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Screening of Inhibitors Using Enzymes Entrapped in Sol−Gel-Derived Materials

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In recent years, a number of new methods have been reported that make use of immobilized enzymes either on microarrays or in bioaffinity columns for high-throughput screening of compound libraries. A key question that arises in such methods is whether immobilization may alter the intrinsic catalytic and inhibition constants of the enzyme. Herein, we examine how immobilization within sol−gel-derived materials affects the catalytic constant (k_{cat}), Michaelis constant (K_M), and inhibition constant (K_I) of the clinically relevant enzymes Factor Xa, dihydrofolate reductase, cyclooxygenase-2, and γ-glutamyl transpeptidase. These enzymes were encapsulated into sol−gel-derived glasses produced from either tetraethyl orthosilicate (TEOS) or the newly developed silica precursor diglyceryl silane (DGS). It was found that the catalytic efficiency and long-term stability of all enzymes were improved upon entrapment into DGS-derived materials relative to entrapment in TEOS-based glasses, likely owing to the liberation of the biocompatible reagent glycerol from DGS. The K_M values of enzymes entrapped in DGS-derived materials were typically higher than those in solution, whereas upon entrapment, k_{cat} values were generally lowered by a factor of 1.5−7 relative to the value in solution, indicating that substrate turnover was limited by partitioning effects or diffusion through the silica matrix. Nonetheless, the apparent K_I value for the entrapped enzyme was in most cases within error of the value in solution, and even in the worst case, the values differed by no more than a factor of 3. The implications of these findings for high-throughput screening are discussed.

An emerging method for immobilization of proteins is their entrapment into a porous, inorganic silicate matrix that is formed via a low-temperature sol−gel processing method.[1] Numerous reports have appeared describing both fundamental aspects of entrapped proteins, such as their conformation,[2−4] dynamics,[5−7] accessibility,[4,8] reaction kinetics,[2,9] activity,[10−20] and stability,[21−28] and their many applications for catalysis, sensing, and affinity chromatography.[29] However, few reports exist describing the use of sol−gel entrapped enzymes for inhibition studies, or as potential targets for high-throughput drug-screening (HTS). This is a particularly important issue, given the emergence of HTS methods based on immobilized enzymes.[30,31]

To date, most studies of sol−gel-entrapped enzymes have made use of the silane precursors tetramethyl orthosilicate (TMOS) or

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tetraethyl orthosilicate (TEOS).[29] However, the production of methanol or ethanol is often detrimental to entrapped proteins and can lead to significant changes in the properties of the enzyme, including the Michaelis constant (K_M), catalytic constant (k_{cat}), and inhibition constant (K_I).[29] The latter is of particular importance in terms of using entrapped enzymes for drug-screening purposes. Recently, a number of new biocompatible silane precursors and processing methods have been reported that are based on glycerated silanes,[1] sodium silicate starting materials,[32] or aqueous processing methods that involve removal of alcohol byproducts by evaporation before the addition of proteins.[33] Furthermore, our group has reported on the development of the new silane precursor diglyceryl silane,[34] which is capable of maintaining entrapped enzymes in an active state for a significant amount of time. A question that arises is whether entrapment of enzymes using this precursor may result in their catalytic and inhibition constants being similar to those in solution.

In this paper, we demonstrate the successful sol−gel encapsulation of the clinically relevant enzymes Factor Xa, dihydrofolate reductase (DHFR), cyclooxygenase-2 (COX-II), and γ-glutamyl-transpeptidase (γ-GT) into DGS-derived glasses and compare the catalytic and inhibition properties of the entrapped enzymes to those obtained in solution. Factor Xa is a serine protease that plays a central role in the blood coagulation cascade,[35,36] and is being promoted as a potentially efficacious target for antithrombosis drug development.[37] DHFR catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate, which is then used as a cofactor in the biosynthesis of thymidylate, purines, and several amino acids.[38−40] DHFR is an essential enzyme in the cell and is the target for antifolate drugs, which act by inhibiting the enzyme in malignant or parasitic cells.[41] COX-II binds to a variety of nonsteroidal antiinflammatory drugs (NSAIDs), which are commonly used for the treatment of pain and inflammation.[42−45] Recently, two specific COX-II inhibitors have been approved, rofecoxib (Vioxx) and celecoxib (Celebrex), which do not alter the function of COX-I, reducing gastrointestinal irritation. γ-GT is a key membrane-associated enzyme involved in glutathione (GST) homeostatis.[46] γ-GT catalyzes the initial step of the transfer of the γ-glutamyl moiety of γ-glutamyl-derived peptides, such as GST, to an acceptor molecule,[46] initiating a cascade of enzymatic reactions that break the tripeptide glutathione into its constituent amino acids so that they can be transported through the cell membrane. It has been reported that some rapidly growing cancer cells express high levels of γ-GT so that their intracellular cysteine requirements can be accommodated.[46−,49] Therefore, inhibition of γ-GT may provide a route to slow the growth of certain metastatic cells.

Herein, we report on catalytic and inhibition studies of the four enzymes in solution and when entrapped in sol−gel-derived materials derived from DGS or TEOS. Given that entrapment can often alter the kinetic rate constant, Michaelis constant, and inhibition constants of enzymes, a primary goal of this study was to ascertain if entrapment into biocompatible silica would result in the maintenance of the solution behavior of the enzyme. In particular, it was important to determine if partitioning of inhibitor between the solution and silica matrix or mass transport limitations would lead to changes in the apparent K_I values of the entrapped proteins and whether such changes would be problematic in terms of the use of immobilized proteins for drug screening.

