Biomimetic Hydrolysis of *p*-Nitrophenyl Alkanoates with Functionalized Mesoporous Silicas

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(Received May 14, 2004; CL-040554)

A biomimetic catalysis of *p*-nitrophenyl alkanoate esters as a function of their alkyl chain lengths through multifunctionalization of molecular self-assembly onto ordered silica nanopores has been investigated in Tris-HCl buffer, and their kinetic parameters were calculated including a Michaelis–Menten kinetics.

Immobilization of enzymes on the surface of solid oxides has been extensively studied because of its immense technological potentials.[1] One method is a formation of chemical bonds between enzymes and a solid support, which often alters the enzymatic activities. Conventional sol–gel processes have been used to trap enzymes and other bioactive agents in an inorganic matrix for biocatalysis and biosensor applications.[2,3] Recently, we have demonstrated that the nanoporous channels can be used as a "nanofactory" to assemble the multifunctional groups and provide catalytic activities.[4] The silica used has a hexagonally ordered nanoporous pattern, high surface area of 950 m^2/g, pore volume of 0.38 cm^3/g as well as narrow pore size distribution of 7.8 nm. Multifunctional groups such as dihydroxyl, imidazole, carboxyl, ethylene diamine, and isobutyl groups were grafted onto this hexagonally ordered nanopore surface mimicking a triads of Ser 195, His 57, Asp 102 of chymotrypsin and these five groups were required for enhanced catalytic activity toward the hydrolysis of esters. The optimum catalytic hydrolysis of *p*-nitrophenyl acetate (PNPA) has been observed on 12% isobutyl grafted catalyst under neutral Tris-HCl buffer.[4] Here we report the hydrolysis of *p*-nitrophenyl alkyl esters (alkanoates) with various alkyl chain lengths using the above-mentioned catalyst. The rate enhancement by hydrophobic association between a catalyst and a substrate has been well documented in the literature.[5,6] However, a systematic examination of hydrolysis as a function of substrate hydrophobicity on immobilized catalysts onto ordered nanopores in micellar catalytic environment has not been reported.

The alkanoates used were acetate (C_2), butyrate (C_4), caprylate (C_8), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), and stearate (C_{18}). In a typical preparation of the nanoporous silica, 4.0 g of Pluronic P123 was dissolved in 30 g of water and 120 g of 2M HCl, and then 8.5 g of tetraethyl orthosilicate (TEOS) was added into the solution at 40 °C. The mixture was aged in a bomb at 120 °C overnight without stirring.[7] The solid product was filtered, washed, and air-dried. Calcination was carried out at 550 °C for 6 h. To the 5.0 g of nanoporous silica suspension in 150 mL of toluene that contained 1.60 mL of deionized water, 0.387 g (1.475 mmol) of 5,6-epoxyhexyltrimethoxysilane was added and refluxed for 6 h. After filtration, the powder was again dispersed in water and the pH was adjusted to 2.2 with hydrochloric acid. The suspension was stirred for 4 h. Solution pH was next adjusted to near 7 through addition of a 20% sodium bicarbonate solution. After filtration and vacuum drying, the dried powder was suspended in a solution of 0.80 mL of deionized water in toluene for 2 h. Then, 0.405 g (1.475 mmol) of *N*-(triethoxysilylpropyl)-4,5-dihydroimidazole, 0.334 g (1.475 mmol) of *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane, 1.157 g (5.9 mmol) of sodium carboxyethylsilanetriol (25%, sodium salt), and 0.263 g (1.475 mmol) of isobutyltrimethoxysilane for 4% isobutyl group (surface area ratio) were also added and refluxed for 6 h. The hybrid material was filtered, washed with toluene and then dried under vacuum before use. The route for the preparation of the enzyme-like nanocatalysts is shown in Figure 1. To achieve the catalyst with surface coverages of 12% (4.425 mmol) of isobutyltrimethoxysilane were combined with the 1.475 mmol of the each functional groups (hydroxy, amine, carboxyl, imidazole) on nanoporous silica of 7.8 nm. The hexagonal packing structure of the nanoporous silica was confirmed by

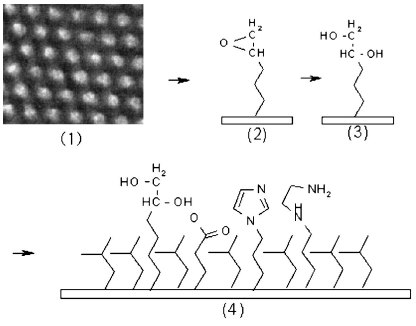

Figure 1. Preparation of multifunctionalized nanoporous silica: (1) TEM image of synthesized nanoporous silica (7.8 nm); (2) 5,6-epoxyhexyltrimethoxysilane graft; (3) Hydrolysis of 5,6-epoxyhexyltrimethoxysilane; (4) Assembly of dihydroimidazole, isobutyl, carboxyl, and ethylenediamine groups.

