Development of Macroporous Titania Monoliths by a Biocompatible Method. Part 2: Enzyme Entrapment Studies

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Although sol−gel-derived silica materials have been extensively used as a matrix to immobilize enzymes and other proteins, the poor pH stability and fragility of silica limits its utility in applications that require operation at pH >8. Herein, we report an alternative matrix, sol−gel-derived monolithic titania, for protein entrapment. The material is prepared from biocompatible precursors using aqueous processing conditions involving the formation of a glycerol−titania composite sol followed by titania condensation and can be made macroporous by the addition of poly(ethylene oxide). The clinically relevant protein γ-glutamyl transpeptidase (γ-GT) was entrapped in monolithic titania, and the effects of the titania sol−gel processing parameters on the retention (leaching), catalytic constant (k_{cat}), Michaelis constant (K_M), and long-term stability of entrapped γ-GT were investigated. It was found that the retention of γ-GT within the monolith was strongly related to the glycerol and PEO concentrations in the starting sol. Under optimal conditions, up to 70% of enzyme initially added to the titania sol was retained in the gel even after copious washing. Entrapped γ-GT demonstrated a higher K_M and lower k_{cat} value than in solution, indicating that substrate turnover was limited by partitioning effects and/or diffusion through the titania matrix. The entrapped enzyme demonstrated better long-term stability than in solution, likely because of protection from unfolding within a rigid titania pocket as well as the liberation of the biocompatible reagent glycerol during the sol−gel process. The entrapped enzyme did not show any loss of activity after storage at 4 °C for 3 weeks, but did show a loss in activity beyond this time. Potential applications of protein-doped titania are described.

Introduction

Inorganic silicate matrixes prepared through the sol−gel route have been widely employed for the entrapment of biomolecules.[1−3] In part, this is due to the optical transparency of mesoporous silica, which makes the silica materials amenable to the development of optical sensors.[4] The ready ability to subtly alter the properties of siliceous materials by the addition of polymers, organosilanes, or other excipients and the ability to form these materials in a variety of formats (e.g., films or bulk monoliths) has been important in expanding their use for protein entrapment. Numerous reports have appeared that describe both fundamental aspects of entrapped proteins, such as their conformation,[5−7] dynamics,[8−12] accessibility,[13] reaction kinetics,[5,14] activity,[15,16] and stability,[17,18] and their many applications for catalysis, sensing, and affinity chromatography.[1−3,19−21]

A serious disadvantage of silica is its solubility at pH values above neutrality[22] and its susceptibility to erosion as a consequence of exposure to phosphate buffers. Silica is also relatively brittle when formed as a macroporous material. By contrast, titania-based materials have excellent pH[23] and thermal stability[24] and superior mechanical strength.[25] Titania

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also has particular advantages for biological applications, because it can selectively adsorb phosphorylated biomolecules, including nucleotides,[26] phospholipids,[27] and sugar phosphates.[28] Thus, in addition to normal chromatography, titania can be used to selectively preconcentrate or isolate phosphate compounds and phosphorylated proteins.[29] Furthermore, titania is amphoteric, allowing it to be an anion and cation exchanger at acidic and alkaline pH, respectively, whereas silica can only act as a cation exchanger.[30]

Precipitation of colloidal titania has previously been used to entrap enzymes in a thin film format for biosensor applications.[31−33] However, there is still no report on the development of protein-doped titania monoliths, even though titania possesses many advantages compared to silica, as noted above. As noted in the preceding article,[34] this is due in part to the nature of the common titania precursor, titanium(IV) isopropoxide ($Ti(OiPr)_4$), the hydrolysis/condensation kinetics of which are difficult to control. Another issue is the inherent ability of the isopropyl alcohol byproduct to denature proteins. Thus, the challenge is to develop a protein-friendly sol−gel route to make titania-based monoliths and to further develop a method that has the potential to provide well-defined pore structures, which could be important in future applications in affinity chromatography.

A number of routes have been reported for the formation of monolithic titania,[35−37] most involving the use of chelating ligands to attenuate the reactivity of the titanium precursor by stabilizing a high coordination state of titanium.[38−48] However, these methods generally utilized large amounts of alcohol to dilute the concentration of titanium precursors, making this sol−gel route unsuitable for protein entrapment. As noted in the preceding paper,[34] glycerol-doped titania sols dispersed in aqueous solvents can be used as a precursor to monolithic titania. Such materials were amorphous and could be made with mesoporous and bimodal or trimodal meso/macroporous morphologies by using the polymer additive poly(ethylene oxide) (PEO). Given the low-alcohol processing conditions and presence of the protein-friendly compound glycerol, we reasoned that such materials should be suitable for protein entrapment.

