Effects of Surface Functionalization and Organo-Tailoring of Synthetic Layer Silicates on the Immobilization of Cytochrome *c*

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The utility of various synthetic hectorites for the preparation of active enzyme complexes by simple adsorption processes was examined. The smectites compared include commercially available Laponite RD, mesostructured hectorites (both with and without organic template), and silane-modified mesostructured hectorite. The complexes were evaluated by the amount of cytochrome *c* that was incorporated and the fraction of enzyme available for redox reaction. The quantity of adsorbed cyt *c* was determined by optical absorption spectroscopy of the supernatant solution, yielding enzyme densities that ranged from 20 to 50 wt %. There was no correlation between weight percent loading and either the surface area or the pore volume of the clay mineral. Rather, other characteristics such as surface compatibility appear to play the major role in this regard. Immobilized enzyme conformation was examined by EPR. Qualitative information regarding protein stability and redox activity was also obtained by chemical reduction experiments. These studies revealed the effects of organo-tailoring of the inorganic surface in terms of steric hindrance of enzyme in active conformation and denaturation within a clay pore microenvironment.

Introduction

The utility of clays has been exploited in a variety of catalytic reactions.[1−4] Coming to the forefront of this field is the immobilization of enzymes for improvements in biocatalysis. Enzyme immobilization proposes to meld the specificity of enzymatic catalysis with the robustness of inorganic clays. While this combination has many proven advantages as compared to free enzyme—enhanced thermal and chemical stability,[5] improved catalytic activity due to spatial concentration of protein and substrate,[6] and retention of protein during separation processes, to name a few—enzyme immobilization has several intrinsic barriers to success. Immobilization within a porous matrix[7−9] or on a solid support[10,11] has been plagued by loss of enzyme activity due to conformational changes of the active site, overall denaturation, steric blockage of the active site, and changes in the microenvironment near the solid surface. Even in cases where activity is high, the enzyme loadings are often extremely low (0.24 wt % in a functionalized SBA-15, for example, where the activity is 84% that of free trypsin).[7] The reported quantity of immobilized protein into porous solids via simple adsorption methods is often quite low. For example, <1 wt % cytochrome *c* was reported encapsulated in mesoporous molecular sieves,[12] in comparison with the large quantities immobilized onto less rigid, organic matrixes such as membranes.[13−15] In this paper, we report the increased immobilization of protein within a mesoporous solid support with retention of significant native biochemical function, resulting in activity comparable to membrane-bound species. Mesostructured synthetic clay minerals provide a matrix stable to extremes of chemical, thermal, and mechanical stresses, are amenable to high protein loading, and can be further tailored for optimal biochemical function via silane modification.

Lamellar silicate clays may be obtained commercially from natural sources or synthesized to provide control over properties such as composition, porosity, and cation exchange capacity (CEC). Under AIPEA nomenclature, only natural materials can be denoted as clays, while a

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synthetic clay is more properly distinguished as a clay mineral.[16] For the purposes of clarity in this particular paper, however, the term clay will be employed for synthetic hectorites with the implication that clay mineral is meant in all cases. Hectorites, which are magnesium silicate smectite (swellable) clays, are especially desirable because of their ease of preparation at high purity (low iron contents, etc.). Smectites contain a 2:1 primary unit consisting of two tetrahedral silicate sheets that sandwich a central metal octahedral sheet, which is separated from another unit via electrostatic interactions that arise from exchangeable cations in hydrated interlayers.[17] Exchangeable cations are bound because of isomorphous substitutions that arise within the inorganic lattice, primarily Li(I) for Mg(II) in the octahedral sheet of hectorite.

Hectorites may be synthesized in the presence of water-soluble organics or polymers using a method developed by Carrado et al.[18−21] which templates porous structures in situ during clay crystallization. This type of organoclay has direct application to biological complexes. The resulting organo-tailored clays (OTC) may be used either with the organic template intact or after removal of the organic template via heating in air. In situ intercalation of different alkylammonium ions or polymers over broad molecular weight and concentration ranges can be achieved. Upon template removal, a purely inorganic mesostructured matrix (mesostructured synthetic clay, MSC) is produced. The individual interlayer spaces collapse but a stable and intact pore system remains from, presumably, the inter- and intratactoid volume. Efforts to create and control the pore size or porosity characteristics of clays in general continue intensively, including MSC systems.[22,23] The use of poly(vinylpyrrolidone) (PVP) molecules or tetraethylammonium (TEA) cations yields materials with different porosity characteristics.

After the desired hectorite has been acquired, it may be further tailored in several ways including variation of the exchangeable cation, incorporation of organic intercalants, or via more complex organo−inorganic nanocomposite formation. This variety of modification may be utilized to provide the optimum interaction between clay surfaces and biological molecules. It has been envisioned that judicious tailoring of clay surfaces may lead to the coupling of clay catalytic processes with subsequent enzymatic reactions.

