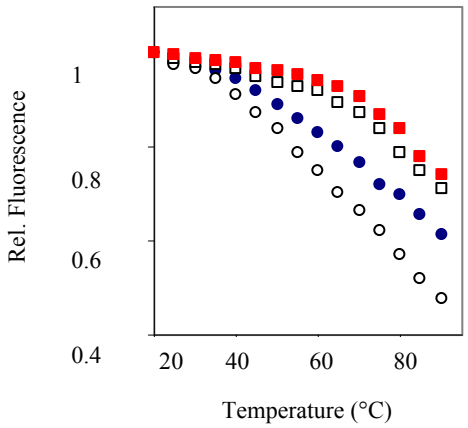

Figure 9: Changes in relative fluorescence intensity for proteins as a function of temperature. (○) Avidin in solution; (●) avidin-biotin in solution; (□) avidin in monolith; (■) avidin-biotin in monolith.

4. CONCLUSION

Avidin was entrapped into a range of silica derived sol-gel matrices with retention of structure and function. Two fluorescence probes of avidin structure and function were evaluated. These probes were used to compare the protein environment in a range of organically modified silicate. Addition of specific organically modified silanes to sol-gel derived materials resulted in a slight

improvement in stability and greater accessibility of avidin to the external reagents.

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