EXPERIMENTAL SECTION

Chemicals. The enzymes Factor Xa (from bovine plasma) and γ-glutamyl transpeptidase (lyophilized powder from bovine kidney) were obtained from Sigma (Toronto, ON). Dihydrofolate reductase (from *E. coli*) was provided by Dr. Eric Brown (McMaster University). Cyclooxygenase-II (holoenzyme in a 1:1 complex with hematin) was donated by Merck Frosst Inc. (Montreal, Canada). Tetraethyl orthosilicate (TEOS, 99.999%) and benzamidine were obtained from Aldrich (Oakville, ON). Diglyceryl silane precursors were prepared from TEOS as described below. The chromogenic substrate S-2222 (for Factor Xa activity assays) was obtained from DiaPharma (West Chester, OH). NADPH, L-glutamic acid γ-(*p*-nitroanilide) (GPN), acivicin, glycylglycine, dihydrofolic acid (DHF), trimethoprim, pyrimethamine, folate, aminopterin, dithiothreitol, arachidonic acid (sodium salt), *N*,*N*,*N*,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), and Triton X-100 were obtained from Sigma (Oakville, ON). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Analogues of celecoxib (termed V2-01, V2-05, V2-06, and V2-09) that were used as inhibitors of COX-II were donated by Dr. Michael Organ (York University, Canada). All water was distilled and deionized using a Milli-Q synthesis A10 water purification system. All other reagents were of analytical grade and were used as received.

Procedures. *Preparation of DGS*. TEOS was distilled to remove any residual water, and a neat mixture of the anhydrous TEOS (2.08 g, 10.0 mmol) and glycerol (1.84 g, 20.0 mmol) was heated at 130 °C for 36 h, during which time EtOH was distilled off. Complete removal of EtOH and unreacted starting materials at 140 °C in vacuo gave DGS as a solid compound that was not contaminated with residual ethanol. Structural characterization of DGS by NMR and the properties of DGS-derived silica are reported elsewhere.[34]

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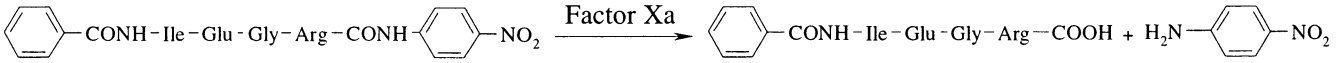
Scheme 1



S-2222 **4-nitroaniline**

Entrapment of Enzymes: Buffered solutions of the enzymes were prepared as follows: Factor Xa was dissolved at a concentration of 0.56 μg mL^{-1} in 50 mM Tris−HCl buffer at pH 8.3 containing 0.5 M NaCl and 5 mM $CaCl_2$; DHFR was dissolved at a concentration of 20 nM in a buffer composed of 100 mM HEPES, pH 8.0, containing 1 mM DTT and 0.01% Triton X-100; COX-II was dissolved at a concentration of 40 μg mL^{-1} in a buffer composed of 20 mM MOPS, pH 7.5, containing 1 mM phenol and 0.01% octyl glucoside. γ-Glutamyl transpeptidase was prepared as a lipid suspension in buffer. Liposomes were first prepared by dispensing 50 mg mL^{-1} chloroform stocks of POPG and POPC in a disposable glass vial and removing the bulk of the organic solvent under a dry nitrogen stream. The remaining solvent was then evaporated under vacuum for 1 h. The dried lipid films were then rehydrated to final lipid concentrations of 2.2 μM with a buffer composed of 200 mM glycylglycine, 20 mM $MgCl_2$, and 50 mM Trizma HCl containing 13 μM of γ-GT to create large multilamellar liposomes with embedded γ-GT. This liposome suspension was then extruded with an Avanti MiniExtruder through 600-nm pores to create a suspension of unilamellar liposomes with a mean diameter of 600 nm.

Entrapment of enzymes into TEOS-derived materials was performed according to a previously reported procedure.[2] Briefly, 4.5 mL of TEOS, 1.4 mL of water, and 0.l mL of 0.1 N HCl were mixed and sonicated for 1 h to produce a homogeneous solution. Equal volumes of the hydrolyzed TEOS solution and buffered solutions of the appropriate enzyme were mixed in a microtiter well (total volume, 50 μL) and allowed to gel (typically 5−20 min). Entrapment of enzymes into DGS-derived materials was accomplished by dissolving 0.20 g of solid DGS in 600 μL of water at pH 7 and sonicating at room temperature (22 ± 2 °C) for 45 min. A 25-μL portion of the DGS solution was then mixed with 25 μL of the appropriate buffered enzyme solution in the well of a microtiter plate. Gelation typically occurred within 10 min. In all cases, the microtiter plate was covered with Parafilm, and a hole was punched through the Parafilm on the top of each well to allow slow drying of the gel to occur. The plate was then stored at 4 °C until tested.

Enzyme Assays: All enzyme assays and inhibition studies were performed in 96-well plates using a TECAN Safire absorbance/fluorescence plate reader operated in absorbance mode. In all cases, sol−gel entrapped enzyme samples were washed three times with buffer solution to remove excess glycerol before the assay commenced. The enzymatic activity of free and entrapped Factor Xa was measured by adding 200 μL of a solution containing varying concentrations of the substrate S-2222. The reaction catalyzed by Factor Xa is shown in Scheme 1. For solution assays, 2 μL of a 5.6 μg mL^{-1} solution of Factor Xa in buffer was also added. The absorbance change at 405 nm was then monitored for the next 20 min (in solution) or 60 min (for entrapped Factor Xa) and was converted to a rate of substrate turnover using a molar extinction coefficient of 10 200 M^{-1} cm^{-1}. For inhibition studies, the free and entrapped enzymes were incubated with varying levels of benzamidine (0−600 μM) for 10 min before the addition of the substrate solution. An incubation time of 10 min was found to be sufficient to achieve reproducible inhibition constants; further incubation did not alter the K_I values.

DHFR uses two substrates in its reaction, and thus, two separate activity assays were performed. To obtain K_M values for NADPH, the enzyme activity was measured using 50 μM DHF and 0−50 μM NADPH in a total volume of 200 μL with a total of 384 fmol of DHFR/well in all assays. To obtain the K_M values for DHF, enzyme activity was measured using 50 μM NADPH and 0−50 μM DHF. To obtain the K_I values of the different inhibitors, the enzyme activity was determined in the presence of different inhibitor concentrations (preincubated with the enzyme for 10 min) using 50 μM NADPH and 0−25 μM DHF. In all cases, the absorbance change of NADPH at 340 nm was monitored for 10 min for solution assays and 30 min for sol−gel-based assays and was converted to a rate of substrate turnover using ϵ_{max} = 12 000 M^{-1} cm^{-1}.

