Chloroperoxidase on Periodic Mesoporous Organosilanes: Immobilization and Reuse

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Six mesoporous materials functionalized with amine groups were developed and screened as potential supports for chloroperoxidase (CPO). A periodic mesoporous organosilane (PMO PA-40) with pore entrances large enough to allow the enzyme entry inside the pores was found to be the best support. When CPO was immobilized onto this material, it could be reused 20 times with retention of activity. On materials with little mesoporous structure and/or pore entrances too narrow to allow the enzyme entry to the channels, immobilized CPO rapidly lost activity upon reuse. Similarly, postsynthetic amino functionalization of a pure silica mesoporous material did not yield a stable biocatalyst.

Introduction

Enzymes are nature's enantioselective, substrate specific catalysts that usually work under mild, environmentally friendly conditions, e.g., in aqueous solutions and at room temperature and atmospheric pressure. However, they are expensive and when used in their water-soluble form are easily denatured and difficult to separate from the reaction mixture for reuse. Immobilization onto a solid support can often overcome these limitations and thus expand the repertoire of conditions under which enzymes will function and the reactions they can catalyze.[1] Different types of solid supports have been used in the immobilization of enzymes, e.g., sol gels,[2] controlled pore glass,[3−4] nanoparticles,[5] and mesoporous materials.[5−7] Mesoporous silicates (MPS), discovered in 1992 by the Mobil research group,[8] offer the possibility of adsorbing or entrapping large biomolecules within their pores. They can have large pore diameters (∼2−40 nm), highly ordered pore structures, with very tight pore size distributions and large surface areas (∼1000 $m^2\ g^{-1}$). These properties, combined with good chemical stability, make them ideal candidate materials for the generation of bioreactors. Additionally, they can be chemically modified with various functional groups. Proteins are adsorbed onto these materials after synthesis, thus avoiding the harsh conditions often required when using other supports, e.g., with microparticles and hydrogels,[9] which may denature the protein. However, careful selection of a suitably functionalized mesoporous structure is key to successful immobilization. The protein needs to bind strongly to the support so that leaching is minimized while still retaining sufficient freedom of movement to perform its catalytic functions.

Chloroperoxidase is a versatile heme peroxidase that exhibits halogenase and peroxidative activity. It has significant potential for use in the fine chemical industry, e.g., for stereoselective epoxidations.[10] Chloroperoxidase (CPO) has been immobilized onto mesoporous materials with some degree of success to date[11−13] but its stability to heat, pH, and denaturants was not greatly enhanced. Aburto et al.[12] reported an increase in the stability of CPO immobilized on pure silica and functionalized SBA-16 compared with free CPO in the presence of the denaturant urea. Hartmann et al.[13] found that immobilized chloroperoxidase (on SBA-15) selectively oxidized indole to 2-oxoindole and was active over a wider pH range than the free enzyme. However, the recycling properties of CPO immobilized onto mesoporous structures have not been reported. Other supports have been used to immobilize chloroperoxidase.[14,15] For example, Kadima and Pickard[14] successfully adsorbed CPO onto porous aminopropyl-glass beads but found that it was easily removed by washing with buffers of high ionic strength. Cross-linking the enzyme with glutaraldehyde after immobilization led to a

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reduction in specific activity but the immobilized enzyme could be reused without loss in activity over four cycles.

Mesoporous structures can be functionalized with organic groups (using organotrialkoxysilanes) by postsynthesis or in situ (i.e., organic groups are included in the reaction mixture during the synthesis of the mesoporous structure) methods. Both methods have disadvantages associated with them. Postsynthesis functionalization can result in low loadings of functional groups and pore blocking, whereas in situ functionalization can disrupt the mesoporous structure formation and phase separation can occur. The use of precursors consisting of alkoxysilanes bridged together by organic groups can eliminate some of these problems. The resulting materials, termed periodic mesoporous organosilanes (PMOs), were first reported in 1999.[16] Different surfactants, ionic strengths, acidic conditions, precursors, additives, and hydrothermal treatments all lead to different pore structure outcomes. Wahab et al.[17] synthesized a propyl-amine-modified PMO (PMO PA) material by co-condensing an amino-functionalized bridged silesquioxane, bis[3-(trimethoxysilyl)propyl]amine (PA), with an ethane-bridged silesquioxane using CTAB as a surfactant. They obtained pore sizes of approximately 4.5 nm with the degree of disorder increasing with the percentage of PA used. However, as noted by Hunks and Ozin,[18] there is always the potential for phase separation when using mixed precursors, resulting in the synthesis of a mixture of two distinct materials rather than one material with a homogeneous distribution of functional groups throughout.