As a first step in evaluating protein-doped titania materials, we have investigated how to control the titania properties to maximize the activity of the model protein γ-glutamyl transpeptidase (γ-GT). This protein was studied because of its importance in many physiological disorders, including Parkinson's disease and perturbation of apoptosis,[49] and because it has been previously studied in both mesoporous TEOS and DGS derived materials[16] and in macroporous diglycerylsilane (DGS) materials,[50] allowing for a direct comparison of enzyme performance in silica and titania materials. A primary goal of this study was to ascertain if monolithic titania gels could be an effective matrix for retaining enzymes with minimal leaching and to determine how entrapment in macroporous titania might alter the catalytic performance and long-term stability of the protein relative to solution. This study lays the groundwork for the development of protein-doped titania-based monolithic columns for applications in biosensing and bioaffinity chromatography.

Experimental Section

Chemicals: The enzyme γ-glutamyl transpeptidase (E.C. 2.3.2.2, lyophilized powder from bovine kidney) was obtained from Sigma (Oakville, ON). Titanium(IV) isopropoxide, L-glutamic acid γ-(*p*-nitroanilide) (GPN), and glycylglycine were obtained from Sigma (Oakville, ON). Anhydrous glycerol was purchased from Fluka (Switzerland). Poly(ethylene) glycol (PEO, MW 10 kDa) was obtained from Aldrich (St. Louis, MO). Cy5-maleimide monoreactive dye was purchased from Amersham Biosciences UK limited (Buckinghamshire, England). All water was distilled and deionized using a Milli-Q synthesis A10 water purification system. All other reagents were of analytical grade and used as received.

Procedures. *Entrapment of γ-GT in Titania Monoliths:* Solutions of γ-GT (1 mg mL^{-1}) were prepared in HEPES·NaOH buffer (pH 7.0, 25 mM). Entrapment of the enzyme in monolithic titania started by adding 1 mmol of titanium isopropoxide (0.30 mL) to anhydrous glycerol at a specified molar ratio (titanium:glycerol = 1:8 to 1:16) and mixing at room temperature for 2 h, followed by adding a specified amount of water to initiate the hydrolysis. To this hydrolyzed sol, aqueous PEO (10 KDa, 6.25 g of PEO/g of H_2O) and γ-GT (1 mg mL^{-1}) in pH 7.0, 25 mM HEPES buffer, was added successively with gentle mixing. The final concentration of γ-GT in the sol was 20 μg/g of gel, whereas the final PEO concentration varied from 0.5 to 3.25 wt %. The mixture containing the enzyme and PEO was subsequently dispensed into microtiter wells (total volume 100 μL, enzyme loading 1.35 μg/well) and allowed to gel (typically 10−50 min, depending on the sol

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composition). The plate was aged in the dark at 4 °C prior to enzyme assays being performed.

Leaching of Entrapped γ-GT: Prior to assaying enzyme activity within monolithic titania, we washed sol−gel entrapped enzyme samples 5 times with buffer solution to remove excess glycerol. The supernatant from each wash was collected to evaluate the immobilization efficiency, which was defined as the ratio of enzyme in the supernatant relative to the amount of enzyme initially added to the sol. Leaching was evaluated by measuring the fluorescence intensity of Cy5-labeled γ-GT in the washing solution, as described by Besanger et al.[50] This method can detect low nanomolar concentrations of labeled enzyme and, under the conditions used in this assay, is able to detect as little as 1% leaching of enzyme. Labeling of γ-GT was accomplished by mixing 1 mL of 1 mg mL^{-1} γ-GT with 1 mg of Cy5-maleimide dissolved in 50 μL of dimethylformamide and allowing the reaction to proceed for 30 min at room temperature and then for a further 18 h at 4 °C. Unreacted dye was separated from the labeled enzyme by passing the mixture though a Sephadex G25 column. The Cy5 labeled γ-GT was then entrapped in titania monoliths as described above. The fluorescence emission intensity of Cy5 was monitored using a TECAN Safire microwell plate reader with excitation and emission wavelengths of 649 and 670 nm, respectively. The emission intensity from the supernatant was summed and normalized to the emission intensity obtained from a concentration of enzyme equivalent to the concentration that would be obtained for 100% leaching.

