Controlling the Material Properties and Biological Activity of Lipase within Sol−Gel Derived Bioglasses via Organosilane and Polymer Doping

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The development of optical biosensors based on sol−gel entrapped proteins requires a detailed understanding of the evolution of the physicochemical properties of the material, their affects on protein function, and how these factors can be tailored by processing conditions. In this study, the polymer additives poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) were dispersed into sol−gel processed materials derived from tetraethyl orthosilicate (TEOS) alone or copolymerized with methyltriethoxysilane (MTES) or dimethyldimethoxysilane (DMDMS), and their effects on the chemical and physical properties of the materials were monitored. In general, the physical properties, including transmittance and resistance to cracking, improved with increasing PEG concentration, but deteriorated with PVA content. The spectroscopic data obtained from entrapped 7-azaindole and 6-propionyl-2-(dimethylamino)naphthalene suggested that the inclusion of polymers and organic moieties into the matrix affected both the homogeneity of the materials and the polarity of the internal environment, with PEG reducing and PVA increasing the internal polarity. In light of these results, preliminary studies were performed on the effects of organic and polymer content on the initial and long-term activity of entrapped lipase. Concomitant with the material data, PVA tended to have a detrimental affect on lipase activity, while PEG provided a concentration-dependent enhancement of the enzyme activity. This study demonstrates for the first time that durable, optically transparent materials with significant lipase activity can be prepared and that optimal materials are produced with TEOS as a precursor and a few weight percent of low molecular weight PEG as an additive, with no need for organosilane precursors.

Introduction

The potential applications of biomolecules entrapped in sol−gel derived materials, particularly in the field of biosensor development, appear to be limitless and consequently this is an expanding area of research. One class of biological molecules that has received considerable attention is proteins, with numerous reports having appeared describing the function,[1] structure,[2] dynamics,[3] accessibility,[2,4] reaction kinetics,[2a,5] initial stability,[6] and long-term stability[7] of entrapped proteins. These studies have established that, in the majority of cases, entrapped biological molecules initially retain at least a portion of their characteristic biochemical functionality, although the variability in function is large, with values between 2%[8] and 100%[9] of solution activity having been reported. It has also been found that many

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biomolecules can remain stable over periods of months.[1] However, encapsulation of some proteins into sol−gel derived glasses has proved less successful. For example, the extreme pH values and high alcohol levels that are encountered during glass formation can destabilize less robust proteins.[10] Alternatively, positively charged amino acid residues located at or near the active site can interact with the negative silicate matrix, as is postulated to occur with lactate and glycolate oxidase.[11] In these cases, encapsulation can result in a substantial or even complete loss of function and can cause significant structural changes in proteins initially and over time.[12] For biosensor development, changes in protein stability with time can lead to changes in sensitivity and consequently drifts in calibration over short time periods.

Despite the apparent success of the sol−gel technique, the fact that some proteins defy attempts to encapsulate them, coupled with the wide variation in reported activity for those that are entrapped, only highlights the complex nature of these biomolecules. It must be appreciated that biological molecules are not 'generic' but form numerous classes of diverse compounds, which not only differ in their function but also in their structure and environment in vivo. With this in mind, it becomes obvious that a single generic method for encapsulation is an ideal that is unlikely to be achieved. Rather, it is imperative that we move toward a more tailored approach to the encapsulation of biological molecules where the composition, higher level structures (i.e., secondary and tertiary conformation of proteins), and modus operandi are all considered.

Advances have already been made in this direction with recent efforts in sol−gel-based protein entrapment being directed toward the use of different silane precursors, such as organically modified silanes,[7e,13] and the co-entrapment of species such as polymers and surfactants along with the protein to modulate material properties and/or protein stability.[11,14] Examples include the entrapment of atrazine chlorohydrolase into methyltrimethoxysilane-based materials,[7e] lipase into polymer-doped materials formed from organically modified silanes (which produced an 8800% enhancement of activity compared to free lipase for esterification reactions but only 40% for hydrolysis reactions involving emulsified oils),[13a,15] lipase and human serum albumin in organically modified silicates (ORMOSILS), with lipase showing up to 50% activity for the hydrolysis of glyceryl tributyrate (GTB),[13b] glucose oxidase and horseradish peroxidase in the presence of a graft copolymer of polyvinylimidazole and polyvinylpyridine,[16] and acetylcholinesterase and butyrylcholinesterase in the presence of poly(ethylene glycol).[17] In each case, the addition of organosilane precursors or polymers resulted in improved function for the entrapped protein. However, in many cases the resulting materials were not suitable for spectroscopic studies (owing to extensive cracking and light scatterring[15]), and often they were not fully characterized in order to better determine the origin of the stabilization.

Although the ability to retain the functionality of the protein upon entrapment is of critical importance, this must not occur at the expense of the materials properties. To date, the majority of studies involving the entrapment of proteins into sol−gel processed glasses have used matrixes derived from either tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS), which form materials that are optically clear, strong, and durable and contain a tunable pore size on the nanometer scale.[1,2] However, the small pore sizes and extensive cross-linking of such materials can lead to problems with capillary stresses which cause the glass to shatter upon immersion into water.[18] This is obviously a problem for biosensor development, which requires the material to be placed in aqueous biological fluids. In addition, such materials have been shown to have extremely long aging times and a large distribution of internal environments,[19] which leads to time-dependent alterations in entrapped protein function.[9b] It is therefore apparent that such problems must be addressed to produce viable biosensors.

The ability to successfully manipulate the properties of sol−gel derived bioglasses, as described above, obviously requires a detailed understanding of the development of the materials. In the present study, we have aimed to understand the effects of organic functionality on the physicochemical properties of sol−gel derived materials and have attempted to correlate this to protein activity by encapsulating the biocatalyst lipase into bioglasses of various composition. Lipase was chosen owing to its applicability to the development of an optical biosensor for triglycerides, which are a key indicator of overall health. The organic functionality has been incorporated into the matrixes in two ways. First, class I materials were prepared by the use of organosilane precursors in the form of the monoalkyl and dialkyl silanes such as tetraethyl orthosilicate (TEOS), methyltriethoxysilane (MTES), and dimethyldimethoxysilane (DMDMS). Second, class II materials were formed by the inclusion of the uncharged polymeric dopants, PEG and PVA. Last, hybrid class I/II materials were formed by using both organosilane precursors and polymer dopants. The material properties that were examined included optical clarity (which is important in the development of optical sensors), pore size, hydra-

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tion stability, and hardness. The internal environment was probed by using the polarity sensitive fluorescent probes 7-azaindole and 6-propionyl-2-(dimethylamino)-naphthalene (prodan), both of which have been used previously for probing the environment of sol−gel derived materials.[13,18,20] In addition, pyranine was used to monitor the retention of PEG dopants in the washed samples by a technique that has previously been applied to the determination of ethanol:water ratios within sol−gel derived materials.[18] Finally, it is shown that the time-dependent changes in the internal environment of the glass can be correlated to the activity of the entrapped lipase, with maximum lipase activity being obtained for samples with the lowest polarity.