In the first syntheses of PMOs, cationic surfactants were used and the resultant mesopores were small (3−4 nm pore diameter).[16] Pluronic surfactants such as P123 or F127 have led to much larger pore PMOs (10 nm)[19,20] opening up the potential of using these materials to adsorb larger molecules, e.g., enzymes. To date, the only published example of protein immobilization onto a PMO is a report describing the adsorption of cytochrome c onto a PMO synthesized with ethane bridging groups.[21,22] In this work, we investigate the immobilization, activity, and reuse of chloroperoxidase on PMO materials and on a postsynthesis amine-functionalized mesoporous silicate.

Experimental Section

Materials. The following chemicals were obtained from Sigma-Aldrich: chloroperoxidase from *Caldariomyces fumago* (34.6 kU/mL, 8.8 kU/mL; all experiments and comparisons of materials were done with CPO of the same activity), tetraethoxysilane (TEOS, 98%), bis[3-(trimethoxysilyl)propyl]amine (PA), trimethylbenzene (TMB), pluronic surfactant F127 ($EO_{106}PO_{70}EO_{106}$), sodium hydroxide, citric acid, sodium citrate, hydrochloric acid (36%), ethanol, monochlorodimedon (MCD), and hydrogen peroxide (11.63 M). Pluronic P123 ($EO_{20}PO_{70}EO_{20}$) was a gift from BASF.

Synthesis and Characterization of Mesoporous Supports. All materials were labeled PMO PA-*x*, where *x* represents the varying percentages of silicon atoms (*x*) originating from the PA precursor. PMO PA-33 was synthesized following a published protocol[20] but using bis[3-(trimethoxysilyl)propyl]amine instead of tris[3-(trimethoxysilyl)propyl] isocyanurate. The surfactant used for this material was the triblock copolymer, P123. The surfactant, water, and acid were stirred at room temperature for 3 h, followed by further stirring at room temperature for 24 h after the addition of the PA and TEOS. The reaction mixture was then autoclaved at 100 °C for 48 h. The molar reactant ratio used for PMO PA-33 was (0.833:0.167:0.017:188:5.8 TEOS:PA:P123:H_2O:HCl) so that 33% of the silicon atoms in the final structure originated from the PA precursor. The surfactant (P123) was removed by Soxhlet extraction with ethanol. A series of materials was synthesized following the low-temperature method reported by Fan et al.[23] using F127, also a triblock copolymer, as the surfactant. The following reagents were stirred together for 2 h at 15 °C: F127 (2.5 g), KCl (12.5 g), TMB (3.47 mL), and 2 M HCl (150 mL). The sources of silica, TEOS and PA (TEOS (18.48 mL) for PMO PA-0, TEOS (14.4 mL) and PA (3.14 mL) for PMO PA-22, TEOS (11.15 mL) and PA (5.45 mL) for PMO PA-40, and PA (13.59 mL) for PMO PA-100) were mixed together and then added to the reaction mixture and left stirring at 15 °C for 24 h. This procedure yielded a 1:0.0024:2.16:0.32:4:100 Si:F127:KCl:TMB:HCl:H_2O molar ratio. The synthesis mixture was then incubated in an autoclave at 100 °C for 24 h. The solid formed was filtered, washed with deionized water, and air-dried before being added to 2 M HCl (∼1 g of as-made product in 60 mL) and placed in an autoclave at 140 °C for 48 h, unless otherwise specified. The surfactant, F127, was removed from the materials by Soxhlet extraction with ethanol. Because of the high salt content in the synthesis mixture, the materials were all stirred in deionized water for 4 h after removal of the surfactant to remove any remaining salt.

The PMO PA-0 material was functionalized, postsynthesis, with amino groups by adding (3-aminopropyl)trimethoxysilane (4.235 mL) to 1 g of the parent material and stirring in 30 mL of 1,4-dioxane. The mixture was refluxed (100−102 °C) for 24 h. The resultant white powder, labeled NH_2−PMO PA-0, was filtered and washed with diethyl ether (3 × 20 mL) and allowed to air-dry.

Nitrogen gas adsorption/desorption isotherms were measured at 77 K using a Micromeritics ASAP 2010 system. Samples were pretreated by heating under vacuum at 348 K for 12 h. The surface area was measured using the Brunauer−Emmett−Teller (BET) method.[24] The pore size data were analyzed by the thermodynamic based Barrett−Joyner−Halenda (BJH) method[25] on the adsorption (pore channels)[23] and desorption (pore openings)[23] branches of the N_2 isotherm. Mesoporous volumes were estimated from the volume of nitrogen adsorbed after the micropores have been filled until after condensation into the mesopores was complete. Particle size was measured using a Malvern 2000 particle size analyzer. Samples were pretreated by sonication for 15 min and stirring in aqueous