Enzyme Assays: The activity of free and entrapped γ-GT was measured in 96-well plates using a TECAN Safire absorbance/fluorescence platereader operating in absorbance mode. Solution assays of γ-GT activity were performed by mixing 25 μL of the diluted γ-GT solution (with a concentration equivalent to that in the titania gel matrix) with 25 μL of a solution containing 200 mM glycylglycine and varying concentrations of GPN (0.25−3.35 mM) in each well and monitoring the change in absorbance with time at 410 nm. The activity of γ-GT in solution was evaluated from the initial rate of product formation during the first 5 min.

Differences in optical transmittance between different titania samples at 410 nm made it impossible to directly measure enzymatic activity in the monolith in situ. Therefore, the activity of titania-entrapped γ-GT was measured by a stop-time assay of the supernatant. Monoliths with or without γ-GT were aged and extensively washed. HEPES·NaOH buffer solution (100 μL, 25 mM, pH 7.0) containing 200 mM glycylglycine and varying concentrations of GPN (0.25−3.35 mM) were added to the top of the monolith. The reaction proceeded for 45 min, followed by transfer of 50 μL of the reaction mixture from the tops of the monoliths to another microwell plate, where the concentration of product formed was measured immediately at 410 nm. Under these assay conditions, measurable activity can be detected from ∼0.05 μg/g of enzyme, or about 2% of the total enzyme loaded.

Results and Discussion

γ-GT Activity vs PEO, Glycerol, or 2-Propanol Levels. The ability to control the formation of amorphous titania monoliths hinged on the utilization of relatively large concentrations of the biocompatible compound, glycerol, to reduce the reactivity of titanium isopropoxide. It is likely that glycerol undergoes transesterification at the titanium precursor, forming a secondary precursor that is less susceptible to hydrolysis and condensation. Upon the addition of water to initiate hydrolysis, part of the glycerol that initially grafted onto the secondary titanium precursor will

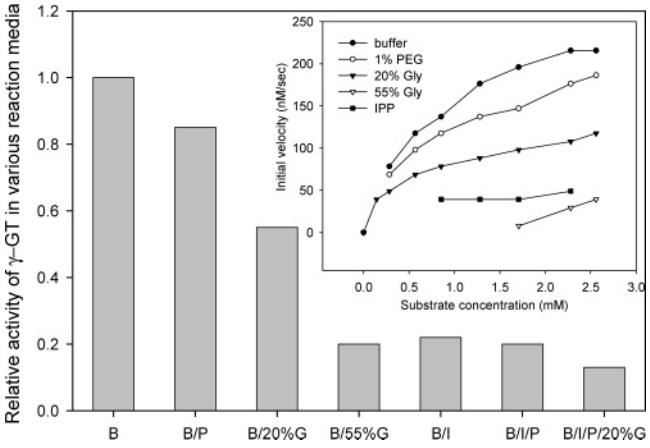

Figure 1. Relative activity and substrate turnover curves for γ-GT in solutions containing PEO, glycerol, and/or 2-propanol. B = buffer, P = 1% PEG, I = 17.6% 2-propanol, G = glycerol.

be replaced with hydroxyl groups, causing glycerol to be released to the solvent phase. Thus, both glycerol and 2-propanol will be present in the sol as hydrolysis processes occur. It was therefore important to establish whether the presence of glycerol could serve to protect proteins present in the sol from denaturation by 2-propanol. To test this possibility, we examined the overall activity and enzyme kinetics of γ-GT in solutions containing PEO, glycerol, and/or 2-propanol at levels similar to those initially present in titania monoliths (Figure 1). PEO decreased the performance of γ-GT in solution only slightly. Visual inspection of the substrate turnover curves (inset to Figure 1) indicated a small decrease in V_{max} and a slight increase in K_M, which is expected given that PEO is a macroviscogen that has been demonstrated to be highly biocompatible.[51] A similar effect was found with chorismate mutase in the presence of PEO 8000.[52] This is because PEO, as a polymeric viscogen, increases the macroviscosity (measured viscosity) of the solution, but has no effect on the diffusion behavior of small molecules; thus, it does not significantly alter k_{cat} or k_{cat}/K_M.[53]

Unlike PEO, the activity of γ-GT was significantly decreased (ca. 50%) in the presence of 20% (w/w) glycerol, although at this glycerol level, Michaelis−Menten (M−M) kinetics were retained ($r^2 = 0.94$ for a Lineweaver−Burke plot). In this case, somewhat larger decreases were observed in V_{max} values and larger increases were observed in K_M values. Glycerol is a commonly used protein stabilizer, and thus it is not likely that glycerol decreases the intrinsic activity of γ-GT. Rather, glycerol is a small-molecule viscogen, and can thus increase both macroviscosity and microviscosity, with the latter decreasing the rates of diffusive processes.[54,55] This hypothesis was further substantiated by measuring γ-GT activity and kinetics in a solution containing 55.3% (w/w) glycerol. In this case, the activity drops to ∼20% of that in buffer and the rate of substrate turnover does not follow M−M kinetics, but rather appears to be diffusion controlled, as the initial rate of substrate

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turnover by γ-GT increases linearly with substrate concentration, where the driving force for diffusion is the substrate concentration gradient.