Experimental Section

Chemicals. Tetraethyl orthosilicate (TEOS, 99.999+%), dimethyldimethoxysilane (98%), poly(ethylene glycol) (PEG, MW 400, 600), and poly(vinyl alcohol) (PVA, MW 13 000−23 000) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methyltriethoxysilane was purchased from United Chemical Technologies Inc. (Bristol, PA). All silanes and polymers were used without further purification. 6-Propionyl-2-(dimethylamino)naphthalene (Prodan) and pyranine were purchased from Molecular Probes (Eugene, OR). Lipase (from *Candida rugosa*, lyophilized powder, 60 000 units/mg), glyceryl tributyrate (GTB), 7-azaindole (7AI), and polymethacrylate fluorimeter cuvettes were purchased from Sigma Chemicals (St. Louis, MO). All water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. All other chemicals were of analytical grade and were used as received.

Procedures. *Preparation of Polymer-Doped Bioglasses.* MTES and TEOS were mixed in volume ratios of 0, 5, 10, or 20% MTES (0−0.28 mole ratio), while DMDMS and TEOS were mixed in volume ratios of 0, 1, or 5% DMDMS (0−0.09 mole ratio). For each sample, a volume of 4.5 mL of the silane mixture, 1.4 mL of distilled deionized water, and 0.1 mL of 0.1 M hydrochloric acid were mixed to give a H_2O:Si ratio (R value) of 4 and sonicated for approximately 1 h until a clear, single phase solution was formed. The hydrolyzed solution was stored for 7 days at −20 °C before use to ensure complete hydrolysis.[13a]

Solutions of the polymers were prepared containing between 1% and 10% (w/v of buffer) of PEG or PVA in phosphate-buffered saline (PBS; 10 mM dipotassium hydrogen phosphate, 100 mM KCl, pH 7.2). Where appropriate, the polymer solutions also contained a fluorescent probe (2 μM prodan, 30 μM 7-azaindole, or 3 μM pyranine) or a protein (18 000 units/mL of lipase). Silicate blocks and slides were prepared by mixing equivalent volumes of the polymer solution and the desired silane solution in a cuvette (2 mL total volume for blocks, 0.6 mL total volume for slides). For preparation of blocks, the mixture was quickly mixed, sealed with Parafilm and then allowed to stand at room temperature until gelation occurred. For preparation of slides, the cuvette was sealed and placed on its side before gelation occurred to produce a thin slide that had initial dimensions of 35 mm × 10 mm × 1.7 mm. After gelation, a small hole was punched in the top of the Parafilm to allow solvent to slowly escape. The resulting materials, containing 0−10% (w/v) of polymer mixed with 0−20.0% MTES, or 0%−5% DMDMS, were either aged in air at 4 °C for a period of several months (unwashed) or washed with buffer 3 times (2 mL per wash, 10 min incubation time) and then aged in air at 4 °C for a period of several months (washed). The physicochemical properties and biomolecule activity of the samples were periodically examined during aging for both types of samples.

Physical Characterization. The resulting materials were characterized by using methods described previously.[13a,18] Briefly, optical transparency was determined by measuring the sample transmittance at 400 nm, dehydration/rehydration behavior was characterized by monitoring the degree of cracking during slow dehydration and rapid rehydration, and hardness was tested in a simple semiquantitative manner by performing scratch tests based on Mohs' hardness scale. The substances used were fingernail (2.5), copper (3.5), iron (4), and a glass plate (5.5). The number given in parentheses increases as the hardness of the compound increases. The number assigned to the sol−gel derived materials is based on the substance for which both the sol−gel product and the substance scratched each other or neither scratched the other. All physical tests were performed on three different samples to obtain average values.

Fluorescence Measurements. Fluorescence spectra were collected by using instrumentation and procedures which are described in detail elsewhere.[2a] Fluorescence emission spectra were obtained on the day after the monoliths were prepared (referred to as day 1 of aging) and intermittently up to 200 days. Samples containing prodan were excited at 370 nm, those containing 7AI were excited at 290 nm, pyranine-doped samples were excited at 351, and finally samples containing lipase were excited at 295 nm. In all cases, emission spectra were collected in 1-nm increments over the appropriate emission range using 0.3-s integration per point and spectral band-passes of 4 nm in the excitation and emission paths. Appropriate blanks were subtracted from all spectra, which were then instrument corrected for variations in monochromator throughput and photomultiplier tube response.

Protein Activity Measurements. Assays of entrapped lipase were carried out using intact slides (i.e., they were not crushed to powders), unless otherwise stated. This was done in order to better model the response of the protein under conditions that were appropriate for optical sensing applications. Given the eventual goal of developing a triglyceride sensor, an assay was chosen that involved the lipase-catalyzed hydrolysis of an emulsion of glyceryl tributyrate (GTB) to butyric acid in aqueous solution, followed by quantification of the fatty acid produced by NaOH titration. In our assay, the intact lipase-doped slide (∼200 μm thick after aging) was incubated in a solution prepared by mixing 0.5 mL of glyceryl tributyrate (0.000 17 mol) with 0.5 mL of distilled water. The assay was run for 30 min at 25 (± 1.0) °C with continuous stirring. The reaction was then quenched by the addition of 2.0 mL of a 1:1 (v/v) mixture of ethanol and acetone, and the free fatty acid content was determined by titration with 0.05 M NaOH to a phenolphthalein end point. The average activity values of the entrapped lipase (over three to five samples) were determined from the volume of NaOH added, and the average volume of NaOH required to reach an end point for nondoped glass was subtracted. All activity values are reported relative to the activity of a freshly prepared lipase solution.