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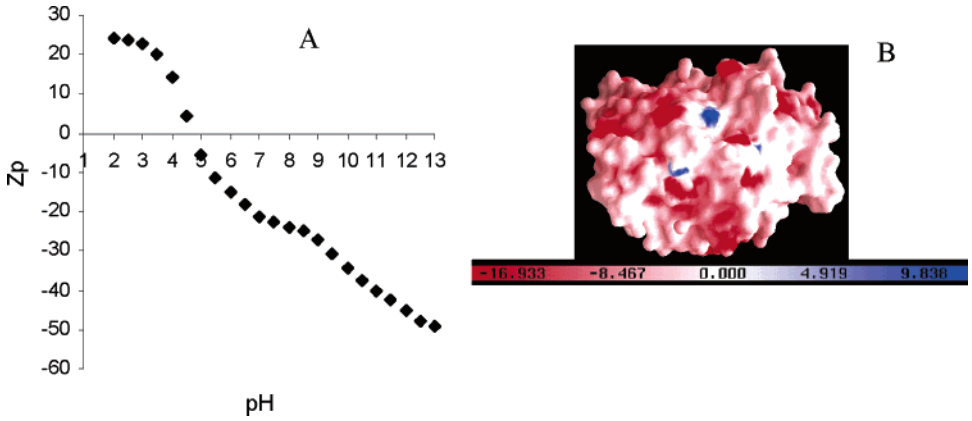

Figure 1. (A) Calculated charge on CPO as a function of pH and (B) Poisson−Boltzmann electrostatic surface potential of CPO as calculated in GRASP at pH 7.0 (blue represents areas of positive charge and red represents areas of negative charge).

solution at room temperature for 24 h. Isoelectric points were measured using a Malvern Zetasizer 3000HSA. Samples were made up in 18.2 MΩ deionized water (Elgastat) at a concentration of ~0.5 mg/mL and sonicated for 15 min before zeta potential measurements were taken. The pH of the solution was manually adjusted by the addition of 0.1 M HCl or NaOH to 10−15 mL of the suspension before the zeta potential was measured.

Adsorption Experiments. CPO concentrations were calculated from the absorbance at 403 nm (ϵ = 91 200 M^{-1} cm^{-1} [26]). A suspension of mesoporous support in the relevant buffer at a concentration of 2 mg/mL was dispersed by sonication for 15 min and stirred for 30 min. Equivalent volumes of the enzyme solution and the suspension were then mixed together and incubated at 25 °C at 160 rpm in a New Brunswick Scientific C24 incubator shaker for 12 h. Samples were centrifuged at 13000 g for 1 min at room temperature, and the absorbance of the supernatant was measured at 403 nm. The concentration of the free enzyme was calculated and hence the quantity of enzyme immobilized was found. Leaching of the immobilized enzyme from the support was monitored by washing 2 mg of enzyme-loaded material with a range of buffers. Unless otherwise stated, enzyme loaded materials were washed (3×) with the immobilization buffer, followed by washing (3×) with a buffer of different pH value and finally with a buffer of higher ionic strength (3×). The enzyme loss for each step was determined by measuring the absorbance at 403 nm.

Chloroperoxidase Activity Assay. Hydrogen peroxide (0.03 mL, 4.8 mM) and 0.025 mL of CPO solution were added to 1.45 mL of 0.1 mM monochlorodimedon and 20 mM potassium chloride in either 100 mM or 10 mM citric acid/phosphate buffer at pH 2.75.[27] The reaction was monitored at 278 nm following the change in the concentration of monochlorodimedon (MCD, $\epsilon_{MCD,\ 278}$ = 12.2 mM^{-1} cm^{-1}). To assay the immobilized enzyme and to determine the loss in activity due to leaching of CPO, 25 μg of CPO−PMO PA were added to the assay after one of the following treatments: washing once with 1 mL of the immobilization buffer, o r washing once with 1 mL of immobilization buffer and once with 1 mL of the assay buffer pH 2.75. Rates of reactions are reported as the number of moles of monochlorodimedon chlorinated per second over the first 30 s. Recycling experiments consisted of adding fresh MCD and hydrogen peroxide to 1 mg of CPO−PMO PA over 20 cycles and the amount of MCD converted after 5 min was measured. The reaction mixture was centrifuged (13 000 rpm, 1 min, 25 °C) and the supernatant was removed between cycles.