Cytochrome *c* (cyt *c*) was chosen as a model for encapsulating active enzyme in this study. Cyt *c* is a small globular protein approximately 30 Å in diameter and weighing less than 13 kD. The active site consists of an iron(III) porphyrin heme covalently bound to the protein backbone and surrounded by several lysine residues on the periphery, which are thought to participate in substrate binding. Biologically, this cytochrome plays a central role as an electron carrier in the respiration of mitochondria; thus, the heme may exist in either oxidized [Fe(III)] or reduced form [Fe(II)]. It was chosen for this model system because of its ready availability, its well-known optical (UV−vis) and electron paramagnetic resonance (EPR) spectroscopy, and the versatile and relatively simple chemical and analytical methods used to assess its conformation and activity.[24]

Several precedents for enzyme immobilization by clays have been published, including general reviews,[25−27] montmorillonite clay-based systems,[28−33] electrochemical studies of myoglobin,[34] hemoglobin,[35,36] and cytochrome *c*,[37,38] and Laponite systems with urease,[39] glucose oxidase,[40,41] and cholesterol oxidase.[42] The interactions of enzymes with clays and their applications to bioremediation also have been thoroughly reviewed recently.[43]

The utility of several hectorites for the preparation of catalytically active enzyme complexes by simple adsorption processes was examined. Clays include the commercially available Laponite RD and various mesostructured hectorites, each with varying degrees of surface area, pore volume, and organic modification. The resulting cyt *c*−clay complexes were evaluated by the amount of cyt *c* incorporated and the fraction of redox-active protein present.

Experimental Section

Materials. Laponite RD, a synthetic hectorite from Southern Clay Products, Gonzales, TX, was used as received. Horse heart ferricytochrome *c* and all synthetic reagents were purchased from Sigma-Aldrich and used as received. A general technique for the preparation of organo-tailored hectorite

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smectite clay minerals (OTCs) via in situ hydrothermal crystallization under atmospheric pressure has been published.[20] Briefly, silica sol, magnesium hydroxide sol, and lithium fluoride (1.5:1:0.25 Si:Mg:Li) were combined with tetraethylammonium (TEA) or poly(vinylpyrrolidone) (PVP, average MW = 29000) polymer (10 wt % of total solids). The reagent mixture was diluted in water to yield 2 wt % total solids, reacted under reflux conditions for approximately 2 days, and then collected by centrifugation and washed repetitively with water.[44] Materials are referred to as either TEA− or PVP−hectorite. Generally, the clays derived from TEA are more crystalline with a greater degree of lamellar stacking order and crystallite plate size.[45] The negative layer charge of these hectorites is neutralized by tetraethylammonium cation (TEA^+) for TEA−hectorite and Li^+ for PVP−hectorite. In the latter, PVP resides in the mesopores between clay tactoids as well as in the gallery interlayers. Both of these hectorites are known to contain up to 20 wt % silica from the starting silica sol material.[20,22] This is thought to act as a disordered pillaring agent and to impart the ultimate mesostructure and some mesoporosity to the system.

To investigate the effect of organic moieties in the organo−hectorites, they were calcined to yield purely inorganic mesostructured synthetic clays (MSCs). The hectorites were placed in quartz boats in an oven under nitrogen flow while heating to 550 °C over 6 h. This temperature was maintained and the flowing gas was then switched to air for approximately 24 h. TEA− and PVP−hectorite were used to produce TEA−MSC and PVP−MSC, respectively. The MSCs exhibit similar pore architecture to the parent materials, although they differ in accessible pore volume and the initial exchangeable cation. Upon calcination of TEA−hectorite, the alkylammonium cations decompose and leave behind H^+. Removal of polymer from PVP−hectorite to form PVP−MSC does not alter the exchangeable Li^+.

Hydrothermal synthesis is not amenable for use with a nonpolar polymer.[20] Therefore, to examine the effect of a nonpolar organic surface species on the quantity and activity of immobilized enzyme, the PVP−MSC was additionally modified by a postgrafting silanation process. Following the method of Okutomo,[46] a C_8−MSC was prepared by reaction of PVP−MSC suspended and refluxed in a mixture of chlorodimethyloctylsilane and rigorously dry toluene under nitrogen flow for 5 days. The solid product was collected by centrifugation and washed sequentially with toluene, acetone, and water.

Cyt *c*/clay complexes were prepared by simple mixing of a concentrated solution of cyt *c* (0.5 mg/mL in phosphate buffer, pH 7) with the dry clay at 4 °C overnight. The complexes were collected by centrifugation; the supernatant buffer solutions were retained for analysis by optical absorbance spectroscopy. Cyt *c* adsorption by the clays was observed by the dramatic movement of its characteristic red color from the supernatant solution to the solid. The quantity of cyt *c* within each composite (wt %) was determined by its difference in concentration in the supernatant compared to the starting solution as measured by its optical absorption at 409 nm (ϵ = 106 000 M^{-1} cm^{-1}) and by thermal gravimetric analysis of the dry powders. The complexes were washed with buffer in triplicate, although this had no significant effect on the quantity of cyt *c*.