Results and Discussion

Physical Properties. *Optical Transparency.* The optical clarity of washed and unwashed PEG- or PVA-doped samples was examined after aging for 21, 80, and 200 days. All samples showed only minor shrinkage and had transmittance values greater than 80% after aging for 21 days at 4 °C, indicating that such samples likely still contained significant amounts of entrapped water (see fluorescence data below). After aging for 80 days, all samples had shrunk to a constant size and mass, and substantial variations in transmittance were observed. No significant changes were observed beyond this point in either size or transmittance.

The transmittance values after 80 days of aging for unwashed and washed samples are provided in Figure 1, charts A and B, respectively. For the unwashed

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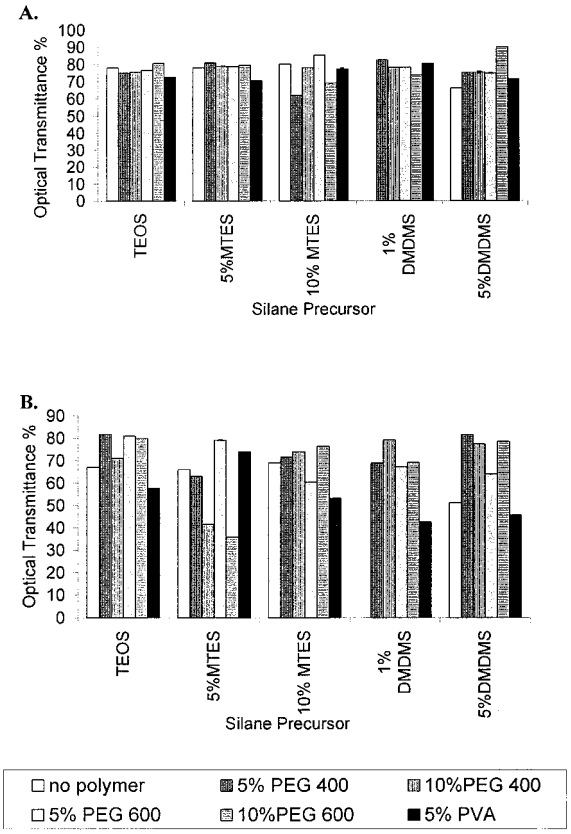

Figure 1. Effects of polymer additives on the optical transmittance of TEOS and organosilane derived sol−gel glasses. (A) Unwashed. (B) Washed.

samples, an optical transmittance in excess of 70% was generally observed in both the presence and absence of polymers. The improvements in optical transmittance observed for the organosilane-doped materials would tend to suggest that PEG may be able to aid in making DMDMS and TEOS more miscible, thereby reducing the degree of phase separation, which has been reported to occur at high organosilane loadings.[13a] For the washed samples it was found that in the absence of polymer additives there was approximately a 15−20% decrease in the optical transmittance compared to unwashed samples. It is known that the washing of sol−gel materials results in larger pore sizes, which would result in increased light scattering and hence a reduction in the optical transparency of the materials.[18] In most cases the presence of PEG was found to ameliorate this problem to some degree, often producing a material of comparable clarity to the unwashed sample.

In contrast, the samples doped with 5% (w/v in the buffer) PVA tended to exhibit reduced transmittance, particularly after washing. Samples containing 10% PVA consistently showed a reduction in optical transmittance of 50% or more (data not shown) and in some cases were opaque. The reduction in transmittance for PVA-doped samples was likely due to the fact that the PVA was a solid in its native state and was very difficult to solubilize. Drying of the materials therefore would have caused the entrapped PVA to concentrate and reprecipitate from solution, making the samples opaque. This was not the case with PEG, as the polymers were of sufficiently low molecular weight to be liquids at room temperature. It should be noted that attempts at entrapping higher molecular weight PEG (1000 Da or greater) resulted in materials that were opaque and chalky and which crumbled when handled. For this reason, only low molecular weight PEG was examined in this study.

Hardness. The hardness of polymer-doped samples was measured after 21, 80, and 200 days of aging (data not shown). At 21 days all of the samples were still very soft and were easily scratched even with soft materials such as talc (classified as 1 on the Mohs' scale). As the samples aged to 80 days, there was an improvement in hardness for all samples tested and discernible differences in the hardness of the various materials. Data for the hardness of the different organosilane precursor derived materials has been reported previously[13a] and indicated that, in the absence of polymer additives, the TEOS derived samples showed the highest hardness value, with a decrease being observed with increasing organic content, i.e., higher organosilane precursor concentration. This decrease in hardness is consistent with the lower degree of cross-linking expected in these materials due to the presence of nonhydrolyzable alkyl groups on the silicon. In most cases, the hardness was found to register at around 3−3.5 on Mohs' scale, which is intermediate between gypsum (or fingernail) and calcite (or copper).

The incorporation of PEG or PVA at low concentrations (i.e., less than 5% w/v buffer) provided no statistically significant affect on the hardness the samples. This is not an entirely unexpected result and confirms that the polymers are unlikely to be intercalated into the siloxane network, but rather they become dispersed within the pores of the materials. Higher levels of PEG also had no measurable effect on hardness, further supporting the suggestion that this polymer did not intercalate into the siloxane network. At the higher PVA concentration of 10% (w/v), it was generally not possible to determine the hardness of the sample, since it crumbled under even a small applied pressure and was easily scratched by soft materials such as talc. Aging the materials to 200 days did not produce any further changes in hardness (even though the internal environment was still evolving), indicating that the durability of the materials did not improve beyond day 80, as might be expected from the constant mass and size of the samples at this point.

Degree of Cracking. The dehydration and rehydration stability of polymer-doped samples was also examined on days 21 and 80 after gelation (see results in the Supporting Information). The results for organosilane derived materials have been reported previously[13a] and indicated that increasing organosilane content resulted in a greater degree of cracking. This can be rationalized by the fact that the TEOS samples were formed from particles of a pure composition and so are relatively durable. The organosilane derived glasses were formed by the oligomerization of two different monomers and hence had a hybrid composition, making them less durable.[13a] In addition, organosilane-doped glasses have a lower degree of cross-linking and hence are not as mechanically stable as TEOS-derived materials.