Analysis of Protein Structure. GRASP, graphical representation and analysis of structural properties (http://wiki.c2b2.columbia.edu/honiglab_public[28]), was used to calculate surface electrostatic potentials of chloroperoxidase. Swiss Deep View[29] and PyMOL[30] were used to estimate the widest diameter. The PDB accession code for chloroperoxidase is 1CPO.[31]

Results and Discussion

Properties of Chloroperoxidase. Applying the scheme previously developed to expedite the immobilization process,[21] we examined the charge and surface properties of CPO by considering the amino acid composition of the enzyme and their relative pKa values (Figure 1A) and by using Poisson−Boltzmann electrostatic potential calculations within the program GRASP (Figure 1B). On the basis of the amino acid composition and structure provided in the Protein Data Bank,[31] GRASP takes into account the shape and curvature of the enzyme molecule in its electrostatic potential calculations.[28] By calculating the protein net charge[32] based on the pK_a values for the individual amino acids, the charges of the enzyme at different pH values can be estimated. Although the effects of the protein environment on the pKa value of each amino acid are not accounted for, the calculated isoelectric point (IEP, Figure 1A) is consistent with the value of 4 reported in the literature.[11] Electrostatic calculations for the surface of CPO indicate that the surface is predominantly negatively charged at pH 7, which is consistent with the low IEP of the protein (Figure 1B). CPO is approximately 6.2 nm in length at its widest dimension.[29−30] This characterization suggests that if CPO was to be immobilized via electrostatic interactions, a positively charged surface on the solid phase, with pore entrances >6.5 nm, would act as a good adsorbent material at pH > 5. This information provides a good starting point for the selection and development of tailored MPS materials for the generation of immobilized CPO.

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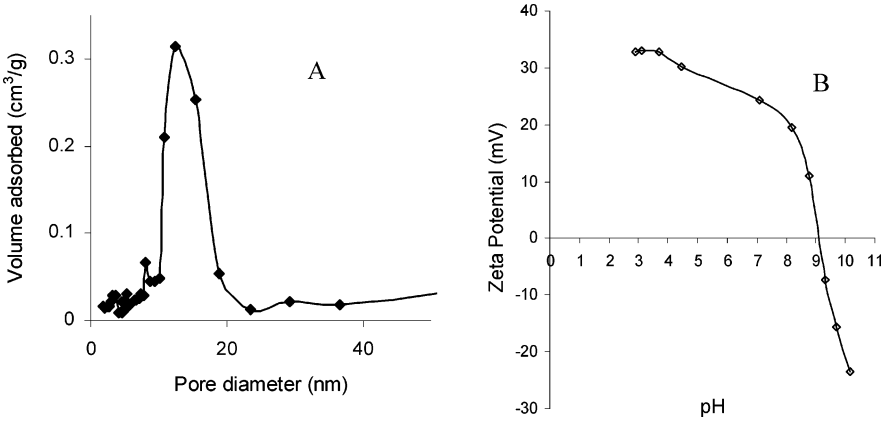

Figure 2. Plots of (A) the pore size distribution and (B) zeta potential as a function of pH for PMO PA-33

Table 1. Physicochemical Properties of PMO-PA Materials

material	surface area (m^2/g)	C_{BET}	V_{total} (cm^3/g)	pore size (des) (nm)	pore size (ads) (nm)	IEP	particle size (μm)
PMO PA-33	660	65	1.28	10−20	9.2−30	9.0	55
PMO PA-0	272	110	0.97	6−17	13−36+	4.1	40
PMO PA-22	468	69	0.6	3−5	12−25	7.6	30
PMO PA-40	335	57	0.65	4−10	7[a]	9.1	18
PMO PA-100	139	47	0.29	[a]	14[a]	10.2	47
NH_2−PMO PA-0	153	41	0.54	7−14	13−30	8.7	24

[a] Macropores.

Table 2. Mesoporous Volume of PMO PA Materials after Sonication for 24 h in 100 mM pH 2.75 Aqueous Buffer

material	initial mesoporous volume (cm^3/g)	mesoporous volume post-sonication (cm^3/g)
PMO PA-33	1.28	0.08
PMO PA-0	0.78	0.7
PMO PA-22	0.41	0.36
PMO PA-40	0.43	0.32
PMO PA-100	0	0

Physicochemical Properties of PMO Materials. PMO PA-33, synthesized with P123 as a surfactant, exhibited a narrow pore size distribution with large pore openings (approximately 10−20 nm, Table 1) that should easily allow the chloroperoxidase molecules entry into the mesoporous channels (Figure 2A). From X-ray diffraction patterns and TEM analysis, PMO PA-33 has a disordered porous structure. The zeta potential of the material as a function of pH (Figure 2B) yields an isoelectric point of 9.1. Over the pH range 4−9, PMO PA-33 is positively charged and should interact in a favorable electrostatic manner with CPO, which should carry a surface negative charge in this pH range. Preliminary adsorption and activity studies indicated that PMO PA-33 would act as a good support for CPO. However, as the immobilization process involves shaking the enzyme and solid support at various pH values, the support must be checked for its ability to withstand shaking in acidic, neutral, or basic aqueous conditions. Such testing should always be performed but unfortunately, this is not usually the case. Chloroperoxidase was stable to shaking (160 rpm) in buffers at pH 5−6 at 25 °C. When PMO PA-33 was stirred in aqueous buffer solutions in the pH range 4−10 for 24 h, it lost much of its porous structure, Table 2. However if the stability of this type of material could be improved, the large pore size and favorable surface charge and composition (free amino groups) of these materials together with preliminary immobilization and activity studies of CPO indicate that PMO PA type materials show good potential as supports for CPO.