Solution- and sol−gel-based assays of COX-II utilized 1 μg of protein/well (for solution, this corresponded to 6.25 μL of buffer containing 160 μg mL^{-1} enzyme), 95 μL of arachidonic acid (0−100 μM), and 95 μL of TMPD (final concentration of 85 μM). Note: these solutions needed to be prepared fresh daily because arachidonic acid is air-, light-, and moisture-sensitive, and TMPD is photosensitive. The absorbance changes of TMPD were monitored at 590 nm for 5 min for solution assays and 30 min for sol−gel-based assays and converted to substrate turnover rates using ϵ_{max} = 11 000 M^{-1} cm^{-1}. For studies of inhibition, 10 μL of a solution containing 0−50 μM of the appropriate inhibitor was added to the sample and allowed to equilibrate for 10 min before introduction of the arachidonic acid (0−95 μM) and TMPD (85 μM).

Solution assays of γ-GT activity were performed by mixing 175 μL of a solution containing varying concentrations of GPN (0.25−3.0 mM) with 5 μL of the enzyme solution (13 μM) to each well. For assays of sol−gel-entrapped γ-GT, the activity was monitored after addition of 150 μL of GPN to the top of a monolith that contained an amount of enzyme identical to that used in the solution assays. In both cases, the absorbance change at 410 nm was then monitored for the next 35 min, and the change in absorbance with time was used to determine the rate of product formation using ϵ_{max} = 10 200 M^{-1} cm^{-1}. Inhibition assays were performed in a similar manner, except that various concentrations (0−78 μM) of the γ-GT inhibitor acivicin were equilibrated with the enzyme (0−180 min) before adding the substrate.

For all assays, the initial rate of product formation (V_0) was evaluated from at least 10 data points over the first 5 min of reaction, where the slope of the response curve was linear. The error margins for the determination of V_0 values were typically ±10%, as reflected in the errors in the k_{cat} values. For all enzymes studied, the K_M and k_{cat} values were calculated by generating double reciprocal (Lineweaver−Burk) plots relating V_0^{-1} to [substrate concentration]$^{-1}$, and fitting these to a linear model.

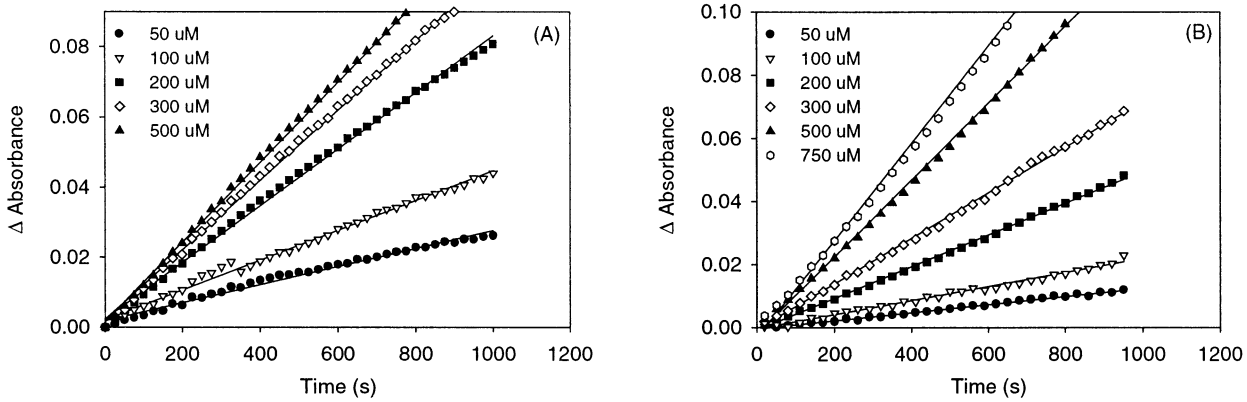


Figure 1. Typical absorbance changes vs time obtained from Factor Xa in solution (panel A) and in DGS-derived glasses (panel B).

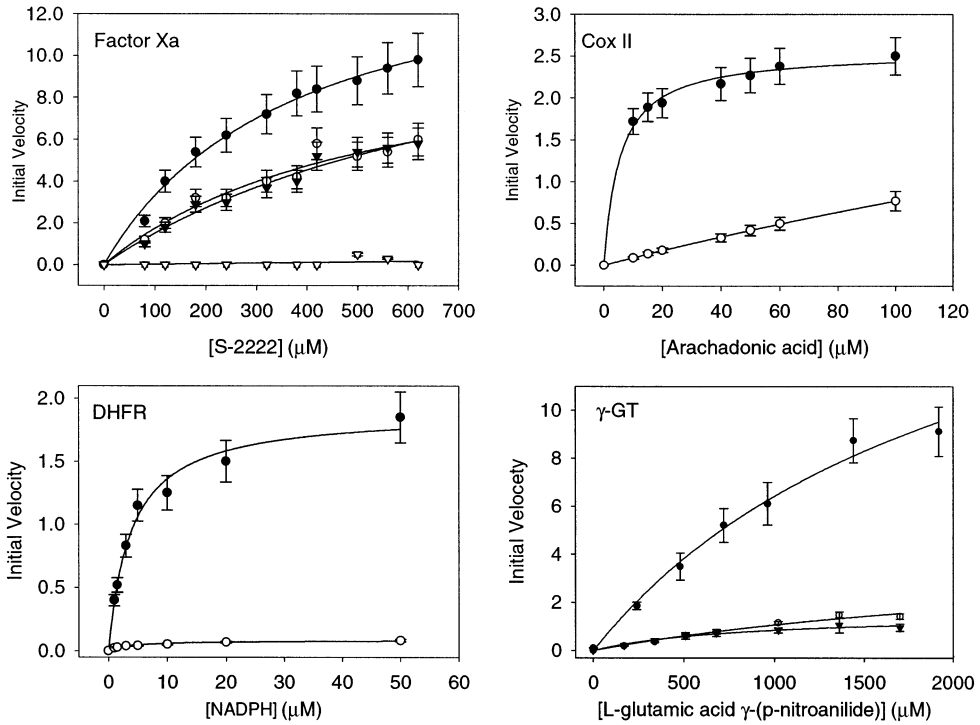


Figure 2. Typical kinetic data obtained from Factor Xa, DHFR, COX-II, and γ-GT in solution (●), DGS-derived glasses (○) and TEOS-derived glasses (▼) after 3 days of aging. The activity of Factor Xa in TEOS-derived materials after 7 days of aging is also shown (▽).