The effect of 2-propanol, the hydrolysis product of titanium isopropoxide, on the activity and rate of substrate turnover by γ-GT in solution was also investigated. Figure 1 indicates that 2-propanol has a significant deleterious effect on the activity of γ-GT (activity is ~20% of that in solution). Furthermore, γ-GT did not follow Michaelis−Menten kinetics in the presence of 17.6% (v/v) 2-propanol (the highest possible concentration of 2-propanol that would be liberated by the titania sol−gel matrix, based on 100% hydrolysis of the titanium precursor). 2-Propanol is a somewhat hydrophilic organic solvent, which has the capability of stripping off essential waters from the γ-GT molecule,[56] thus it is likely that this compound resulted in partial denaturation of γ-GT. This result suggests that removal of either glycerol or 2-propanol from the titania monolith is necessary to minimize the viscosity and the detrimental effects of the alcohol, and thus maximize enzyme activity.

Finally, we note that testing of combinations of additives, including PEO+IPA or glycerol + IPA, which was done only at the highest substrate concentration, led to an additive effect, where the activity of PEO + IPA was similar to that of IPA alone, whereas the activity of 20% glycerol + IPA was only 60% of the activity observed for IPA alone. The lack of a protective effect for PEO is likely caused by the limited amount of PEO associated with enzyme, because PEO is present at only 1% (w/v). Similarly, glycerol does not impart any stabilization of the enzyme, but rather decreases activity because of increased viscosity relative to IPA alone. Overall, the data show that glycerol and IPA should be removed prior to assaying the enzyme activity, which is why all activity assays of biocomposites are done after extensive washing to remove these constituents. Note that the data in Figure 1 serve only to predict the potential effects of additives on protein stability during entrapment and aging and should not be directly compared to the activity of the biocomposites after washing.

γ-GT Leaching from Titania Monoliths. Given the need to remove glycerol and 2-propanol, a washing step was performed prior to activity assays of entrapped γ-GT to minimize the effects of these matrix components on enzyme activity. However, as noted in the previous report,[34] titania monoliths prepared with glycerol alone were mesoporous, whereas those containing PEO were typically macroporous; thus, it was important to assess how the washing step might affect the retention of the entrapped enzyme.

The leaching of γ-GT was tested using titania gel monoliths containing various glycerol and water levels (1:16:16; 1:12:16, or 1:8:12 titanium:glycerol:water molar ratios), prepared according to composition **1** in Table 1. Samples contained 1 wt % PEO to produce a material that contained both mesopores (~7 nm diameter) and macropores (ca. 700 nm diameter),[34] because such meso/macroporous materials are representative of the materials typically used for fabrication of monolithic bioaffinity columns.[50] Initial attempts to monitor protein leaching by measurement of the activity of the leached enzyme were unsuccessful, mainly because of large differences in the solvent composition (i.e., levels of glycerol, PEO, and 2-propanol) present in the wash solutions obtained from the different titania monoliths. This had a direct effect on enzyme activity, making it impossible to quantify the amount of leached enzyme using activity. For this reason, the fluorescence of Cy5-labeled γ-GT was measured to evaluate the leaching of γ-GT from titania monoliths.

Table 1. Composition of Starting Sol Used to Produce Sol−Gel-Derived Titania Materials

composition	titanium isopropoxide (mmol)	glycerol (mmol)	water (mmol)	PEO (10 KDa) (wt %)
1	1	8−16	12−16[a]	1
2	1	12	16	0−3.25
3	1	16	16	1
4	1	12	16	2

[a] Materials of composition **1** were prepared with 1:16:16; 1:12:16, or 1:8:12 titanium:glycerol:water molar ratios.