Fan et al.[23] synthesized a stable large pore cubic pure silica mesoporous silicate by stirring a neutral surfactant F127, a swelling agent (trimethylbenzene), and tetraethoxysilane together at low temperatures, followed by a high-temperature hydrothermal treatment for 48 h at 140 °C in 2 M HCl to widen the entrances to the mesoporous channels. This was observed by a shift in the position of the desorption branch of the nitrogen isotherm after the hydrothermal treatment, resulting in an increase in the pore entrance size from <4 to 16.7 nm. Initial attempts in this study to synthesize a more stable PMO PA type material used this synthesis procedure with ∼22% of Si emanating from the PA precursor and 78% from TEOS, yielding PMO PA-22. This material did not lose its mesoporous structure upon sonication and stirring in buffer over the pH range 2.75−10, Table 2. However, the high-temperature hydrothermal treatment, which expanded the pore entrance size of the pure silica materials, did not yield the same results with PMO PA-22. With this material, the desorption branch gave a pore entrance size distribution of 3−5 nm: too small to allow CPO molecules to enter into the mesoporous channels. Subsequent syntheses of PMO PA materials used hydrothermal treatments for longer times at 140 °C in an attempt to expand the pore entrance size. A series of PMO PA-22 materials were made, treating at 140 °C for 24, 48, 96, and 192 h; however, longer hydrothermal treatment did not significantly or uniformly widen the pore entrances for the PMO PA-22 material (Figure 3A).

A series of PMO PA materials (PMO PA-0, PMO PA-22, PMO PA-40, and PMO PA-100, Table 1) was made by altering the composition ratio of the silica precursors (TEOS and PA), with 48 h of hydrothermal treatment at 140 °C. It is clear from the shape of the nitrogen adsorption isotherm that PMO PA-100 has little mesoporous structure (Figure

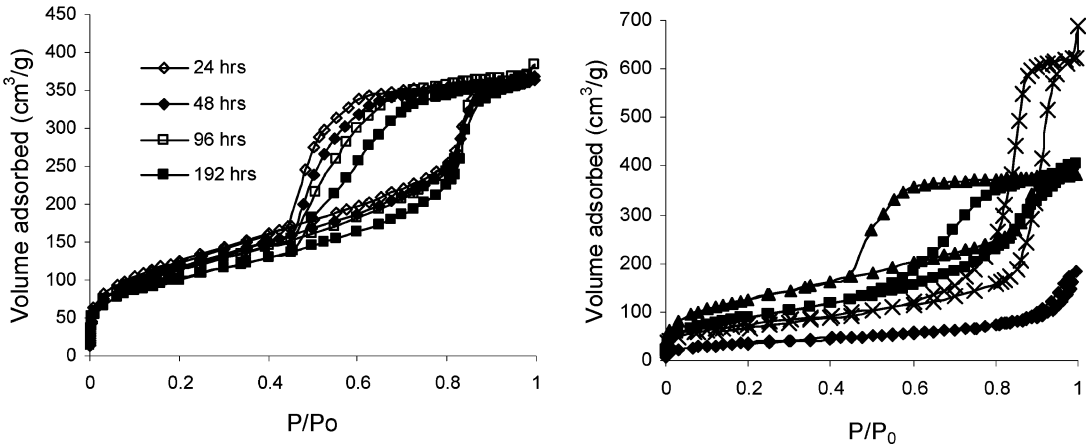

Figure 3. Nitrogen adsorption isotherms for (A) PMO PA-22 with hydrothermal treatments at 140 °C for 24, 48, 96, and 192 h and (B) PMO PA-0 (×), PMO PA-22 (▲), PMO PA-40 (■), PMO PA-100 (◆), hydrothermal treatment at 140 °C for 48 h.