Inhibition constants were calculated by assessing the changes in the K_M values of the enzyme in the presence of varying levels of inhibitor, according to the equation

$$K_I = \frac{[I]}{(K_M'/K_M) - 1} \quad (1)$$

where K_M is the Michaelis constant in the absence of inhibitor, K_M' is the Michaelis constant in the presence of inhibitor, and [I] is the concentration of inhibitor.

RESULTS AND DISCUSSION

Catalytic Efficiency of Free and Entrapped Enzymes. Figure 1 shows plots of absorbance vs time for Factor Xa in solution (panel A) and when entrapped in DGS-derived silica (panel B) in the presence of varying amounts of substrate. In both cases, there is a broad linear range from which initial rate data could be obtained, and it is clear that the slope of the response curve increases with substrate concentration, as expected. Similar absorbance−concentration plots were obtained for all other enzymes tested, both in solution and in DGS-derived materials. In no case did we observe a lag time before the increase in absorbance, indicating that the rate of diffusion of substrate into the silica matrix was relatively fast. Examination of the activity of the solution surrounding the silica matrix provided no absorbance changes (within error), ruling out the possibility of the responses being due to protein that had leached from the matrix.

Figure 2 shows typical kinetic data (V_0 vs [S]) obtained from Factor Xa, DHFR, COX-II, and γ-GT in solution, DGS-derived glasses, and TEOS-derived glasses after 3 days of aging (note: COX-II and DHFR showed no activity in TEOS-based glasses). In all cases, the enzymatic reactions followed Michaelis−Menten kinetics, allowing for the extraction of kinetic data. Tables 1 and 2 provide the k_{cat} and K_M values and the overall catalytic efficiency

Table 1. Kinetic Parameters of Free and Entrapped Enzymes

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Factor Xa in solution	360 ± 40	37 ± 3	10^5
Factor Xa in TEOS	400 ± 40	21 ± 4	3.5×10^4
Factor Xa in DGS	500 ± 50	27 ± 3	4.5×10^4
COX-II in solution	5.0 ± 2.0	9 ± 2	1.8×10^6
COX-II in TEOS	no activity	no activity	no activity
COX-II in DGS	6.0 ± 2.0	4 ± 2	9.5×10^5
γ-GT in solution	1950	3.9×10^4	2.0×10^7
γ-GT in TEOS	1420	5.2×10^3	3.6×10^6
γ-GT in DGS	5690	1.7×10^4	3.0×10^6

Table 2. Kinetic Parameters of Free and Entrapped DHFR

	NADPH			DHF		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
solution	3.0 ± 0.5	6.8	2×10^6	2 ± 1	11.8	5×10^6
TEOS	no activity			no activity		
DGS	20 ± 2	1.5	5×10^5	5 ± 3	1.6	8×10^5

(k_{cat}/K_M) for each of the enzymes in solution and in DGS- and TEOS-derived materials. In all cases, entrapment into a sol−gel-derived glass reduced the k_{cat} value of the enzymes, which can be related to a reduced rate of delivery of substrate to the enzyme. This is expected on the basis of the tortuous path that must be taken to allow diffusion of small molecules through the porous network of the silica,[9] along with possible substrate−silica interactions, which would also slow the movement of molecules through the matrix.[2] Previous studies have noted similar effects for a series of enzymes entrapped in TEOS- or TMOS-derived glasses, where k_{cat} values for the entrapped enzymes, such as alcohol dehydrogenase,[50] bovine carbonic anhydrase II,[51] glucose-6-phosphate dehydrogenase,[27] and oxalate oxidase,[52] were in the range of 3−4000-fold lower than the corresponding values in solution. An important point to note from the kinetic data is that the k_{cat} values of enzymes entrapped in DGS-derived materials were in all cases within a factor of 7 of the value in solution, and in most cases, were within a factor of 2 of the solution value (Factor Xa, COX-II, γ-GT). The only enzyme that appears to be significantly affected immediately after entrapment into DGS is DHFR, and even in this case, the catalytic constants are within an order of magnitude of those in solution. On the other hand, entrapment into TEOS-derived glasses either led to complete protein denaturation (COX-II, DHFR) or produced decreases in k_{cat} values beyond those obtained in DGS-derived materials, providing evidence for improved enzyme performance in DGS-derived materials.

The strength of substrate binding, as reflected by the K_M value, was also altered significantly upon entrapment. In general, the K_M values of entrapped enzymes increased relative to solution, indicative of weaker binding of substrates to the enzyme. This is expected, owing to the reduction in the rate of transport of the substrate to the enzyme, and is consistent with the reduced k_{cat} values. Since K_M is defined as $(k_2 - k_{-1})/k_1$ for the reaction $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$, restrictions in mass transport would not affect off rates (k_{-1} and k_2) but would be expected to lower the value of k_1 (slower on-rate), causing the value of K_M to increase relative to the solution value, as was observed in upon entrapment of most of the enzymes.[53]

Mass transport limitations may also explain why K_M values appeared to be more severely affected upon entrapment into DGS relative to TEOS. This effect is likely due to residual glycerol in the DGS samples that lead to decreases in transport of the substrate to the enzyme. Indeed, examination of enzyme kinetics in solutions containing 25% glycerol (v/v) (to increase viscosity and slow diffusion) confirmed that k_{cat} values were lowered and K_M values increased in the presence of glycerol. For example, γ-GT in 25% glycerol showed a k_{cat} value of 2.86×10^4 s^{-1} (70% of the solution value) and a K_M value of 8.2 mM (~4-fold higher than in solution), leading to a decrease of 6-fold in catalytic efficiency. Similar effects were observed for each of the other enzymes (Factor Xa: $K_M = 700$ μM, $k_{cat} = 24$ s^{-1} corresponding to a 3-fold decrease in efficiency; COX-II: $K_M \sim 2.3$ μM, $k_{cat} = 7$ s^{-1}, ~2-fold decrease in efficiency; DHFR: $K_M = 29$ μM, $k_{cat} = 1.6$ s^{-1} for NADPH, 9-fold decrease in efficiency). These results confirm that the presence of residual glycerol within DGS-derived materials, coupled with the inherently slow diffusion of materials though such matrixes,[2,9] likely contributed to the decrease in catalytic efficiency of the enzymes upon entrapment as a result of mass transport limitations, but at the same time glycerol improved enzyme stability, thereby resulting in an overall improvement in enzyme performance relative to TEOS-based glasses.