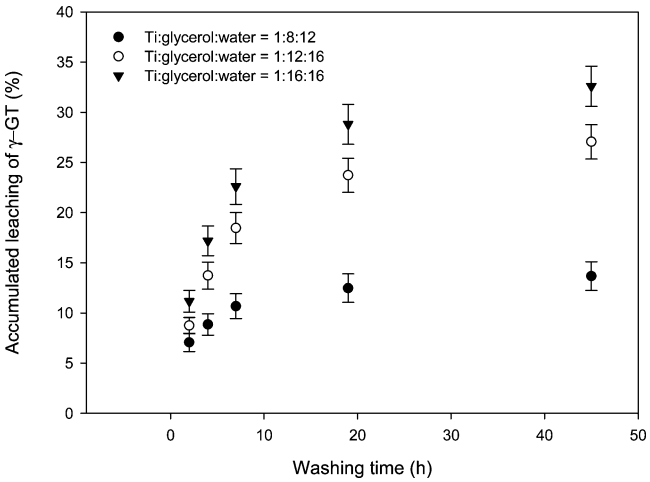

Figure 2. Leaching vs glycerol and water levels for monoliths containing an initial loading of 20 μg of γ-GT per gram of gel. All gels contain 1 wt % PEO. The data were obtained from three measurements on independent samples, and the error bars represent one standard deviation from the mean.

The leaching profile of labeled γ-GT from titania gels containing various levels of glycerol and water is shown in Figure 2. The leaching profile demonstrated significant losses in entrapped enzyme over the first four washes, which then reached a plateau in subsequent washing cycles. The loss of protein in the initial washing cycles is consistent with γ-GT leaching being driven by diffusion. Interestingly, the accumulated leaching of γ-GT increased from 13.7 to 32.6% when glycerol and water concentration increased, although increased water content was the predominant factor controlling leaching. This is consistent with the expected increases in average pore size as a function of increased water content.[57] Higher glycerol concentrations may also promote a more porous structure from which a larger proportion of γ-GT may leach upon washing.

As noted in the previous manuscript,[34] the addition of PEO leads to alterations in both meso- and macroporosity, with total meso- and macropore volume increasing rapidly as PEO

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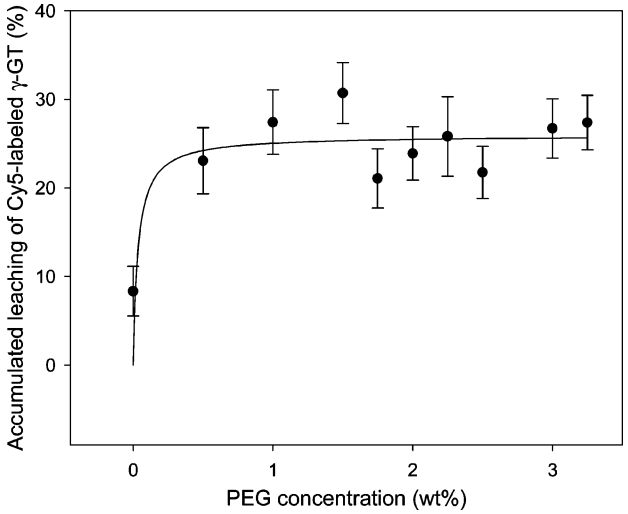

Figure 3. Effect of PEO concentration in gel on the amount of γ-GT leaching from a monolith containing a molar ratio of 1:12:16 Ti:glycerol:water as a result of exhaustive washing. Initial protein loading is 20 μg of γ-GT per gram of gel. The data were obtained from three measurements on independent samples, and the error bars represent one standard deviation from the mean.

concentration is increased from 0 to 2.5 wt % PEO and then decreasing slightly as PEO concentration is further increased to 3.25%. To more carefully assess the role of PEO in controlling leaching, we prepared titania gel monoliths containing various PEO (10 kDa) concentrations according to composition **2** in Table 1, with PEO concentrations ranging from 0 to 3.25 wt % and a total of 20 μg of entrapped protein per gram of gel. The accumulated leaching of γ-GT from titania gels containing various concentrations of PEO is shown in Figure 3. The leaching of γ-GT increases from 8 to 23% upon the addition of as little as 0.5 wt % PEO, consistent with the formation of macropores at low levels of PEO. This hypothesis is substantiated by the observed increase in meso- and macropore volume at such levels of PEO,[34] and by the opaque appearance of the titania monolith in the presence of 0.5 wt % PEO. The overall degree of leaching by diffusion remained essentially constant over the range of 1−3.25 wt % PEO, suggesting the presence of PEO primarily affects macroporosity but not mesoporosity, as confirmed by the BET data provided in the previous report.[34] Proteins entrained within mesopores would be expected to be retained during washing. Importantly, even for the macroporous materials, a total of ~70% of the initially added protein remains within the titania matrix when using an initial enzyme loading of 20 μg/g of gel.