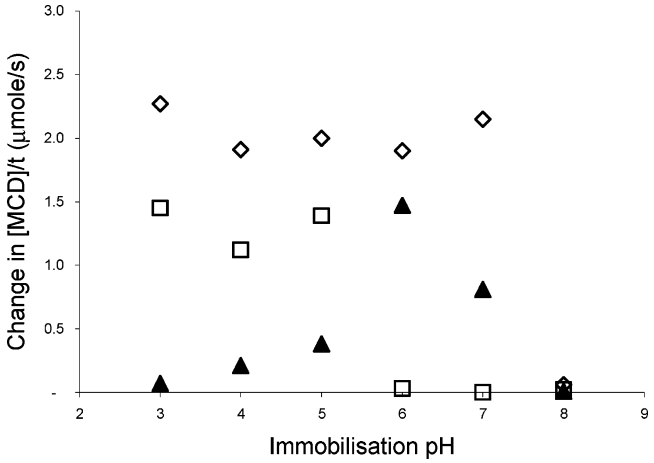

Figure 4. Activity of free CPO (◇), CPO−PMO PA-33 (▲) and the supernatant (□) at pH 2.75, initial [CPO] = 0.46 μM, [PMO PA-33] = 1 mg/mL, 25 °C.

3B). PMO PA-40 exhibited larger pore openings than PMO PA-22 (Table 1). Longer hydrothermal treatment again resulted in an increase in the disorder of all of the materials (data not shown). From the nitrogen adsorption isotherms, the percentage of PA precursor in the synthesis mixture strongly influenced the final structure formed, but not simply by increasing the disorder as was expected.[34] PMO-PA-22 has much narrower pore entrances than PMO-PA-40. PMO-PA-100 has the lowest surface area and total pore volume. XRD analysis showed that, with the exception of the pure silica material PMO PA-0, which exhibited an ordered cubic structure, the remaining materials were disordered (data not shown).

The physicochemical properties of the postsynthesis functionalized material, NH_2−PMO PA-0, are also listed in Table 1. This material was a pure silica cubic porous structure that was functionalized after the surfactant had been removed by refluxing it with 3-aminopropyltrimethoxysilane in 1,3-dioxane. The degree of functionalization is difficult to ascertain, as is the location of the functional groups; the amino groups may not be homogeneously dispersed on the surface of the mesoporous channels. The surface area, pore volume, and pore size distribution (Table 1) were reduced in comparison to the parent materials. However, the pores should have entrances wide enough to allow the CPO molecules inside the channels.

Table 3. Specific Activity[a] of CPO in the Conversion of Monochlorodimedon (0.1 mM) by Free and Immobilized CPO after 30 s, in 10 mM Buffer pH 2.75[b]

	Δ[MCD]/*t* (μmol/(μmol × s)) after 1 wash immobilization buffer	Δ[MCD]/*t* (μmol/(μmol × s)) after 1 wash assay buffer[a]
free	30	
CPO−PMO PA-33	17.1	2.3
CPO−PMO PA-22	10.3	2.3
CPO−PMO PA-40	20	8.4
CPO−PMO PA-100	22	1.3
CPO−NH_2−PMO PA-0	12.4	4.7

[a] Assumes no leaching occurred during washings. [b] Loading of CPO: 1.1 μmol/g onto PMO PA-40, PMO PA-100 and NH_2−PMO PA-0, 1.4 μmol/g onto PMO PA-33, 0.35 μmol/g onto PMO PA-22.

PMO PA-100 had the highest isoelectric point, at pH 10.2, whereas the IEP of the remaining PMO PA materials decreased with decreasing PA content (Table 1). The stability of the PMO PA-*x* series was tested by sonication and stirring the materials in phosphate/citric acid buffer at pH 2.75, the conditions for the activity assay for CPO. The materials synthesized with F127 were found to be reasonably stable under these conditions. Only small losses in the mesoporous volumes of PMO PA-22 and PMO PA-40 were observed, and both materials retained distinct mesoporous structures (Table 2).

Immobilization of Chloroperoxidase. Initial immobilization studies indicated that the optimum activity of CPO−PMO PA-33 occurred when immobilization was conducted at pH 6 (Figure 4). At lower pH values of 3−5, not all of the CPO was immobilized (the supernatant exhibited CPO activity). There was no CPO activity in the supernatant when adsorption was performed at pH values between 6 and 8. However, the activity of CPO immobilized at pH 7 was greatly reduced and completely eliminated if immobilization was performed at pH 8. This is consistent with the fact that free CPO was inactive at pH 8. Complete adsorption of CPO occurred at pH 6, and the immobilized enzyme retained 75% of the activity of free CPO.