Another potential effect that could alter the observed K_M values is partitioning of the substrate between the solution and the silica matrix. Silica is polar and anionic; thus, partitioning would be expected to occur for charged analytes (increased partitioning of cations, partial exclusion of anions). Partitioning will also depend on ionic strength. In this work, we used >0.1 M ionic strength in all assays to minimize partitioning effects (ionic strength was 0.55 (Factor Xa), 0.1 (DHFR), 0.12 (COX II), or ~ 0.3 M (γ-GT)). S-2222 and DPN are both zwitterionic and should show no partitioning; thus, only mass transport should affect the turnover of substrates by Factor Xa and γ-GT. Given that the k_{cat} values for these enzymes are not tremendously altered (less that a factor of 2 in DGS vs solution), mass transport limitations should be minimal, and the K_M value should be relatively similar to solution, as is observed for both enzymes.

Cox II used arachadonic acid (anionic), which should be excluded from the matrix, leading to a somewhat higher apparent K_M value, since more substrate is needed in solution to provide an adequate level of entrapped substrate for the reaction to proceed (k_{cat} and V_{max} are not affected by partitioning). However, partial charge screening likely plays a role in offsetting this effect; thus, the K_M value is not much higher than that obtained in solution. The most dramatic changes in K_M values were for DHFR, which used NADPH (dianionic owing to the phosphate groups) and dihydrofolic acid (anionic) as substrates. Exclusion of anions

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(51) Badjic, J. D.; Kostic, N. M. *Chem. Mater.* **1999**, *11*, 3671.
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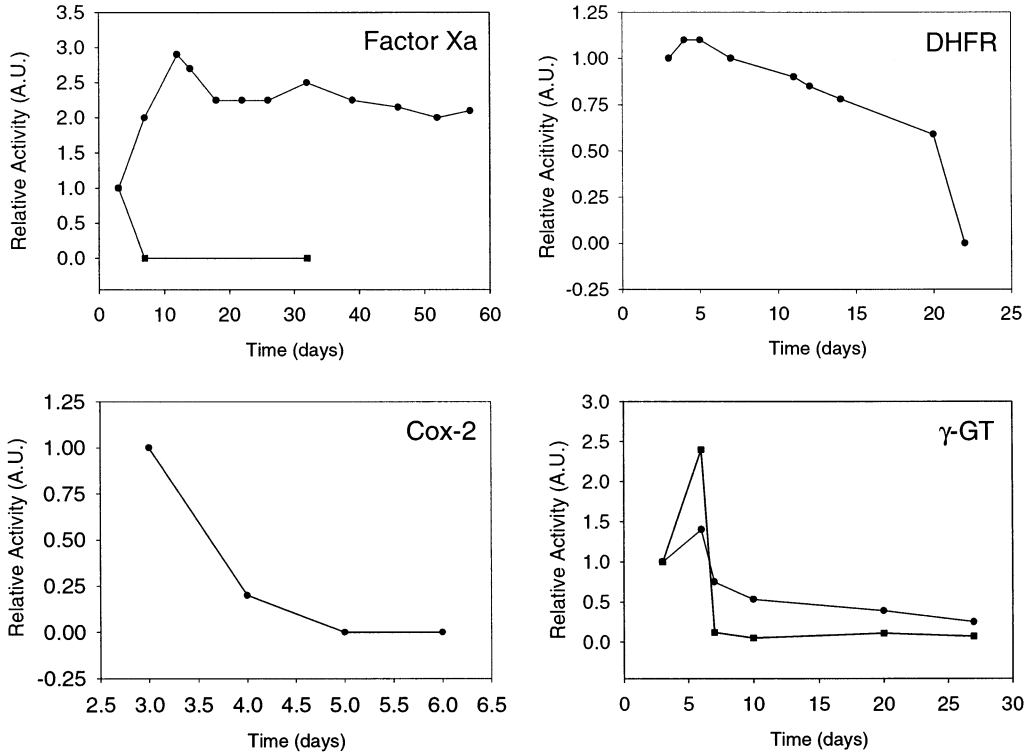

Figure 3. Changes in activity of the enzymes with storage time in DGS- (●) and TEOS-derived (■) materials for Factor Xa, DHFR, COX-II, and γ-GT.

from the matrix leads to a lower than expected level of substrate in the vicinity of the enzyme and an increase in K_M, as was observed for both DHF and NADPH. Thus, partitioning effects likely contribute significantly to changes in K_M values, suggesting that higher ionic strength should be employed when possible to offset this effect.

Long-Term Stability of Entrapped Enzymes. The data presented above provide insight into how enzyme performance is affected at early times after entrapment. Another issue in the use of entrapped enzymes is how their performance is altered over time after entrapment. Figure 3 shows changes in the relative activity of the enzymes with storage time in DGS- and TEOS-derived materials. In both cases, the samples were stored in buffer solution at 4 °C until tested. The activity was measured as the initial rate of production of product, and was normalized to the value obtained during the initial test of enzyme activity 3 days after entrapment. No detectable leaching of any enzyme from either of the sol−gel matrixes was observed during the time course of the aging study; thus, all changes in activity are due to alterations in the catalytic efficiency of the entrapped enzyme.