Characterization. Thermal gravimetric analysis (TGA) measurements were obtained on an EXSTAR6200 from Seiko Haake Instruments. The samples were measured in a 100 mL/min O_2 flow with a temperature ramp of 10 °C/min to 900 °C. Total combustible organic content was calculated by measuring the weight loss over the approximate temperature range of 200−600 °C.[47] Nitrogen sorption isotherms were collected on a Micromeritics ASAP 2010 system at 77 K with samples outgassed at 120 °C under high vacuum for at least 4 h. Surface areas were determined by multiple point BET calculations (C = 100−300) and pore volumes were calculated from the desorption branch of the isotherm.

Solution optical absorbance spectra were collected using a Shimadzu UV-1601 spectrophotometer in the usual manner. Optical absorbance spectra of cyt *c*/clay complexes were obtained by vigorous mixing of the composite within pure buffer immediately before data collection. Absorption peak heights were calculated by subtracting background scattering from the solid composite particles manually using the absorbance at λ = 409 nm. The calculated error was taken as 3Ω from the measurement of three sets of sample preparations. Derivative electron paramagnetic resonance (EPR) spectra of the high-spin Fe(III) within the oxidized heme of the cytochrome were acquired using a Bruker X-band (9 GHz) spectrophotometer using 2 mW power with a 10 cT microwave modulation amplitude at 4 K. Samples were packed within liquid chromatography tubing in the presence of phosphate buffer at pH 7. The liquid chromatography tubing has no background signal in the microwave region scanned. Filled tubes were then inserted into a conventional glass EPR tube and placed into the microwave cavity for analysis.

Results

Characterization of Unmodified Clays. Synthesis of hectorites was confirmed by XRD (patterns not shown). The XRD patterns showed all of the expected diffraction peaks for hectorite (see refs 20 and 22 for details). In accordance with the size of the organic gallery species, the d(001) basal spacings were 1.4 nm for TEA−hectorite and 2.0 nm for PVP−hectorite. Upon calcination of the hectorites to form TEA− and PVP−MSC, the (001) diffraction collapsed to 1.0 nm (the size of a single 2:1 clay lattice sheet) while the remaining clay peaks were unaltered. The silane-grafted product C_8−MSC also showed the typical clay peaks.

Representative nitrogen sorption isotherms are shown in Figure 1. They are of type IV with primarily H2 hysteresis loops, as is typical for smectite clays that contain mesoporous networks of broad structure.[22,48,49] Only C_8−MSC deviates from this somewhat. While still displaying a type IV isotherm, it displays hysteresis that is more reminiscent of either an H3 or an H4 loop. Such loops are indicative of platelike aggregates with slit-shaped pores, although the mesoporosity for this sample is still evident in the broad uptake values between 0.6 and 0.8 P/P_o. Reliable interpretation of these isotherms is limited to quantitative measurement of specific surface area and pore volume,[50] and these data are provided in Table 1. Both parameters are observed to increase after calcination, as expected, due to template removal. The values decrease again after silane modification of the PVP−MSC, which is also expected.

Figure 2 contains representative TGA data. For a typical sample, the loss of a small amount of adsorbed and interlayer water or solvent is observed below 200

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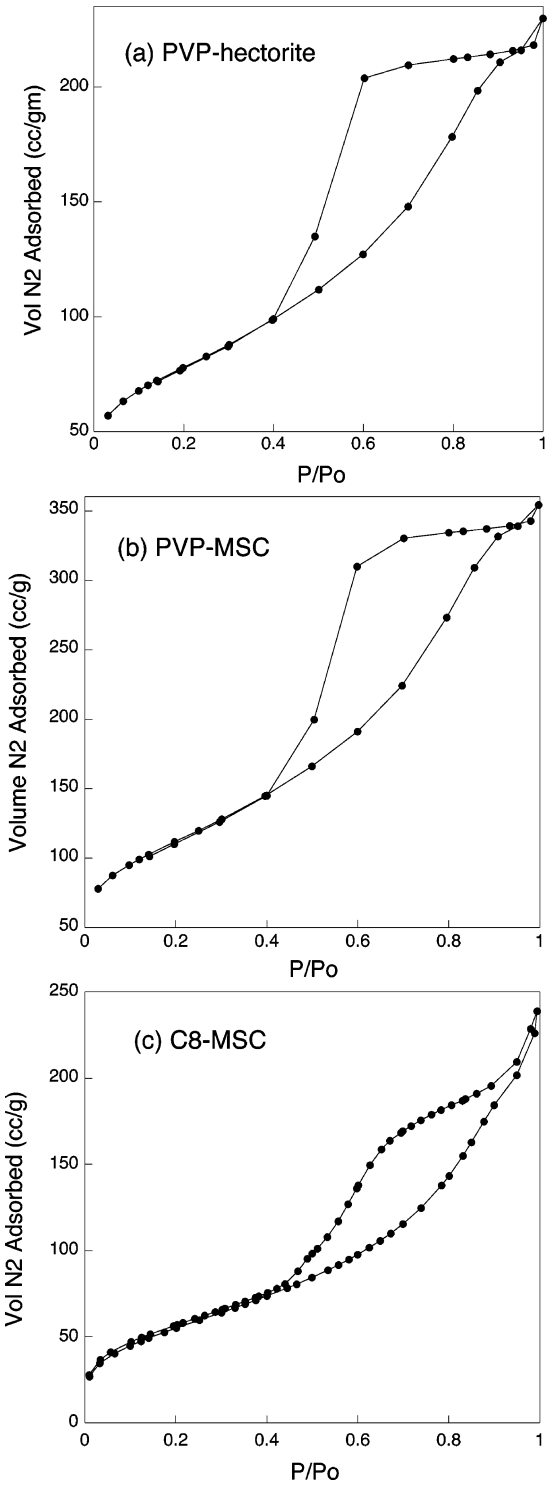