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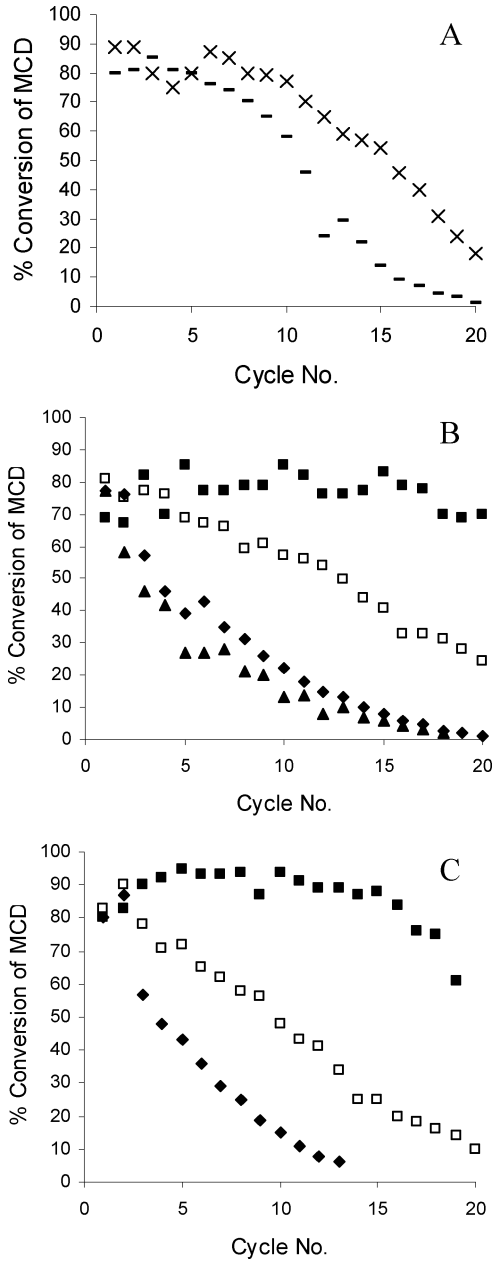

Figure 5. Percentage conversion of MCD (0.1 mM) by 1 mg of (A) CPO−PMO PA-33 material over 20 cycles in 10 mM (x) and 100 mM (-) citric acid/phosphate pH 2.75 buffer, loading of 0.94 μmol/g, and (B) CPO−PMO PA-x 10 mM pH 2.75 buffer and (C) CPO−PMO PA-x in 100 mM pH 2.75 buffer. Loading of 1.1 μmol/g: NH2-PMO PA-0 (□), PMO PA-40 (■), PMO PA-100 (◆). Loading of 0.35 μmol/g: PMO PA-22 (▲).

Thus, immobilization studies were all performed at pH 6. At an initial CPO concentration of 0.95 μM, PMO PA-0 adsorbed little CPO, whereas PMO PA-100, which exhibited little mesoporous structure, adsorbed all of the CPO present (0.95 μmol/g). This indicated that the amino functional groups on the surface of the material participate strongly in the adsorption of CPO. A lower loading was obtained on PMO PA-22 (0.35 μmol/g), which may be due to there being fewer available amino groups on the surface of the material and its smaller pore entrances. PMO PA-33, PMO PA-40, and NH_2−PMO PA-0 adsorbed all of the CPO present (0.95 μmol/g).

Leaching tests were carried out on the immobilized CPO using the immobilization buffer (10 mM phosphate buffer, pH 6) and the assay buffer (10 mM citric acid/phosphate buffer, pH 2.75). Low levels of leaching were observed from all five materials (PMO PA-33, PMO PA-22, PMO PA-40, PMO PA-100, and NH_2−PMO PA-0) when they were washed with the immobilization buffer, but approximately 40% of the adsorbed enzyme was leached during the first wash with the assay buffer with low levels of leaching (<2%) during further washings. A similar leaching pattern was observed for all materials when the assay buffer concentration was increased from 10 mM to 100 mM. Similarly, Kadima et al.[12] found that when CPO was adsorbed on to amino-functionalized porous glass beads, at loadings of approximately 10.7 μmol/g, 5% of CPO was leached when it was washed with low ionic strength (5 mM, pH 6) buffer, whereas 90% was leached on washing with high ionic strength (300 mM, pH 6) buffer. Because of this high amount of leaching, the activity of covalently immobilized CPO was examined.[12] The highest specific activity was observed at the lowest enzyme loadings and was 36% of that of the free enzyme. It was hoped that the confined space of the mesoporous channels in PMO PA materials would eliminate the need for a covalent linker but still enable recycling of the immobilized enzyme.

Activity of CPO at pH 2.75: Free and Immobilized. The activity of free and immobilized CPO was compared for all PMO PA materials. Control reactions showed that none of the PMO PA materials reacted with MCD in the presence of hydrogen peroxide. At high peroxide concentrations, 2.3 mM, CPO can be deactivated by peroxide[34] but it retains activity at a lower peroxide concentration of 0.096 mM. Some of the activity of CPO−PMO PA bioreactors may result from leaching, especially as 40% of the enzyme was leached off during the first washing with the assay buffer. All of the bioreactors exhibited much lower activity rates after being washed once with 10 mM assay buffer (Table 3).