In general, all enzymes showed increased activity when entrapped in DGS-derived glasses when compared to their activity in TEOS-derived materials. Indeed, neither COX-II nor DHFR showed any activity in TEOS-derived materials, whereas Factor Xa lost all activity after only 1 week of aging. The only enzyme that demonstrated significant activity in TEOS-derived materials was γ-GT, and even then, the activity was in most cases a factor of 2 lower than in DGS-derived materials. This may reflect the fact that γ-GT is inherently membrane-associated and, thus, may be less susceptible to denaturation by hydrophobic species. Hence, the protein is more likely to maintain its active conformation, even in the presence of ethanol.

The activity of enzymes entrapped into DGS-derived materials was in most cases far superior to the activity obtained in TEOS-based materials. For example, both COX-II and DHFR, which were inactive in TEOS-based materials, showed at least some activity in DGS-derived glasses. COX-II was by far the least stable of the enzymes tested, showing complete loss of activity in as little as 5 days, even in DGS-derived materials. However, this enzyme was found to be inherently unstable, even in solution, showing a complete loss of activity in as little as 1 day when stored at 4 °C in solution; thus, the activity in DGS-derived materials is actually significantly improved relative to solution. COX-II contains Fe^{3+}-protoporphyrin IX as a cofactor, which can dissociate from the protein during storage, leading to a mixture of apo- and holoenzymes.[54] To obtain maximal activity, hematin is added to the mixture to minimize the loss of the protoporphyrin group. It is likely that the presence of ethanol (in TEOS-derived materials) either promoted dissociation of the protoporphyrin, or deactivated the hematin, leading to loss of function of the COX-II enzyme.

In the case of DHFR, the enzyme retained up to 60% of its solution activity in DGS-derived materials for a period of 3 weeks, at which point it underwent a catastrophic loss in activity. It is not clear why the activity was lost so suddenly, but it is important to note that the ability to maintain significant activity over a period of 3 weeks is still likely to be sufficient for many applications, such as the development of sol−gel-based microarrays. Finally, in the case of entrapped Factor Xa, the enzyme remained stable for a period of at least two months when entrapped in DGS-derived materials, showing that such materials have the potential to serve

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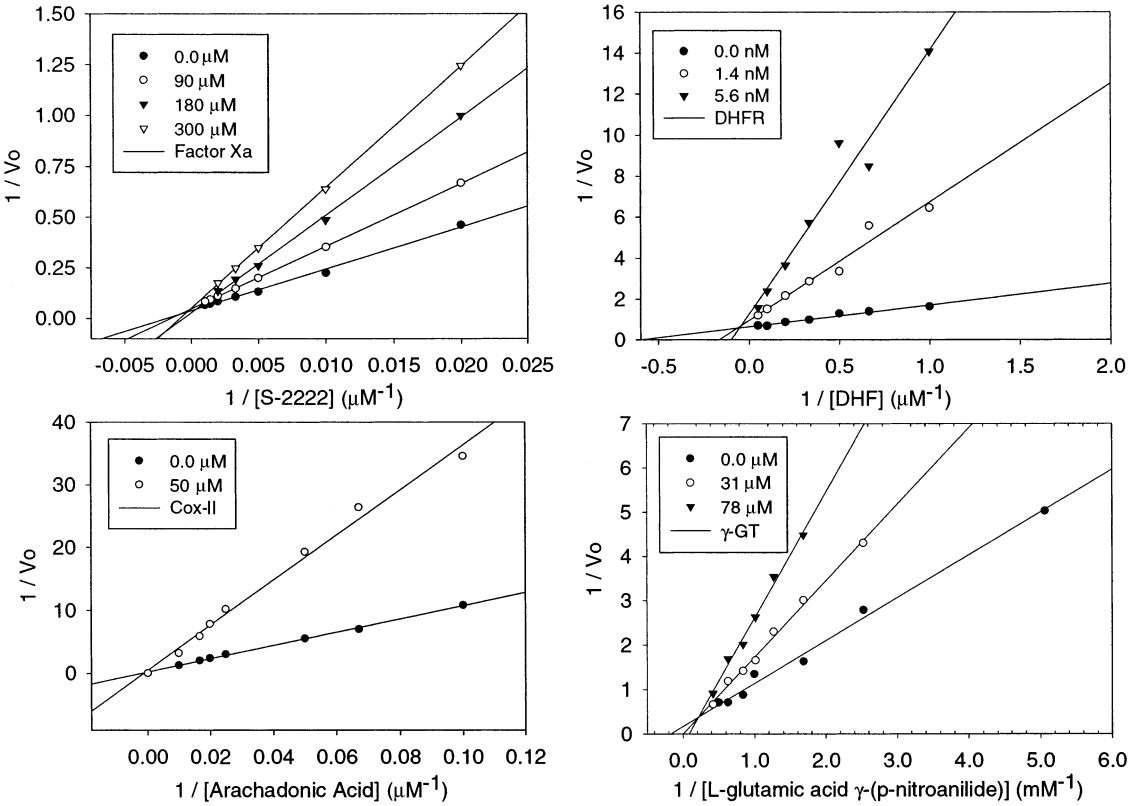

Figure 4. Typical inhibition plots for Factor Xa (benzamidine inhibition), DHFR (trimethoprim inhibition), COX-II (V2-01 inhibition), and γ-GT (acivicin inhibition) in solution DGS-derived glasses after 3 days of aging. The concentration of inhibitor used is shown on the plots.

as useful platforms for the development of immobilized enzyme columns or microarrays.

An unexpected finding was that, with the exception of COX-II, the activity of the entrapped enzyme increased during the first 1−2 weeks of storage, followed by a decrease of the enzyme activity over a period of several days to weeks. The origin of the increased activity at early storage times is not clear. All assays were performed after washing to remove glycerol; thus, the level of glycerol present during the assay is not likely to be a major factor in determining the observed activity. The reasons for the observed increases in activity are likely to be more complex and may include alterations in surface polarity and charge, pore size, protein accessibility, molecular crowding, and entrapped water structure (i.e., protein hydration) as the sample aged.[55,56] We are currently investigating this phenomenon in more detail and will report on our findings in a future manuscript.