Figure 1. Nitrogen adsorption (bottom trace of the curves) and desorption (top trace of the curves) isotherms of clay supports prior to cyt *c* loading: (a) PVP−hectorite organo-tailored clay, (b) PVP−MSC after calcination, and (c) C_8−MSC after organosilane grafting of PVP−MSC.

°C. The water content correlates with the type of organic modifier; it is slightly higher in those samples with the water-soluble template intact, less for those that are calcined, and significantly less for the C_8−MSC. Between 200 and 600 °C the loss of organic, either template or organosilane in the case of C_8−MSC, is observed. There is insignificant weight loss over this region for a clay that has no organic present (see Figure 2a for PVP−MSC). Chemisorption of the alkylsilane is confirmed by the TGA of C_8−MSC from which was calculated a total organic content of just over 14 wt % (see Figure 2b). A small weight loss near 750 °C, typically 0.5−2 wt %, is observed for smectites due to the loss of structural lattice hydroxyl groups via dehydroxylation. This peak is completely missing from the

Table 1. Physical Data for Various Hectorites and Cyt *c*/Hectorite Complexes[a]

matrix	S.A. (m^2/g)	P.V. (cm^3/g)	wt % cyt *c*[b]	% reducible cyt *c*[c]	wt % active cyt *c* in matrix[d]
C_8−MSC	240	0.43	21.0	30.7	6.5
PVP−hectorite	277	0.36	38.5	14.2	5.5
TEA−hectorite	334	0.36	37.6	9.6	3.6
PVP−MSC	404	0.57	26.1	10.6	2.8
TEA−MSC	396	0.64	28.1	6.2	1.7
Laponite	348	0.28	47.3	2.8	1.3

[a] N_2 physisorption data taken prior to cyt *c* loading; S.A. = surface area, P.V. = pore volume. PVP− and TEA−hectorites are organo-tailored clays (OTCs); MSCs are mesostructured synthetic clays with no organic template remaining; C_8−MSC is an organosilane-modified PVP−MSC. [b] Total wt % cyt *c* immobilized as determined by optical spectroscopy. [c] [mg of reduced cyt *c* from 550 nm band]/[mg of total cyt *c*] × 100 after $Na_2S_2O_4$ reduction. [d] Determined by multiplying the previous two columns/100.