Figure 4 shows the amount of γ-GT retained in the titania gel matrix (formulation **3**, Table 1) after washing vs the amount of protein loaded in the gel matrix, after accounting for leaching. The amount of γ-GT retained is almost linear with the amount of γ-GT initially entrapped over the concentration range studied, although there appears to be some offset at low enzyme loading that suggests higher leaching in such cases. At higher enzyme loading, up to 70% of the protein is retained, which should allow for the use of this material in applications such as frontal affinity chromatography,[19] biosensor development, or as immobilized biocatalysts for bioreactors.

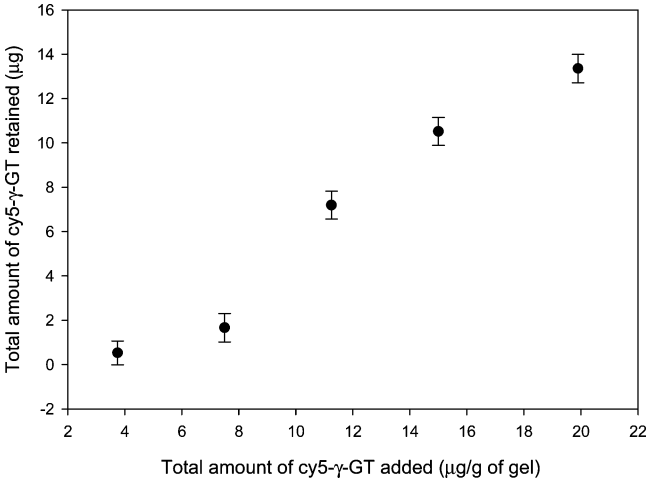

Figure 4. Effect of enzyme loading on the amount of enzyme retained in a titania gel monolith containing a molar ratio of 1:16:16 Ti:glycerol:water as a result of exhaustive washing. All gels contain 1 wt % PEO. The data were obtained from three measurements on independent samples, and the error bars represent one standard deviation from the mean.

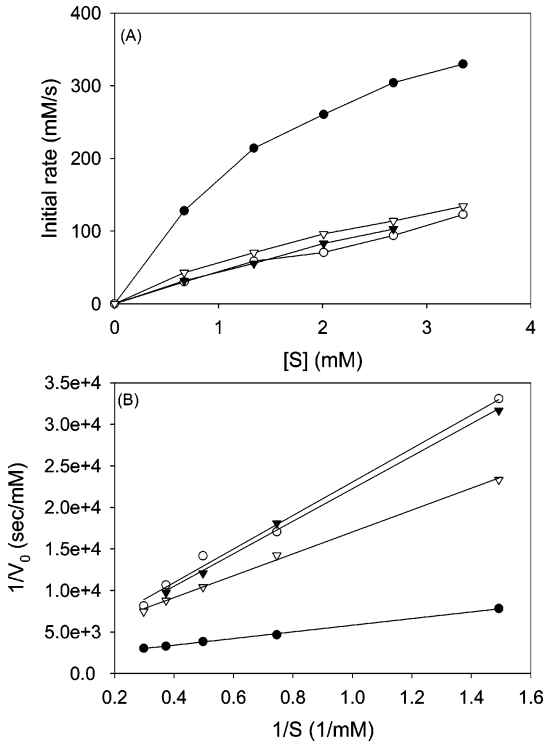

Figure 5. (A) Substrate turnover plots and (B) Lineweaver−Burke plots for γ-GT in solution and when entrapped within titania biocomposites. (●) solution (r^2 = 0.999); (○) 1:8:12 (r^2 = 0.992); (▼) 1:12:16 (r^2 = 0.997); (▽) 1:16:16 (r^2 = 0.997). r^2 value denotes correlation coefficient for fitting to the Lineweaver−Burke equation.

Catalytic Performance of γ-GT in Titania Gel Monolith. The kinetics of substrate turnover by γ-GT entrapped in titania monoliths derived from various molar ratios of the titanium precursor and glycerol were examined (Figure 5 and Table 2). All materials were formed with 2 wt % PEO to produce a bimodal meso/macroporous morphology that was appropriate for fabrication of columns and were washed prior to kinetic assays to remove entrained glycerol and 2-propanol. In all cases, entrapped γ-GT demonstrated Michaelis−Menten kinetics (see Lineweaver−Burke plots in Figure 5b), which allowed for the determination of kinetic parameters for the entrapped enzyme. As shown in Table 2,

Table 2. Summary of Enzyme Kinetic Properties of γ-GT in Solution and When Entrapped into Titania Materials Derived from Different Sols[a]