Overall, the long-term stability studies showed that all enzymes had improved activity in DGS-derived materials relative to TEOS-derived materials. This is not surprising, since TEOS-derived materials may contain up to 35% ethanol immediately after gelation,[57] which is likely to promote denaturation of entrapped enzymes. On the other hand, DGS-derived materials evolve glycerol, a known stabilizer of proteins, which may cause the protein to adopt a more compact and rigid conformation as a result of preferential hydration and, thus, decrease the specific volume of the protein.[58,59] Glycerol may also act as a humectant, providing greater retention of entrapped water, and it may also coat the walls of the pores within the silica material, reducing direct contact of the protein with the sol−gel matrix. It may, therefore, be concluded that DGS is a more suitable precursor for sol−gel matrixes than TEOS for the encapsulation of the enzymes.

Inhibition of Free and Entrapped Enzymes. Inhibition studies were initiated for all enzymes in solution and DGS-derived glasses. It was not possible to generate useful inhibition data for either COX-II or DHFR within TEOS-derived glasses, owing to the poor activity of these enzymes within such materials. However, inhibition constants were obtained for Factor Xa and γ-GT in TEOS-derived glasses. In the case of Factor Xa, the K_I value for inhibition by benzamidine was 2.1 mM, a factor of 10 higher than in solution. In the case of γ-GT entrapped in TEOS-derived materials, the K_I value for inhibition of the enzyme by acivicin was 450 μM, which is also >10-fold higher than the value obtained in solution. These results indicate that entrapment of enzymes in TEOS-derived materials is likely to be inadequate for the purposes of drug screening. In both cases, it is likely that both changes to the active site of the protein and interactions of the inhibitors with the silica matrix may have led to the need for much higher levels of inhibitor to alter the protein function.

Figure 4 shows representative inhibition plots for each of the four enzymes when entrapped in DGS-derived materials. Table 3 shows the structures of the inhibitors and the inhibition constants generated for each enzyme in solution and in DGS-derived

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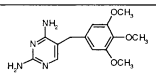
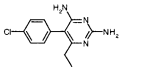
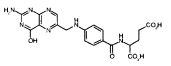
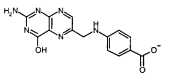
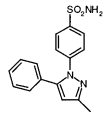
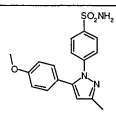
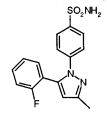
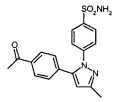
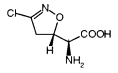
Table 3. K_I Values for Inhibition Studies in Solution and in DGS-Derived Glasses

Inhibitor	Structure	Target enzyme	K_I in solution	K_I in DGS
Benzamidine		Factor Xa	158 ± 5 μM	200 ± 30 μM
Trimethoprim		DHFR	1.5 ± 0.5 nM	3 ± 2 nM
Pyrimethamine		DHFR	37 ± 5 nM	46 ± 5 nM
Folic Acid		DHFR	16 ± 5 μM	13 ± 2 μM
Pteroate		DHFR	none	none
V2-05		COX-2	10 ± 3 μM	27 ± 1 μM
V2-01		COX-2	80 ± 15 μM	87 ± 12 μM
V2- 06		COX-2	106 ± 2 μM	92 ± 5 μM
V2-09		COX-2	47 ± 1 μM	84 ± 35 μM
Acivicin		γ-GT	39 ± 3 μM	40 ± 4 μM

materials. Although many of the enzymes, such as Factor Xa and DHFR, have proteinaceous inhibitors, all inhibitors in this study were chosen to be small molecules, because these are representative of the types of molecules obtained from combinatorial libraries and also because larger protein-based inhibitors would not be able to enter into the silica matrix and interact with the entrapped protein. As shown in Table 3, the majority of the inhibition constants obtained in DGS-based glasses were within error of those obtained in solution. The only exceptions were the COX-II inhibitors V2-05 and V2-09, and even in these cases, the largest difference in inhibition constants was a factor of 3. Overall, the data show that reliable inhibition constants could be obtained over a K_I range spanning low nanomolar to high micromolar concentrations, suggesting that entrapped enzymes should be useful in high-throughput drug screening studies.

Previous studies of enzymes immobilized on charged surfaces have shown that mass transport limitations tend not to alter K_I values, provided that sufficient time is allowed for the inhibitor to reach equilibrium with the enzyme.[53] Stated another way, K_I is related to the ratio K_M'/K_M. Both K_M' and K_M should be altered to a similar degree by mass transport; thus, the *ratio* should remain constant regardless of mass transport effects. Thus, any variations in K_I values upon entrapment of an enzyme are likely to be due to partitioning effects, which can change the equilibrium concentration of the inhibitor in the vicinity of the enzyme. Such effects have previously been demonstrated to occur for charged analytes within sol−gel-derived silica,[9] wherein cations were preconcentrated in the matrix while anions were partially excluded. Since inhibitors compete with substrate molecules for binding to the active site, partitioning of both species must be considered to evaluate the overall affect on inhibition constants. If the inhibitor and substrate are of the same charge or uncharged, no differential partitioning occurs, and thus, no changes in inhibition will be observed relative to solution. If the matrix is anionic (as for the silica matrix), then inhibition will be greatest when the inhibitor is cationic and the substrate is anionic; inhibition will be least effective when using anionic inhibitors and cationic substrates.

However, substrate charge must also be considered. For Factor Xa, the inhibitor benzamidine is cationic, and the substrate is

zwitterionic. Thus, a partitioning of benzamidine into the matrix and a concomitant lowering of the inhibition constant would be expected, as is observed. For DHFR, the inhibitors pyrimethamine ($pK_a = 7.34$) and trimethoprim ($pK_a = 7.13$) would be cationic at pH 8.3 (the pH at which assays were performed), while the substrates are anionic; thus, one might expect preferential partitioning of the inhibitors into the silica, leading to lower inhibition constants relative to solution. However, at the ionic strength employed for these studies, the partitioning effect would be minimized, leading to the reasonable agreement between the inhibition constants in solution and silica. Folic acid (pK_a 4.7) would be anionic and, thus, would undergo a similar degree of partitioning relative to dihydrofolate. Thus, the K_I values for folic acid should not be significantly affected by partitioning, as observed. In the case of γ-GT, both the substrate (DPN) and the inhibitor (acivicin[60]) are zwitterionic; thus, no partitioning would be expected, and indeed, no changes are observed in the inhibition constants upon entrapment in DGS-derived silica. A further point to note in the case of γ-GT is that the enzyme is present on the surface of a phospholipid liposome, and it is possible that the lipid may act to increase the porosity of the matrix through a templating effect and may also aid in passivating the silica matrix through lipid/silica interactions. Together, these effects would likely lead to a more "solutionlike" environment for the enzyme and produce a more reliable inhibition constant.