The leaching studies raise the question of whether the CPO activity arises from the immobilized enzyme or from the slow release of the remaining adsorbed enzyme into the reaction solution. CPO−PMO PA-40 and CPO−NH_2−PMO PA-0 retained the highest activity rates after washing with both the immobilization and assay buffers (Table 3). If phase separation occurs during the synthesis of PMO PA-40, it would consist of a mixture of PMO PA-0 and PMO PA-100. If this occurred, the CPO activity from CPO−PMO PA-40 would originate from CPO immobilized on the PMO PA-100 phase, as CPO does not adsorb onto the pure silica PMO PA-0. However, CPO−PMO PA-40 retained much higher activity than CPO−PMO PA-100 after being washed with the 10 mM assay buffer, indicating that the CPO was immobilized onto PMO PA-40 in a different manner than in PMO PA-100. Thus, phase separation is not likely to have occurred. Adsorption of the enzyme into the mesoporous channels of PMO PA-40 allows the enzyme to retain activity, unlike in CPO−PMO PA-100. The instability of PMO PA-33, the narrow pore entrances of PMO PA-22, the absence of a mesoporous structure in PMO PA-100, and the

heterogeneous distribution of amine functional groups in NH_2−PMO PA-0 may account for the greater loss in activity compared to PMO PA-40.

Immobilized CPO: Reuse. The activity of a milligram of support loaded with CPO was monitored over 20 cycles (Figure 5). With CPO−PMO PA-33 (∼0.94 μmol/g), when the assay was conducted in the lower ionic strength assay buffer, activity was retained for longer than at higher strengths (Figure 5A). Even at the lower ionic strength, however, the activity fell by ∼70% after 20 cycles. This may be due to leaching but also to the decomposition of the PMO PA-33 material, which would release the enzyme into the reaction solution. PMO PA-40 was the optimal support material for CPO with the enzyme retaining its activity over 20 cycles (Figure 5B). However, at higher concentrations of buffer (100 mM), losses in CPO activity occurred after ca. 15 cycles. CPO immobilized on to NH_2−PMO PA retained 20% of its initial activity after 20 cycles. The enzyme appeared to be desorbed more easily from the remaining two materials, PMO PA-100 and PMO PA-22. As mentioned above, PMO PA-100 does not have any mesoporous structure and the entrance sizes of the mesopores in PMO PA-22 were too small to allow the CPO molecules access to the sheltered interior, allowing them to reside only on the external surface area. When the assay buffer concentration was increased from 10 to 100 mM, the rate of loss of activity was increased slightly for PMO PA-100 and NH_2−PMO PA-0 (Figure 5C).

The ability of CPO−PMO PA-40 to convert 80% (0.12 μmole) of the monochlorodimedon present in the reaction mixture in 5 min over 20 cycles indicates that a significant amount of active CPO is retained on the PMO PA-40 surface. In these experiments, equimolar amounts of hydrogen peroxide and monochlorodimedon were added, 100% conversion of the substrate should be possible by altering the MCD:peroxide concentration ratio. This result demonstrates that an amino-modified support can be used to develop a stable biocatalyst without the requirement for covalently linking the enzyme to the support. The PMO-PA structure is synthesized in one step, with a known extent of functionalization. In contrast, the extent to which NH_2−PMO PA-0 was functionalized with amino groups in the postsynthesis grafting of amino groups is difficult to estimate, as is the final location of the amino groups. Lei et al.[35] reported that mesoporous silica functionalized with only 2% amino and 2% carboxylic functional groups exhibited higher loadings and better activity when supporting organophosphorus hydrolase than when functionalized with 20% of the functional groups. Overall, the postsynthesis grafting of functional groups is a more complicated process and depends largely on the reaction conditions during functionalization and the final material can be difficult to fully characterize. The location and percentage of amino groups in the NH_2−PMO PA-0 material used here were not established and would have a strong influence on activity.

Conclusions

CPO was successfully immobilized on a variety of newly synthesized PMO PA materials. Studies using CPO−PMO PA-33 showed significant losses in activity over 20 cycles in a low ionic strength assay. However, some of this loss in activity could be attributed to the inherent instability of this support material. The more stable PMO PA material, PMO PA-40, with pore entrances large enough to allow the enzyme entry into the shelter of the pores was found to be a better support for CPO. If the CPO molecules do not have access to the shelter of mesopores of the support, as with PMO PA-100 and PMO PA-22, the immobilized enzyme biocatalyst lost all activity upon reuse. When the enzyme was immobilized onto this PMO PA-40 material, it could be reused 20 times with little loss in activity. Thus, much work is required to fine-tune the properties of a mesoporous material as a suitable host for an active enzyme. The important factors include pore size, stability, and distribution of functional groups of the material and size, surface properties, and stability of the enzyme.

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