Ti:glycerol: water ratio	K_M (mM)	V_{max} (μM/s)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
free enzyme	2.23	0.445	3.92	1.76×10^3
1:8:12	7.04	0.349	2.84	0.403×10^3
1:12:16	7.39	0.377	3.64	0.492×10^3
1:16:16	3.41	0.259	2.71	0.797×10^3

[a] All materials contain 2 wt % PEO and an initial loading of 20 μg of γ-GT per gram of gel.

the entrapment of γ-GT led to a higher apparent K_M than free γ-GT. A similar situation was observed when γ-GT was entrapped in a glycerol-derived silica material.[16] K_M is a parameter reflecting the affinity of the enzyme for its substrate, where higher K_M values indicate a lower affinity between the substrate and enzyme. In most cases, K_M values of entrapped enzymes increase compared to their solution values, indicating weaker binding of substrates to the enzymes. The entrapment matrix can also impose a barrier for mass transfer of substrate into the matrix, reducing the on-rate of the substrate, k_1, in cases where the reaction is diffusion controlled and thereby leading to an increase in the K_M value ($K_M = (k_2 - k_{-1})/k_1$), where k_{-1} is the off-rate of the substrate from the substrate:enzyme complex and k_2 is the rate of conversion of bound substrate to product.

Table 2 also gives the apparent k_{cat} value of γ-GT entrapped in the different titania monoliths. Entrapped γ-GT demonstrated up to 90% of its solution k_{cat} value, indicating that the activity of γ-GT is largely retained in the titania gel. Note that the k_{cat} value was obtained by taking into account the amount of γ-GT that leached upon washing while assuming that the entrapped enzyme is active and fully accessible. This assumption will likely result in an overestimation of the enzyme concentration and hence an underestimation of k_{cat} ($k_{cat} = V_{max}/[E]$) where V_{max} is the maximum rate of substrate conversion under conditions of substrate saturation at a given enzyme concentration [E]. Also note that the observed kinetic parameters were dependent on monolith size, with k_{cat} decreasing and K_M increasing as monolith size increased (data not shown). This is expected because diffusion of substrate to/within the matrix plays a role in the apparent kinetics of the entrapped enzyme. The role of mass transfer and substrate partitioning on the kinetics of entrapped enzymes has been discussed in detail in an earlier paper by our group.[16]

A key point to note is that it is generally difficult to obtain the true k_{cat} of an entrapped enzyme because it is not typically possible to quantitate occluded protein.[1] Furthermore, the enzyme may be either active, partially active, active but with altered kinetic behavior, or totally denatured upon entrapment.[4] Thus, both k_{cat} and K_M must be treated as apparent values. Even so, the (k_{cat}/K_M) for entrapped γ-GT, which reflects the overall catalytic efficiency of the enzyme, was 23−45% of the solution value, suggesting that the entrapped protein retained up to half of its catalytic efficiency upon entrapment. This is significantly better than the catalytic efficiency of γ-GT in silica materials (15% of the solution value[16]) and far superior to the activity of many other proteins in silica materials, which often show up to 4000-fold lower

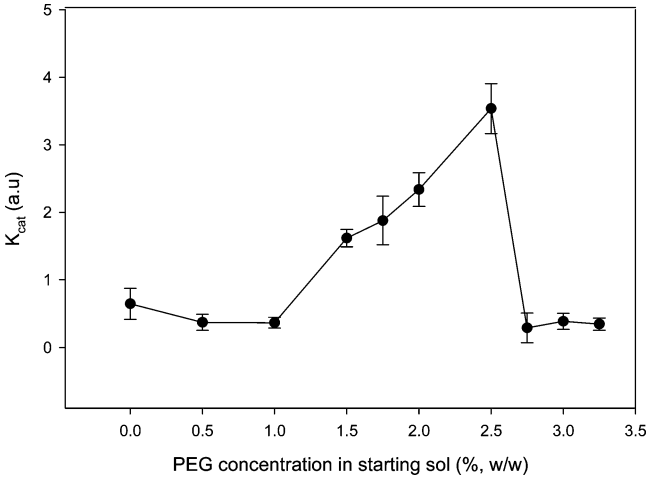

Figure 6. γ-GT activity in titania gels as a function of PEO concentration. Gels contain a molar ratio of 1:12:16 Ti:glycerol:water and have an initial loading of 20 μg of γ-GT per gram of gel. Error bars denote one standard deviation for samples tested in triplicate.

catalytic efficiency relative to their solution values.[1] Although it is not possible to conclude that such improvements in activity are applicable to other enzymes, it is encouraging to find that entrapment of the model enzyme γ-GT within titania did not lead to a significant reduction in activity relative to solution.