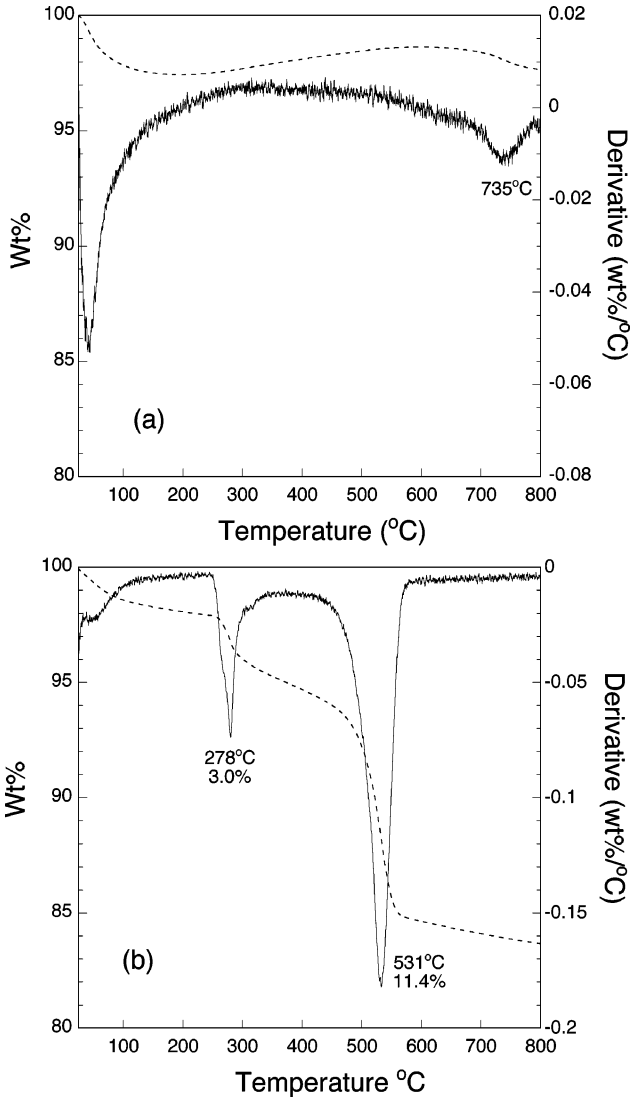

Figure 2. TGA wt % loss (dashed lines) and first derivative (solid lines) curves for (a) PVP−MSC and (b) C_8−MSC.

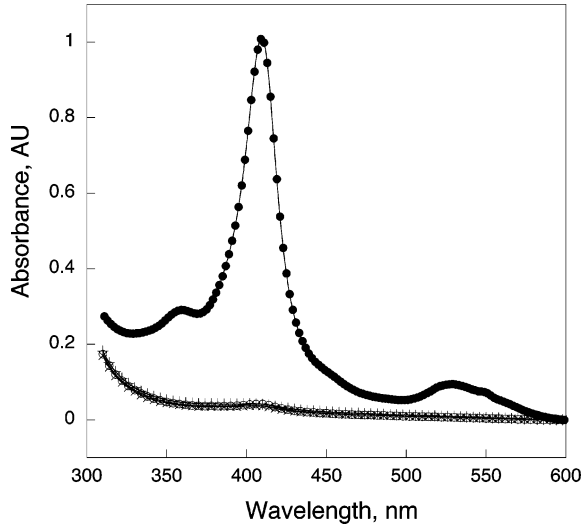

Figure 3. Optical absorbance spectra of the supernatant (solid circles) and wash solutions (wash #1 = ○; wash #2 = +; wash #3 = X) in the preparation of cyt *c*/TEA−hectorite complex.

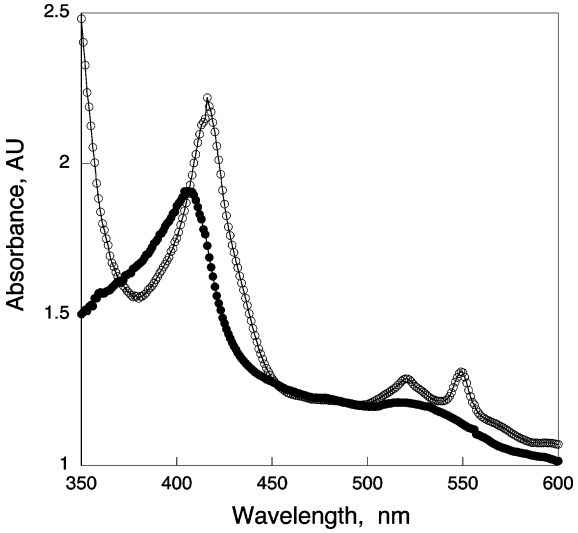

Figure 4. Optical absorbance spectra of oxidized (solid circles) and reduced (open circles) cytochrome *c* within a PVP−hectorite complex. The reduction was performed chemically using sodium hydrosulfite.

C_8−MSC surface-modified composite, which demonstrates the successful grafting of the silane via the lattice hydroxyl groups.

Characterization of Cyt *c*/Clay Complexes. Cytochrome *c* was readily adsorbed into all of the hectorites by simple mixing of a concentrated protein solution with a predetermined amount of clay at 4 °C overnight. For Laponite, significant adsorption of the protein was apparent from an almost complete loss of color from the supernatant solution and subsequent concentration of color in the solid. All of the protein and clay mixtures showed some adsorption of protein from the supernatant solution by the solid that was obvious by visual examination. The quantity of protein in the supernatant and wash solutions was determined by optical absorbance spectroscopy (see Figure 3). These spectra were not complicated by scattering from the solid matrix. With use of low ionic strength buffer solutions, no leaching of enzyme from the composites was observed. TGA was not used to determine cyt *c* loadings due to uncertainties between organic template vs protein combustion events.

The cyt *c* content verified by optical absorption spectroscopy of the supernatant solutions is provided in Table 1. From this measurement, relatively high loadings of cyt *c* were calculated for all of the clay complexes, with the highest for Laponite at nearly 50 wt %. The complexes prepared using OTC hectorites with template intact incorporated almost 40 wt % cyt *c*. The calcined clays bound slightly less than the parent materials at 26−28 wt %. The silane-modified MSC bound the least at 21 wt %.