The COX-II enzyme provided the most challenging system for inhibition studies (owing to the poor stability of this enzyme) and also generated the largest differences in inhibition constants upon entrapment of the enzyme. For Cox II, the V2 compounds are cationic, and the substrate is anionic; thus, we may expect increased inhibition, as is, indeed, observed for V2-05 and V2-09. However, at the ionic strength used for these assays (0.12 M), partial charge screening would be expected to occur, decreasing the partitioning effect. This may explain why V2-01 and V2-06 show inhibition constants for entrapped enzymes that are within error of the values obtained in solution. Overall, the inhibition constants for entrapped COX-II were all within a factor of 3 of the values in solution (as was the case for all other enzymes tested); thus, it appears that the use of enzymes entrapped in DGS-derived silica may be amenable to high-throughput screening studies.

Application of Entrapped Enzymes to HTS. An important finding from this study was that a wide variety of different substrates and inhibitors of varying size and charge were all able to enter the DGS-derived glasses and interact with the active site of the entrapped enzyme. This finding suggests that the enzymes are essentially fully accessible to externally added reagents and that the active sites are not significantly altered so as to greatly affect the binding constants of either substrates or inhibitors. Rather, it appears that variations in either K_M or K_I values between solution and entrapped enzymes are likely to be based on mass transport limitations (K_M) or partitioning of substrates/inhibitors between the solution and glass (affecting both K_M and K_I). Specific interactions of substrates or inhibitors with the silica matrix may also play a role, although at the ionic strength employed, such interactions should be minimized.[9]

In general, the inhibition constants obtained for entrapped proteins were within a factor of 3 of the solution value, and in many cases, the inhibition constants for free and entrapped proteins were within error of each other. It must be noted, however, that such good agreement in K_M and K_I values occurred only in DGS-derived materials; significant deviations in k_{cat}, K_M, and K_I values were obtained when TEOS-derived materials were used to entrap the enzyme, and in many cases, the entrapped protein did not remain active in these materials. This finding shows the advantages of using biocompatible silane precursors for entrapment of proteins.

Overall, the inhibition studies suggest that the encapsulation of enzymes within DGS-derived materials may be useful for the development of a HTS platform that is based on immobilized enzymes. Of the four enzymes tested, all provided useful catalytic and inhibition data, although the COX-II enzyme proved to be a problematic system that was not particularly amenable to entrapment, even in DGS-derived materials. However, this enzyme is inherently unstable, even in solution and, thus, when it is used for any type of HTS studies, it may require special precautions be taken, such as short assay times and minimal exposure to room temperature before use. The small differences in the inhibition constants of the entrapped enzymes are not likely to be particularly significant in high-throughput drug screening trials, where typically, changes of a factor of 10 or more are needed to separate a "hit" from a nonlead. Indeed, all inhibitors that would be considered candidates for further study (i.e., those with submicromolar inhibition constants, such as trimethoprim and pyrimethamine) were correctly identified, indicating that entrapment did not have a deleterious effect in HTS studies. Given that both mass transport and partitioning effects can alter K_M and K_I values, it is clear that materials with greater porosity should be used to maximize diffusion rates and that high ionic strength should be employed to offset partitioning effects. Second generation materials that have surfaces modified to be either neutral or zwitterionic may also prove advantageous for HTS applications, since these materials would eliminate electrostatic partitioning effects.

Other requirements for a robust HTS method include good reproducibility, good stability of the enzymes, ability to distinguish changes in K_M or k_{cat} upon inhibition, and ideally, the potential for multiple use. As shown herein, the majority of enzymes tested (except COX-II) are sufficiently stable to be used over a period of weeks for HTS studies. Furthermore, the kinetic and inhibition data obtained from the entrapped enzymes was highly reproducible, providing values that had precision similar to those obtained in solution. At this stage, all inhibitors tested were competitive inhibitors; thus, we have examined only changes in K_M values upon inhibition. Further studies on the effects of entrapment on noncompetitive, allosteric and mixed inhibitors are needed to better assess the capabilities of the entrapped enzymes for HTS applications. Finally, multiple use capability, while not examined herein, has been demonstrated for many sol−gel- entrapped proteins,[12] and thus, we expect that it should be possible to run multiple assays on the same entrapped enzyme system with good reproducibility.

CONCLUSIONS

The clinically relevant enzymes Factor Xa, DHFR, COX-II, and γ-glutamyl transpeptidase were successfully entrapped in DGS-derived materials. In general, the k_{cat} values for all enzymes decreased by a factor of 2−7, while the K_M values of the enzymes

(60) Allen, L. M.; Maria, V. C.; Meinking, T. *Chem-Biol. Interact.* **1981**, *33*, 361.

increased upon entrapment into DGS-derived materials, leading to an overall lowering of the catalytic efficiency of the entrapped enzyme by 2−10-fold. These changes are predominantly due to slower diffusion and partitioning of the substrate within the microporous material, suggesting that increases in porosity may be one route to increase the catalytic efficiency of entrapped enzymes. Inhibition studies indicated that the interaction of the inhibitor with the entrapped enzyme was remarkably similar to that in solution, with all but one K_I value being within a factor of 2 relative to the value in solution. The minor changes in inhibition constants reflect interactions of the inhibitors with the silica surface that likely alter the concentration of free inhibitor in the sol−gel matrix. However, in no case was the inhibition constant for the entrapped enzyme more than a factor of 3 different from the value obtained in solution, indicating that immobilized enzymes are likely to be adequate for high-throughput drug screening. Efforts to both understand and control the partitioning of small molecules between solution and the matrix are ongoing, and the results of these studies will be presented in due course.

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