Conversely, the presence of PEG had no such detrimental affect. For the TEOS-derived samples, no negative effect was observed, irrespective of concentration

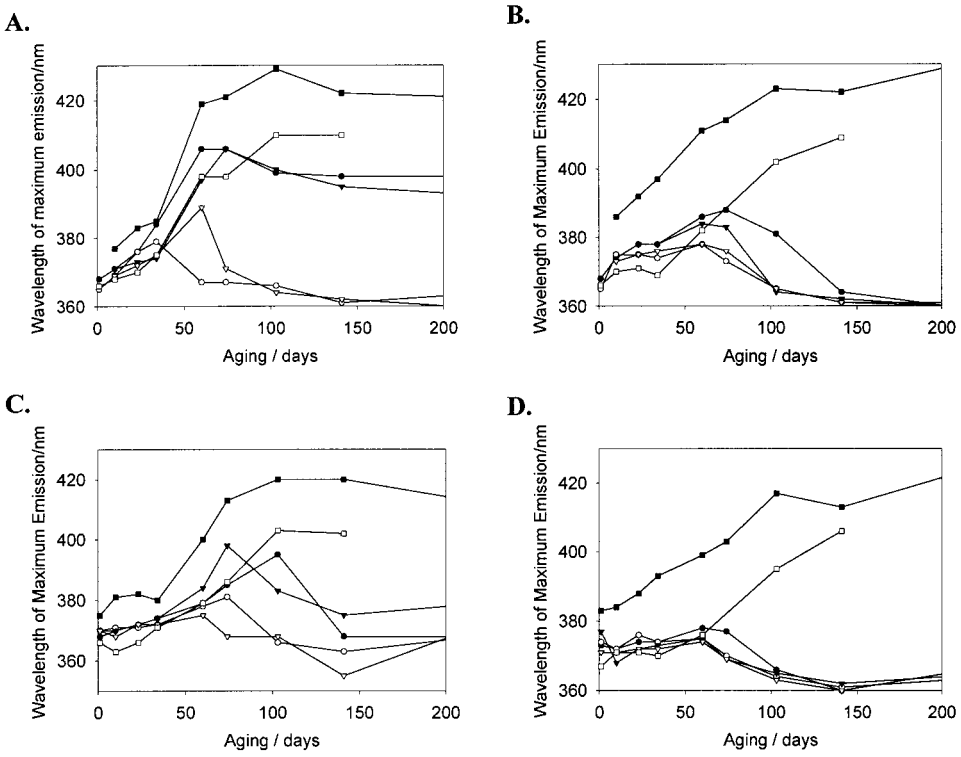

Figure 2. Change in wavelength of emission maximum of 7AI as a function of matrix aging in (A) TEOS-derived unwashed glasses, (B) TEOS-derived washed glasses, (C) 10% MTES-derived unwashed glasses, and (D) 10% MTES-derived washed glasses. (□) No polymer; (●) 5% PEG 400; (○) 10% PEG 400; (▼) 5% PEG 600; (▽) 10% PEG 600; (■) 5% PVA.

or molecular weight of PEG. The presence of PEG in organosilane derived samples resulted in an improvement in material stability, suggesting that the polymer is able to negate the negative affects of organosilanes to yield a material that is comparable to TEOS-derived samples. Similarly, at low concentrations (5% w/v buffer), doping with PVA was also able to ameliorate the effects of organosilane content on stability. In contrast, at higher concentrations of PVA there was again a tendency to reduce the durability of the sol−gel derived materials.

An interesting trend was that samples that were washed immediately after gelation showed substantially better resistance to cracking. This trend is in agreement with previous reports[18] and is consistent with the role of excess water in promoting coarsening, resulting in larger pores and a higher degree of cross-linking within the silicate material.

In general, it was found that the use of organosilane precursors for the formation of sol−gel materials tended to have a somewhat detrimental effect on the material properties. However, this could be negated by the use of a PEG dopant in the matrix, allowing for the preparation of durable materials that were suitable for spectroscopic studies. The fact that PEG demonstrated no deleterious affects on the physical properties of the materials indicated that PEG additives could be used to prepare sol−gel derived materials via a process that was amenable to biomolecule entrapment (i.e., no addition of ethanol to promote hydrolysis and gelation at physiological pH).

Fluorescence Studies of Internal Environment. To better understand the role of polymer dopants on both material properties and biomolecule behavior, the effect of PEG and PVA on the internal polarity of the materials was examined. Some controversy exists concerning the effect that polymers such as PEG have on the internal environment of bioglasses. For example, in some cases, small amounts of PEG have been reported to enhance the local dipolarity and the reorientational mobility of entrapped probes and to also increase the dynamics of entrapped biomolecules.[14a] Conversely, other studies have suggested that PEG leads to decreased polarity and less rotational mobility, depending on the molecular weight of the polymer and the specific probe studied.[14b] Hence, one goal of the present study was to further explore this issue using two polarity sensitive probes, 7-azaindole and prodan.

7AI. Representative charts showing the change in wavelength of maximum emission as a function of material aging for different preparation protocols are provided in Figure 2. In most cases the samples prepared with and without polymer dopants displayed wavelengths of maximum emission in the range 366−370 nm, with the exception of PVA doped materials, which had emission maxima in the range 375 nm (washed samples) to 385 nm (unwashed samples). These initial wavelength values are consistent with those reported previously for undoped materials and reflect an internal polarity between that of ethanol (355 nm) and water (385 nm). As the matrix aged at 4 °C, the probe slowly underwent a shift in its emission maximum, the direction of which depended on the internal composition of the glass and the preparation protocol. For unwashed TEOS-derived samples (Figure 2, panel A), all samples showed a red-shift in their emission maximum at early aging times (i.e., over the first 30 days), consistent with a slow loss of ethanol from the internal environment.[18] TEOS-derived samples continued to shift beyond this time and eventually reached

an emission maximum of ~410 nm after approximately 80 days of aging, remaining constant thereafter.