After cyt *c* adsorption within each clay matrix, the composite was collected by centrifugation and the optical absorbance spectrum was acquired. These composites exhibited optical absorption spectra consistent with oxidized cytochrome *c*, which is the expected Fe(III) form. Some broadening of the spectral peaks was visible over the large background scattering. A typical absorption spectrum is shown in Figure 4 (line with solid circles). Background scattering increased the measured absorbance most significantly at the blue end of the spectrum, which affected the most intense peak of the oxidized species at 409 nm. The complexes were then

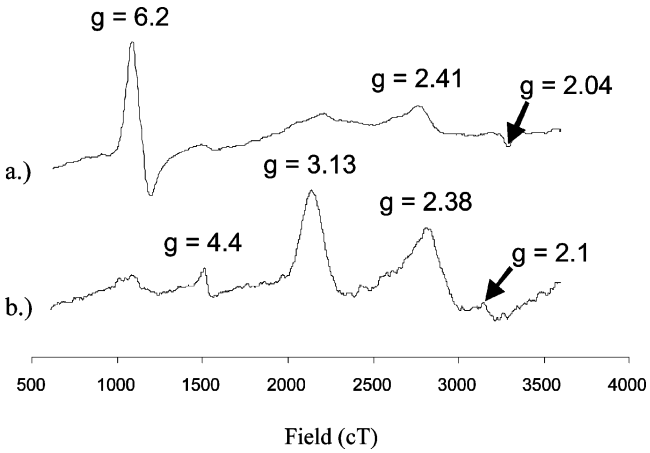

Figure 5. EPR spectra of cyt *c* in solution at (a) pH 2.2 and (b) pH 7.0.

subjected to chemical reduction using sodium hydrosulfite ($Na_2S_2O_4$) in order to reduce Fe(III) to Fe(II) in the cyt *c* active site. Optical absorbance spectra of the reduced complexes display peaks indicative of reduced cyt *c*, with major peaks at 420, 520, and 550 nm (see Figure 4, line with open circles). Because of the large background scattering from the solid clay particles, the peak at 420 nm was not useful for quantification of the reduced enzyme. Therefore, this quantity was calculated based on the absorbance at 550 nm (ϵ = 27 000 M^{-1} cm^{-1}). No oxidized cyt *c* was detectable after chemical reduction using optical absorption. However, the calculated quantity of reduced enzyme was not equal to the total immobilized cyt *c* as measured by loss from the supernatant solution. In other words, the reduction of immobilized cyt *c* was not exhaustive, but the oxidized species was not detectable due mainly to the large background scattering at the blue end of the spectra.

The coordination state and structure of the ferric heme site was qualitatively probed by EPR. As shown in Figure 5, characteristic differences between active cyt *c* and the species denatured (non-native, biochemically inactive conformation) in acidic solution can be observed using this technique. The main components of the active

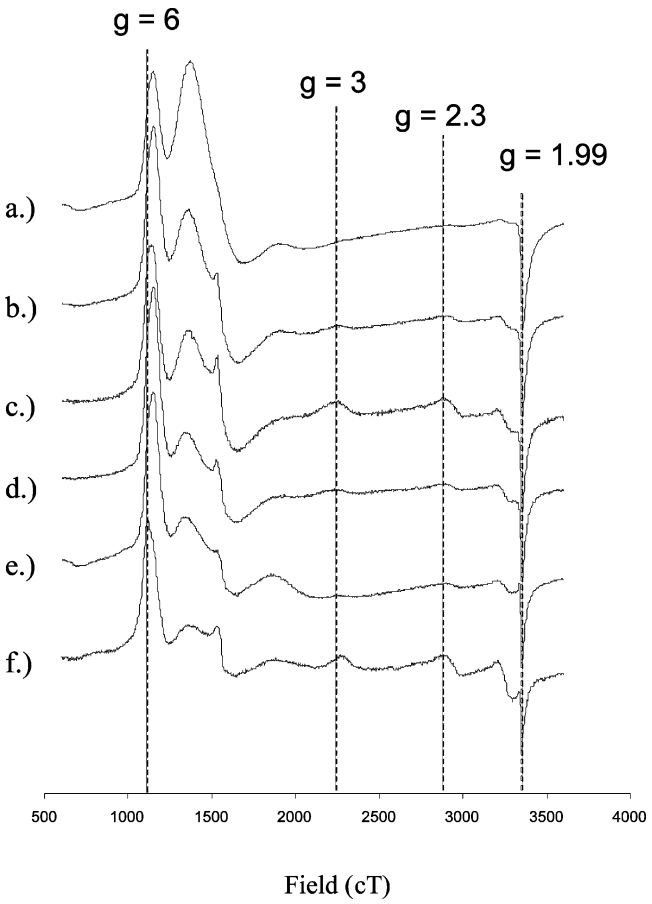

Figure 6. EPR spectra of cyt *c*/clay complexes: (a) Laponite RD, (b) TEA−hectorite, (c) PVP−hectorite, (d) TEA−MSC, (e) PVP−MSC, and (f) C_8−MSC.

cyt *c* spectra occur at about $g_z = 3$ and $g_y = 2.4$, with smaller spectral features at $g_z = 4.4$ and $g_y = 2.1$, presumably due to small amounts of iron-containing contaminants or denatured protein. The denatured cyt *c* spectrum displays one major feature at approximately $g_z = 6$ and a minor resonance at $g_{x,y} = 2.0$, characteristic of high-spin ferric iron induced by replacement of one of the Met/His axial ligands with water. In the acid-denatured cyt *c* spectrum, weak signals are observed which are indicative of the active conformation. It is assumed then that there is an equilibrium condition between the two species in solution.