Knowing that varying the PEO concentration would alter the porosity of gel matrix, it was of interest to examine how the gel properties related to the kinetic properties of entrapped γ-GT (Figure 6). At low PEO concentrations (<1 wt %), PEO has no statistically significant effect on the apparent k_{cat} of entrapped γ-GT (after accounting for leaching). However, the k_{cat} increased by a factor of 2.5−7 when the PEO concentration in the titania gel monolith increased from 1 to 2.5 wt %, whereas further increases in PEO concentration lead to a dramatic decrease in the apparent k_{cat}. As noted in the previous paper,[34] increasing the PEO concentration initially resulted in the development of large mesopores and significant increases in pore volume. However, further increases in PEO concentration beyond about 2.5 wt % lead to a decrease of both meso- and macropore volume and the disappearance of large mesopores. Thus, it is likely that the increase in the apparent k_{cat} value with increasing PEO concentration over the range 1−2.5 wt % is due to facilitated mass transfer of substrate into the mesoporous gel matrix. Indeed, at 2.5% PEO, γ-GT exhibited a k_{cat} in titania gel (3.54 s^{-1}) that was comparable to that in solution (3.92 s^{-1}), demonstrating that entrapment process did not significantly alter the γ-GT kinetic properties and that mass transfer limitations were minimized in gel with micrometer-sized macropores. Above 2.5% PEO, the decrease in mesopore diameter and pore volume likely hinder access of the substrate to the entrapped γ-GT, thus decreasing the k_{cat} value.

The activity of γ-GT entrapped in titania monoliths (composition **4**, Table 1) was measured after storage for the specified time period. For many applications, aging is required to allow the hydrolysis and condensation to proceed to develop a robust matrix with a strong gel structure, which is necessary for eventual applications such as the develop-

ment of monolithic capillary columns.[19] The samples were aged in air in the presence of their original pore solvent (i.e., without first washing the matrix) so as to retain the protein-stabilizing compound glycerol during aging. However, extensive washing was done just prior to performing the activity assay to remove glycerol, PEO, 2-propanol, and extractable γ-GT. The activity of γ-GT entrapped in titania remained constant over a period of three weeks, and then slowly decreased to ~50% of its original activity after 7 weeks under the conditions used for aging. This activity profile vs aging time is similar to what was reported for γ-GT entrapped in diglycerylsilane (DGS); however, it must be noted that the specific activity of γ-GT in titania was ~3-fold higher than in silica at all aging times.[50] Because aging should lead to decreases in pore size, it is not likely that the loss in activity is due to increased leaching at longer aging times. Rather, the loss in activity is related to slow denaturation of the entrapped enzyme as the pore solvent evaporates[16] and indicates that although entrapment does not lead to a loss in initial activity relative to solution, it also does not prevent denaturation during storage. It must be noted that these preliminary results reflect the effects of entrapment for only one enzyme under a single set of storage conditions and thus cannot be generalized to all proteins. However, the ability to retain activity for the entrapped enzyme that is similar to solution does bode well for the use of titania as a support for enzyme immobilization.

Conclusions

A biocompatible sol−gel route for fabrication of enzyme-doped monolithic titania was developed using glycerol as a chelating ligand to control the reactivity of titanium isopropoxide. Importantly, it is not necessary to remove the 2-propanol liberated during the hydrolysis process as the concentration of glycerol apparently overcomes the detrimental effects of this compound on enzyme activity. The gelation behavior and pore morphology of protein-doped titania monolith could be easily controlled by altering the concentrations of glycerol, water, or PEO, resulting in materials with mesoporous or meso/macroporous morphologies. Under ideal conditions it was possible to retain up to 70% of the initially entrapped protein.

Activity assays showed that entrapped γ-GT could retain up to 90% of its solution k_{cat} value, although the K_M value also increases, leading to an overall catalytic efficiency of 23−45% relative to the solution value, which is approximately 2 to 3-fold better that was obtained for the same protein in sol−gel-derived silica materials. Optimal activity was obtained with 2−2.5% PEO (10 kDa), which is likely due to optimal mass transfer in such monoliths. The activity of the entrapped enzyme was retained over a period of at least 7 weeks (50% loss of activity), demonstrating that titania materials have potential as a medium for fabrication of bioanalytical devices such as biosensors and bioaffinity chromatography columns. Further examination of the potential of titania for entrapment of a broader range of proteins and demonstration of such materials for the development of bioassay devices will be reported in future manuscripts.

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