Inclusion of 5% (w/v) PVA produced an increase in local polarity as compared to undoped TEOS-based samples, as evidenced by the red-shift in the emission maximum to beyond 420 nm over the first 100 days. This result is consistent with the interaction of the probe with the hydroxyl groups of PVA and appears to be due to hydrogen bonding between the PVA and the pyrrole nitrogen of 7AI.[18,21] Addition of PEG resulted in significantly lower internal polarity as compared to undoped TEOS-derived samples. Inclusion of low levels of PEG (5% w/v of PEG) produced materials that initially shifted to values as great as 385 nm at day 60, while samples with 10% PEG remained at approximately 375 nm for the first 60 days. All of these materials underwent substantial blue-shifts beyond this point, eventually reaching 360 nm by day 140. These data indicate that PEG results in an overall decrease in internal polarity, consistent with the report by Bright et al.[14b] This is not unexpected given that the PEG used had only a terminal hydroxyl group present and hence would not be as polar as the PVA dopant.

The long time over which the samples evolved is a direct result of the temperature of aging. Previous studies involving the aging of sol−gel derived materials at room temperature generally showed that the sample environment evolved over a period of 40 days or less.[18] While this is beneficial in terms of producing materials that attain a steady-state composition relatively rapidly, aging at room temperature can be detrimental to proteins, which often require storage at lower temperatures. Clearly, aging of glasses at lower temperatures lengthens the time over which the material evolves, and this must be considered when such materials are used for applications such as biosensing, where such an evolution in the internal environment can lead to a continual drift in calibration.

In the case of samples that were washed after gelation, all samples initially showed a red-shift in the emission maximum, which was consistent with the preferential evaporation of ethanol and the enhanced level of water as compared to unwashed samples. TEOS-derived samples underwent a red-shift attaining a maximum wavelength of approximately 410 nm over a period of 140 days (see panel B).

Inclusion of low levels of PVA (5% w/v) resulted in a significant red-shift in the emission wavelength as compared to TEOS-derived samples, with the emission maximum shifting to 420 nm over the first 60 days and remaining relatively constant beyond that point, in agreement with the data from unwashed samples. Addition of PEG to TEOS-derived materials that were washed after gelation resulted in significant differences in the internal polarity, depending on the level and molecular weight of PEG employed. Samples containing only 5% (w/v) PEG displayed behavior that was similar to that shown by TEOS-derived samples. Monitoring of the PEG content of the washed samples by pyranine fluorescence indicated that a portion of the entrapped PEG was removed, resulting in very low levels of doping. Hence, the significant blue-shift, which was observed for the unwashed samples containing similar levels of PEG, was not seen. Addition of 10% PEG resulted in materials that showed slight increases in polarity at early stages of aging (red-shift from 365 to 380 nm over 30 days for PEG 400 and 60 days for PEG 600), followed by significant decreases in internal polarity over the next 30 days or more (blue-shift from 380 to 360 nm by day 100). These results clearly show that samples containing 10% PEG provide low polarity environments and that washing does not remove all of the PEG dopant from these samples, which was again confirmed by the pyranine fluorescence spectra.

Addition of polymer additives to MTES derived samples results in the creation of a hybrid class I/II material, having both covalently attached and dispersed organic components. Both unwashed and washed materials containing 10% MTES were characterized with and without PEG and PVA dopants present. The fluorescence data for the MTES samples is shown in Figure 2, panels C and D. In general, both the washed and unwashed samples behaved in a manner that was similar to the respective TEOS derived samples. For example, PVA produced large red-shifts in the emission maxima of the entrapped 7AI over a period of 100 days, indicative of increased polarity. On the other hand, PEG-doped samples remained unchanged for 60 days, at which point the internal environment shifted to a lower polarity over a period of further 40 days. It was also determined that washing of MTES-derived samples produced noticeable wavelength shifts, particularly for samples that contained the lower level of PEG 400 or 600. These results agree with those obtained for TEOS-derived samples and support the suggestion that the washing step removes a significant portion of the PEG. As with the TEOS-derived samples, the MTES-derived materials reached a maximum polarity at approximately day 100 after gelation, with samples containing more PEG having a lower overall polarity. The samples then slowly decreased in polarity, reaching a steady-state by day 140. Hence, the inclusion of organosilane precursors does not alter the overall aging time or characteristics.

One interesting finding was that the addition of PEG to MTES-derived samples did not result in any further decreases in polarity compared to undoped MTES samples, indicating that the effects of organosilanes and polymer dopants were not additive. In fact, the emission wavelength maxima for a given dopant level and type were generally within 5 nm of each other, regardless of the nature of the silane used to form the glass. This suggests that the probe may have associated preferentially with the polymer dopant rather than the organosilane.

Further support for this conclusion comes from the fwhm values for the 7AI-doped samples. An unexpected finding was the apparent increase in the values for 5% PEG-doped materials, regardless of whether the samples were washed or not. This result is consistent with low PEG concentrations solvating only a fraction of the probe molecules, while the remainder are surface adsorbed to the matrix silanols, thereby producing the

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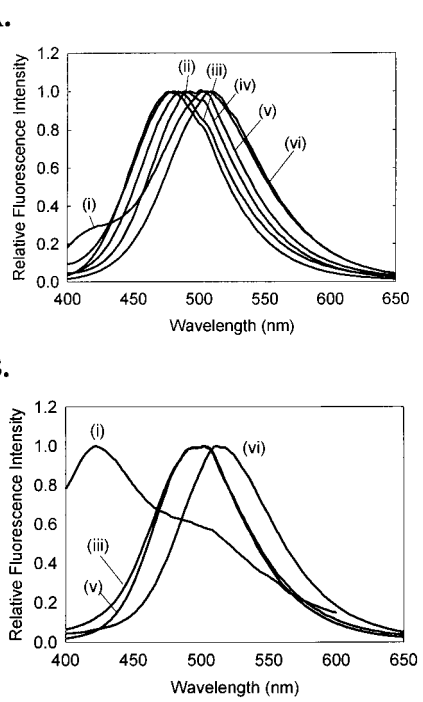

Figure 3. Emission spectra of prodan entrapped in (A) unwashed and (B) washed, polymer-doped materials prepared by using TEOS. (i) No polymer; (ii) 5% PEG 400; (iii) 10% PEG 400; (iv) 5% PEG 600; (v) 10% PEG 600; (vi) 5% PVA.

apparent increase in the distribution of environments. With an increased concentration of polymer, it is likely that the majority, if not all, of the probe molecules are solvated by the PEG and so the material appears more homogeneous with a reduced distribution of environments. The fwhm values for 7AI in the presence of PVA were comparable to those observed in undoped glass, indicating this polymer did not affect the homogeneity of the probe environment.