Such an equilibrium is also observed for the enzyme within the clays as demonstrated in Figure 6. The multitude of EPR peaks indicates the transformation of the native cytochrome Fe(III) coordination environment (planar heme, axial Met/His ligation) into a variety of non-native configurations. These include high-spin EPR absorption peaks at $g = 6$ and $g = 2.0$ observed for all samples corresponding to the removal of one of the Met/His axial ligands, as well as EPR signals in the $g = 4$ region characteristic of high-spin Fe(III) in non-heme environments. In addition, an ensemble of low-spin configurations are observed with g_z peaks ranging from $g = 3.4$ to $g = 2.9$ that are likely to correspond to a variety of planar heme, His/His, and Met/His axial ligand coordination geometries. The fact that the amplitudes of the native low-spin conformations do not appear to coincide with the levels of active enzyme assayed by reduction (Table 1) is consistent with at least partial Fe(II)-induced (reduction-induced) refolding of denatured configurations, as has been observed in solution.[51]

Discussion

Encapsulation. The first major hurdle in preparation of a solid biocatalyst is the incorporation of enzyme in significant quantity within the solid support. Since several previous reports have calculated this quantity based on the loss of enzyme from the supernatant solution,[12,52] the weight percent of cyt *c* derived from the change in the optical absorbance (at 409 nm) of the enzyme in the supernatant solution of the composite preparation is presented. These data are included in Table 1. As an alternative, the amount of organic species within a sample may be easily measured by TGA; this method has also been used by others.[53] This method was employed here only when it was certain that cyt *c* was the only possible organic species possible. Overall, the results obtained by TGA are more conservative. The most consistent results occur for PVP−MSC, where the loading is 25.0 wt % via TGA vs 26.1 wt % via optical methods. For TEA−MSC and Laponite, the values are 22.4 vs 28.1 wt % and 30.1 vs 47.3 wt % by TGA and optical methods, respectively. There is not currently a satisfactory explanation for the large discrepancy in Laponite loadings. No matter which method is employed, however, even the more conservative loading values are considered quite high for cyt *c* protein immobilization by adsorption techniques within solid inorganic matrixes.

There is no correlation observed in Table 1 with either the surface area or the pore volume of the clay matrix with weight percent enzyme loading (by either optical or TGA methods). Rather, the nature of the surface itself may be the critical factor, including such features as particle size, whether a template is present or not, and the nature of the template or surface-modifier (grafting) molecule. This latter factor will be discussed in more detail in the following section. One variable that stands out for these materials that makes them unique from other clays that have been employed for enzyme immobilization is that they are synthetic hectorites. The fact that they are magnesium silicates rather than more commonly used silicates or aluminosilicates may have dramatic effects on surface chemistry. For example, the inherent Lewis acidity of a hectorite is lower than that of a montmorillonite. Even more important, however, may be a simple particle size effect. All of the synthetic hectorites have particle sizes that are significantly smaller than natural clays. A natural smectite, including hectorite, will typically have 1−2 μm particle sizes. Laponite platelets on the other hand have been measured at just 40 nm[54] and the OTC/MSCs are approximately 100−200 nm.[22] Such a dramatically reduced particle size will naturally raise the accessible surface area (the surface area of a natural hectorite is approximately 80 m^2/gm).

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Stability of Protein Conformation. The second major hurdle regarding bioinorganic catalysts is the retention of a significant portion of enzyme activity after immobilization. Both conformational changes and steric hindrance may participate in the inactivation of an enzyme. EPR is a very sensitive measure of the local environment of a paramagnetic species, which is in this case the oxidized heme. Using this technique, one is able to probe the structure of a protein at its active site and detect either an active or a denatured configuration. It has been shown for cyt *c* that changing the pH to a very acidic level changes the coordination state of the heme and that similar coordination states are observed in unfolded conformations.[55] Therefore, the EPR spectra of a solution of cyt *c* at neutral pH (active conformation) and that of a solution of cyt *c* at very acidic pH (denatured cyt *c*) were compared to the spectra obtained from the cyt *c* within the clay complexes (Figures 5 and 6). Proof of active ferric cytochrome conformation was evident by the peak at $g = 3$. Each composite also clearly contained the denatured conformer as indicated by the large low field resonance.