Prodan. Representative spectra of prodan in polymer-doped TEOS-derived materials are shown in Figure 3 (the emission maxima as a function of aging time are provided in the supplementary data). As was observed with 7AI, both the washed an unwashed materials showed an initial emission maximum reflective of an internal polarity between ethanol (496 nm) and water (531 nm). A general trend for the TEOS- and MTES-doped samples was a slight red-shift in the wavelength of emission maximum over a period of 60−80 days, presumably due to loss of ethanol, followed by a blue-shift owing to the loss of entrapped water. This trend is consistent with the 7AI study and also agrees with previous studies on the aging of TEOS and tetramethyl orthosilicate (TMOS)-derived sol−gel materials. In the presence of PEG or at higher organosilane loadings, this initial red-shift is not observed owing to a higher degree of hydrophobicity imparted by the organic moieties. The blue-shift continues to be evident until an environment of medium polarity is obtained at approximately day 100, indicated by a maximum emission wavelength in the region of 450−460 nm (reflective of prodan in acetone (452 nm) or dimethylformamide (462 nm)[22]).[22]

For PVA-doped materials, prodan showed a red-shift with respect to the undoped or PEG-doped samples, again reflecting the presence of additional hydroxyl functionality in the matrix. However, the magnitude of this affect decreased inversely with organosilane content, indicating that increasing organic functionality counteracts the presence of the hydroxyls on the polymer. Such an effect is expected, since prodan is extremely insoluble in polar media,[23] and would therefore be expected to partition into the more hydrophobic regions of the matrix that result from the presence of MTES.

Interestingly, the prodan doped materials showed very little difference in internal polarity between the washed and unwashed samples. Both types of processing methods produced materials that demonstrated a sequential red-shift followed by a blue-shift, and samples with similar levels of polymer dopants generally attained a comparable degree of hydrophobicity. The only significant difference between washed and unwashed samples was observed for undoped TEOS-derived materials, which showed a peak at 420 nm that was larger for washed samples. This peak has been observed previously for prodan-doped glasses and is attributed to the emission from prodan aggregates.[13a] Such aggregates would be present at a higher concentration in washed samples owing to the loss of ethanol, which reduced the solubility of the prodan.[13a,23] The lack of significant emission intensity at 420 nm for the polymer-doped samples indicates that the polymer is able to solvate the probe. This finding suggests that a less polar environment is present within the polymer-doped materials as compared to the polymer-free samples.

Overall, the results suggest that aging of samples at 4 °C results in extended drying times compared to aging at room temperature, with the samples requiring up to 140 days to fully age. It was also found that PVA tends to enhance the local dipolarity within sol−gel materials, while PEG decreases polarity. The spectroscopic data suggest that 7AI is a superior probe for studies of microenvironment within sol−gel derived materials and is more sensitive to a variety of processing parameters than is prodan.

Effects of Dopants on Protein Behavior. *Free Lipase.* Before examining entrapped lipase in the presence of polymer dopants, the effects of PEG and PVA on the activity and conformation of free lipase was determined. Solution studies indicated that even in the absence of any polymer additives, lipase remained fully functional for at least 35 days when stored at 4 °C and decreased in activity only slightly (<10%) over a period of several months. The addition of small amounts of PVA (5% w/v) to a freshly prepared solution of lipase reduced the enzyme activity to less than 5% of its activity in the absence of the polymer. This result is likely due to the presence of hydroxyl groups on the PVA molecules interacting with the protein and thus causing lipase to adopt a "closed" conformation in which the active site is inaccessible to the solvent.[24] Tryptophan (Trp) emission spectra of lipase in a 5% (w/v) PVA solution indicated that the polymer produced a decrease in the Trp emission intensity but no shift in maximum

(22) Weber G.; Farris, F. J. *Biochemistry* **1979**, *18*, 3075.

(23) Sun, S.; Heitz, M. P.; Perez, S. A.; Colon, L. A.; Bruckenstein, S.; Bright, F. V. *Appl. Spectrosc.* **1997**, *51*, 1316.

(24) Cygler, M.; Schrag J. D. *Biochim, Biophys. Acta* **1991**, *1441*, 205−214.

emission wavelength (data not shown). These results suggest that PVA did not produce the required conformational change in the protein to create the active "open" state.

PEG, on the other hand, was found to have no deleterious effect on enzyme activity at levels of up to 10% for PEG 400 and 600. Addition of PEG to the free lipase in solution resulted in only a minor decrease in Trp emission intensity but also produced a slight red-shift in the emission maximum, indicative of a conformational change that is consistent with the open form of the protein. It is known that less polar environments promote the open conformation of lipase,[24] and this likely allowed for increased interaction between the enzyme and substrate.

Entrapped Lipase. Lipase was encapsulated into sol−gel-processed glasses derived from pure TEOS, 20% MTES, or 5% DMDMS both in the presence and absence of PEG and PVA, with and without a washing step before aging. The enzymatic activity was determined from intact monoliths relative to a fresh solution of lipase at several times after gelation had occurred, and the results were correlated to the internal environment of the glass at the different aging times. It must be noted that the activity values are apparent activities and are determined by a combination of factors, including protein accessibility, protein turnover number, the fraction of active protein, mass transfer of the substrate to the glass, partitioning of substrate into the glass via a solid-phase microextraction (SPME) process, diffusion of substrate and product within the glass (which is known to be slow for intact monoliths),[2a] and partitioning of product out of the glass. Hence, the values do not directly reflect the enzyme activity but rather report on the overall effectiveness of the entrapped catalyst and support.

PVA Doping. Our PVA-doped samples consistently showed little or no hydrolytic activity relative to solution (<5%), regardless of which silane precursors were used to prepare the glass or how long the samples were aged (data not shown). While this result was expected given the effect of PVA on free lipase activity, it was not clear why Reetz and co-workers had been able to achieve 40% activity (relative to solution) when using this additive.[13b] Comparison to the work of Reetz revealed several differences with respect to the present study. First, the molecular weight and degree of hydroxylation of the PVA used in the present study was different than that used by Reetz. Second, their samples were prepared by base-catalyzed hydrolysis, while ours were prepared by acid-catalyzed hydrolysis, likely producing a smaller average pore size in our samples. Third, a different source of lipase was used in the study by Reetz (*Psuedomonis cepacia*) as compared to the present study (*Candida Rugosa*). Finally, their assay involved hydrolysis of olive oil, while ours utilized hydrolysis of GTB. Hence correspondence between the results of Reetz's study and ours should not be expected. The differences in activity for the entrapped lipases clearly highlight the potential problems that can arise as a result of the variability in protein properties and entrapment conditions and suggests that "generic" entrapment protocols may not be possible.

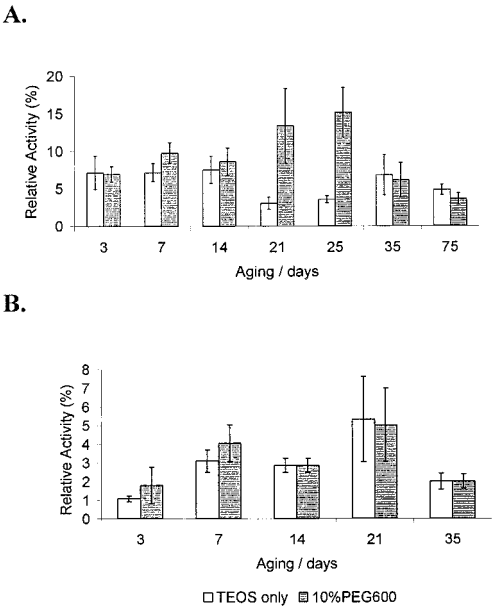

Figure 4. Time course depicting the change in activity of lipase entrapped in TEOS derived monoliths in the presence and absence of 10% PEG 600. (A) Unwashed bioglasses and (B) washed bioglasses.

PEG Doping. Figure 4 shows the effect of aging time on activity for a TEOS-derived samples containing no polymer or 10% PEG 600. Several features deserve special mention. First, the results indicate that the apparent activity generally improves with time up to 25 days, which indicates that the internal environment becomes more suitable for maintaining lipase in an open form or that SPME of the substrate is enhanced as the glass ages. Second, the inclusion of PEG in unwashed samples eventually provides enhanced stability compared to undoped samples, but only after a sufficient aging time (21 days or more). Third, washing of the samples results in decreased activity values, which has been attributed to the removal of PEG upon washing, as confirmed by a fluorescence study of pyranine/PEG-doped slides. Overall, the results clearly indicate that PEG provides a beneficial effect on the apparent activity of lipase.

Increased aging of the entrapped lipase in PEG-doped TEOS samples beyond 25 days caused the apparent activity of the protein to decrease, suggesting that the continued evolution of the matrix eventually resulted in deactivation of the protein, even at 4 °C. Such a situation is consistent with decreased pore sizes, which would be expected to decrease the rate of partitioning of the substrate into the glass. Even so, it should be noted that even after 75 days a significant portion of the activity still remained in unwashed glasses that were either polymer-doped or undoped, which confirms that the entrapment of lipase in optically clear glasses is a suitable technique for the development of an optical biosensor for triglycerides. Long-term studies were not done for the washed samples as very little change in lipase activity was observed over the first 21 days of aging and because it was established that the polymer dopants were removed during washing.

Figure 5 shows the effect of the various organosilane precursors and different levels and molecular weights of PEG on the activity of entrapped lipase after 21 days

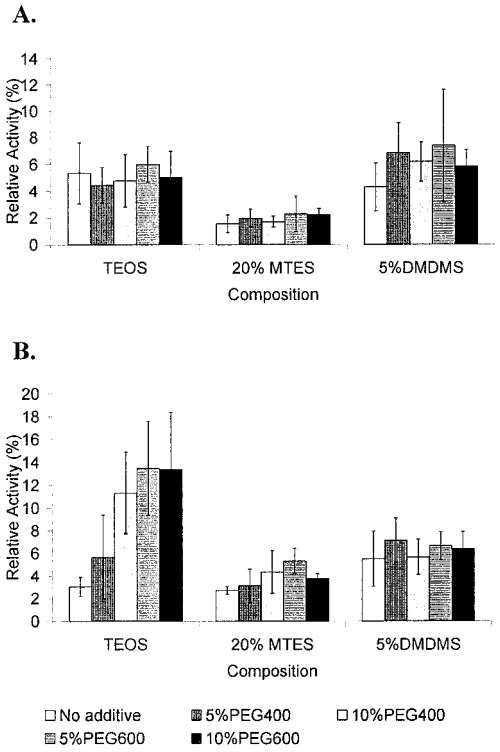

Figure 5. Relative activity on day 21, compared to solution, of lipase entrapped in different sol−gel matrixes. (A) Washed bioglasses and (B) unwashed bioglasses.

of aging. For the washed samples (panel A) the activities of lipase are relatively consistent at around 2−6% relative to solution, with samples derived from TEOS and DMDMS showing slightly better stability than those derived from MTES. Comparing samples of the same organosilane composition shows that PEG had no statistically significant affect on enzymatic activity, consistent with the leaching of PEG during the initial washing stages. The study of internal environment indicated that there was little difference in polarity between the washed samples at day 23, regardless of composition, hence the similarity in lipase activity is not unexpected.

Unwashed samples (panel B) derived from TEOS showed increases in the apparent activity of lipase that were dependent on the level and molecular weight of PEG present. Increasing the level of PEG 400 from 5% to 10% resulted in a doubling of the apparent lipase activity, suggesting that PEG may behave in a concentration-dependent manner. On the other hand, the inclusion of PEG 600 at either 5% or 10% (w/v) was found to provide similar enhancements of activity of approximately 5-fold as compared to the nondoped materials, producing some samples that attained up to 15% of the activity measured in solution. Increased levels of PEG 400 or 600 produced no further enhancements in activity. Thus, 15% relative activity may represent the maximum value attainable for intact glasses. The improvement in activity for the PEG-doped samples as compared to undoped samples is likely due to a combination of possible factors, including: (1) improved protein accessibility or function, (2) enhanced partitioning of the hydrophobic substrate (GTB) into the less polar matrix via a SPME process, and/or (3) larger pore sizes (polymer-doped monoliths did not shrink as much as the undoped samples). The low apparent activity compared to solution suggests that the rate of diffusion of materials in and out of the glass is restricted and may produce activity numbers that do not directly reflect protein behavior.