EPR assays provided a qualitative assessment of the retention of intact cytochrome structures in the clays by relating the relative intensities of the $g = 3$ and $g = 6$ peaks. It was then reasoned that the amount of cyt *c* able to participate in substrate-driven oxidation−reduction reactions is a function of both steric access to external reductants and existence of the proper protein conformation. Therefore, a quantitative examination of cyt *c* reducibility (as a marker for activity) was carried out through chemical reduction of the cyt *c* composites. Upon reduction, optical absorbance spectra show complete conversion of the encapsulated cytochrome to the reduced state. Although oxidized species are not detectable under the broad background (as discussed earlier), the quantity of reduced cyt *c* could be determined from these spectra based on the optical absorbance peak at 550 nm. This quantity was further used to calculate the relative amount of active cyt *c* within each matrix with respect to the total quantity of enzyme as measured optically. These results are included in Table 1, with activity = [reduced cyt *c* (wt % of complex) from 550 nm band]/[total cyt *c* (wt % of complex) from optical absorption] × 100%. A final column is included that displays the total weight percent of cyt *c* that is available in its oxidized (or "active") form by multiplying the previous two columns.

Possible sources of denaturation are through contact with the solid surfaces (including protein−surface as well as protein−pore architecture interactions) or due to changes in the microenvironment of the aqueous solution within the clay. These effects are most significant in the Laponite complex, which shows no evidence of active enzyme in the EPR spectrum (Figure 6a). Parts (b) and (c) of Figure 6 show improvement in relative activity for the TEA−hectorite and PVP−hectorite OTC cyt *c* composites, respectively. Either a smaller portion of the protein is in direct contact with a very active solid surface or the surface of an OTC is more compatible with cyt *c* than Laponite. More favorable protein compatibility could be due to the increased organophilicity of the organo-containing clays. Quantification of the redox activity verifies this dramatic improvement, from 3% in Laponite to 14% in PVP−hectorite. TEA−hectorite is not quite as high, however, at about 10%, even though the total cytochrome loadings are nearly identical (about 38%). This comparison reflects the difference in organic modification behavior from the small TEA molecule, which is present exclusively in the interlayer environment, to the large, hydrophilic PVP polymer, which is present in both interlayers and on the external surfaces of the clay. The MSC counterparts, which are devoid of organic compatibility, both display lowered loadings and activities.

The effect of inorganic surface incompatibility has been overcome by further modification of the clay surface by organosilane, as can be seen by comparison of parts (e) and (f) of Figure 6. A greater portion of active heme was detected by EPR in Figure 6f; cyt *c* activity as measured by chemical reducibility also increased significantly, jumping from about 11% activity within the PVP−MSC to almost 31% activity within the C_8−MSC. This modification from MSC to its grafted counterpart increased the organophilicity and lowered the surface area of the matrix, resulting in an overall improvement of the compatibility of the protein within the solid support. The organosilane may be responsible for buffering the protein from specific adsorption onto the clay siloxane surface, and thus has an effect on surface compatibility as well as the architecture of the matrix. Consistent with the EPR results, Laponite fared the worst with only 3% of the total cyt *c* retaining its activity after adsorption. This amounts to less than 2 wt % active cyt *c* within the complex as a whole despite the high protein density. In the remainder of the complexes, the amount of activity measured was considered significant.

While amounting to 6.5 wt % active cyt *c* within the C_8−MSC composite, this clearly shows the marked effect that tailoring the microenvironment of the solid substrate may have on the compatibility with the sorbed protein. Others have seen dramatic improvements in retention and activity by functionalizing surfaces. The use of purely siliceous mesoporous molecular sieves to immobilize enzymes is known to result in considerable leaching due to weak interactions. Functionalization of silica gel[56,57] and sieves such as SBA-15[7] is known to strengthen interactions between enzyme and support, and therefore improve leaching characteristics. In experiments not concerning functionalization, improvements in enzyme stability and activity have been achieved in mesoporous sieves by changing the framework composition (e.g., to FSM-16 from SBA-15) and by careful control of pore size.[58] Future studies of the cyt *c*/hectorite system will focus on reuse of the biocatalyst over several reduction−oxidation cycles and on increasing their stability.

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Summary

A variety of clays have been utilized in the preparation of catalytically active enzyme complexes by simple adsorption processes using cytochrome *c* as a spectroscopically amenable model enzyme. High protein loading, dictated by organic tailoring and optimum architecture, leads to high concentrations of enzyme in the active conformation. The use of synthetic organo-templated clays (OTC) imparted high loading and high activity, a vast improvement over the commercially available hectorite called Laponite. Calcination of OTC gives a fixed system of stable mesopores (MSC), but the now less organophilic surfaces preclude higher adsorption of large macromolecules. Therefore, loading was slightly attenuated and activity was reduced. Subsequent silane organo-modification of the MSC restored a portion of the clay's ability to house active enzyme without significant loss of loading.

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