To determine the effect of reduced diffusion rates through intact monoliths, lipase activity was measured for samples entrapped in PEG-doped TEOS monoliths (10% PEG 600) that were crushed to a grain size of approximately 25−50 μm. These samples provided a lipase activity of 79% (± 8%), relative to solution, or better than 5-fold higher than was obtained for intact slides, confirming that restricted diffusion through monoliths was a major contributor to the apparently low activity values. The increased activity in crushed samples is likely due to an increased surface area for the interaction of lipase and substrate, combined with a reduction in the path length required for diffusion of substrate and products in and out of the glass. These results suggest that the use of thin films (<1 μm thick) may be appropriate for eventual sensor development to help reduce diffusion times and enhance reaction rates.

Unwashed samples prepared using organosilane precursors tended to show little or no improvement compared to washed samples, and the inclusion of PEG did not significantly alter the function of the entrapped protein. The fluorescence study clearly indicated that such samples were relatively hydrophobic and showed that the addition of PEG to unwashed samples did not produce any significant alterations in the final polarity (as compared to undoped samples, see Figure 2). Hence, it is not likely that the reduced activity was the result of changes in the partitioning of the substrate into the matrix. Furthermore, previous studies of organosilane- and PEG-doped materials have suggested that such species have limited effects on pore diameter[14a] (although our PEG-doped samples did show less shrinkage). Hence, substantial changes in protein accessibility are not likely to be the basis of the poor activity (we are currently examining protein accessibility for a series of entrapped proteins and will report our findings in a future manuscript).

A more likely explanation is that the organosilane is able to interact directly with the polymer, causing the polymer to be unable to interact with and stabilize the protein. Such a result, taken together with the finding that removal of PEG by washing removed the activity enhancements, is consistent with a stabilization process wherein the PEG operates directly on the protein via osmotic stabilization, as has been widely reported for proteins in solution.[25] Such compounds reduce the solubility of the peptide backbone relative to water and hence destabilize the unfolded state of a protein. Therefore, in the presence of osmolytes the protein remains folded in order to reduce the exposure of the backbone to the osmolyte solution. It is also possible that the PEG protected lipase from denaturation and subsequent aggregation resulting from the ethanol encountered during entrapment or aids in maintaining the "open" form of the protein, thus helping to stabilizing the active form of the protein.

When compared to the results of other groups, it becomes apparent that entrapment of lipase into PEG-

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doped materials provides activity values that are on par with most other groups. For example, Reetz and co-workers reported hydrolysis rates for emulsified olive oil of 40% relative to solution for crushed powders composed of lipase entrapped in a 4:1 TMOS/poly(dimethylsiloxane) matrix containing approximately 4% (w/w) PVA.[13b] More recently, Gill and co-workers reported hydrolytic activities of up to 185% relative to free lipase (the most active immobilized lipase to date) for hydrolysis of emulsified triolein by 100−300 μm granules of a lipase−silicone biocomposite.[26] On the other hand, Kuncova et al. obtained activity values of only 2−6% relative to solution for lipase entrapped in thin films derived from TEOS, MTES, or DMDMS without PEG additives.[15c] When compared to the activity values obtained by other groups, the activities of lipase in our crushed samples (ca. 80%) are better than those obtained by Reetz and co-workers[13b] and Kunocova and co-workers.[15c] On the other hand, our values are approximately half the value obtained by Gill and co-workers using silicone biocomposites, suggesting that further improvements in lipase activity may be possible by further manipulating the internal environment of the sol−gel derived composite. While entrapment of lipase into intact monoliths produces significant decreases in apparent activity, judicious choice of additives does provide reasonable enzyme activity (particularly when compared to undoped materials) while still maintaining desirable material properties such as optical transparency and good durability. In our opinion the potential advantages of intact transparent glasses, particularly for spectroscopic analyses and sensor applications (i.e., for a triglyceride sensor in the present case), outweighs the activity improvements obtained from using powders. The results do, however, suggest that thinner monoliths or films may be advantageous for eventual sensor development. The application of such glasses for optical biosensing of triglycerides in biological samples is currently under investigation and will be reported in due course.

Conclusions

We have shown that the copolymerization of TEOS with organically modified silane precursors and the entrapment of polymer additives into sol−gel processed materials are useful methods for modifying the internal environment and hence the activity of biomolecules entrapped in sol−gel derived biomaterials. The addition of PEG to TEOS and organosilane-derived samples generally offered enhanced material properties in terms of dehydration/rehydration stability and optical clarity. However, samples doped with PVA tended to be opaque and thus were not useful for optical measurements. The incorporation of PEG also produced materials that had a much narrower distribution of internal environments as compared to nondoped samples, and this allowed good control of the internal polarity of the materials.

The entrapment of proteins into TEOS derived materials in the presence of low molecular weight PEG resulted in a significant improvement in the medium term stability of lipase, as compared to entrapment in the absence of polymer additives. The results also suggested that the PEG acts via the direct stabilization of the protein, rather than by altering the material properties. A serious problem is that PEG can be removed from the material on prolonged soaking in fresh buffer. Extensions of this work will require that methods be developed for retaining the polymer within the network, using either covalent attachment of PEG to the matrix or direct attachment of the polymer to the protein. Both of these methods are currently under investigation in our lab.

Even though PEG leaching is currently a serious problem, this research demonstrates that by using combinations of copolymerization and polymer doping, it is possible to fine-tune the physicochemical properties of sol−gel processed material. Furthermore, this work is the first to demonstrate that substantial activity can be obtained from sol−gel entrapped lipase in the absence of organosilane precursors if hydrophobic polymer additives are incorporated into the glass. The resultant materials show superior physicochemical properties when compared to materials prepared with high levels of organosilanes and hence with appropriate modifications, such as covalent linkage of PEG to the silicate material to avoid leaching, may be amenable to the development of optical sensors for compounds such as triglycerides. Bearing in mind the diverse nature of biological molecules that have the potential to act as qualitative and quantitative biosensors, this less "generic" and more tailored approach to biomolecule entrapment will prove invaluable.

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Supporting Information Available: Two tables of material property data (cracking) and four tables of wavelengths of maximum emission for 7AI and prodan in both washed and unwashed sol−gel derived materials (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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