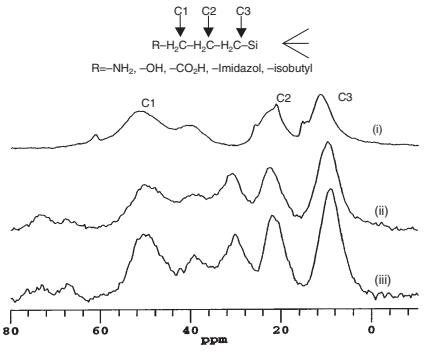

Figure 2. ^{13}C CP/MAS spectra of the multifunctionalized samples: (i) $(OH)_2$, (ii) $(OH)_2$–NH_2–Imidazole, and (iii) $(OH)_2$–NH_2–Imidazole–CO_2H.

Table 1. The calculated Michaelis–Menten parameters

Alkyl Chain Length(C_n)	C_2	C_4	C_8	C_{12}	C_{14}	C_{16}	C_{18}
K_m (μM)	464	558	1540	1028	843	1041	3414
V_{max} ($\mu Mmin^{-1}$)	97	110	204	81	29	34	44
k_{cat} (min^{-1})	1.93	2.19	4.07	1.63	0.58	0.68	0.89
V_{max}/K_m (min^{-1})	0.21	0.20	0.13	0.08	0.04	0.03	0.01
k_{cat}/K_m (times10^4 $\mu Mmin^{-1}$)	41.6	39.3	27.1	15.8	6.9	6.5	2.6

TEM showing the uniform size packed in a well-ordered and interconnected arrays (Figure 1(1)).

Once the chemical groups were bound to the silica pore surfaces, both the overall surface area and pore size were reduced to 170 m^2/g and to 4.5 nm, respectively, depending on the surface concentration of the modified silanol groups used.

Thermogravimetric analysis up to 500 °C (10 °C/min) indicates a large endothermic dissociation at 314 °C of organic silanes. Chemically functionalized surfaces have been characterized using ^{13}C CP/MAS solid-state NMR spectroscopy with respect to molecular conformation in the bound state and clustering of hydrophilic molecules at island-like domains within the nanoporosity. The most apparent trends seen in spectra (Figure 2) relate to the intensities of the CH_2 peaks of the ethoxy group, C2 and C3 at 10 and 21 ppm, respectively. Close examination of the spectrum reveals that the resonance peaks are sharper than those of the monofunctionalized material attributed to synergistic functional confinement in which the multifunctional groups appear to show enhanced mobility as confirmed by relaxation time measurements (data are not shown here), and show a shift of intensity from higher to lower shielding. In case of low surface coverage, the chemically functionalized silanes interacted with the free hydroxy group comprising the hydrophilic domains on the porous SiO_2 surface. Although the bending of the bounding molecule end to the surface formed ring-like structures identified by NMR chemical shifts and line intensity changes, at the higher surface coverage, the bending was inhibited, and the end of the bounding molecules became available to participate in the nitrophenyl alkanoates hydrolysis. The mimicking catalytic hydrolysis was demonstrated with a 5.0 times 10^{-3} M of substrates stock solutions in THF. A suspension of 50 mg of catalyst in 4.6–4.9 mL of 0.1 M Tris-HCl buffer (pH = 7.6) was put into a 15 mL centrifuge tube. Then 0.4–0.1 mL of the substrates stock solutions were added to bring the total volume in each tube to 5.0 mL. Four different substrate concentrations were examined: 100, 200, 300, and 400 μM. The reaction mixtures were shaken in an Environ Shaker at 30 °C, filtered through a 0.2 μm filter, and the kinetic products were measured on a UV–vis spectrophotometer at 400 nm. The amount of *p*-nitrophenol released was calculated from a calibrated curve of pure *p*-nitrophenol. The reaction rates were obtained by plotting of $[\ln(C_\infty/(C_\infty - C_t))]$ vs time (t) in which the $[\ln(C_\infty/(C_\infty - C_t))]$ term is modified from $[\ln(A_\infty/(A_\infty - A_t))]$.

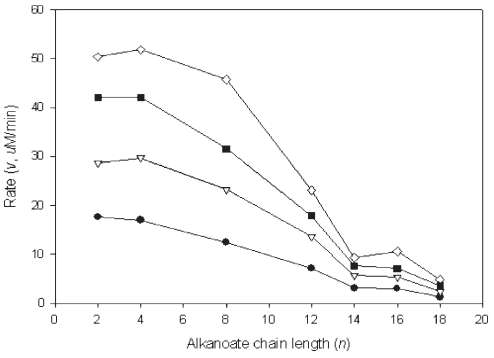

Figure 3. Biomimetic catalytic efficiency for *p*-nitrophenyl alkanoates as a function of alkanoate chain lengths: ●, 100 *u*M; ▽, 200 *u*M; ■, 300 *u*M; ◇, 400 *u*M.

The Michaelis–Menten parameters (K_m, V_{max}, and V_{max}/K_m) are shown in Table 1. The values for C_2 and C_4 are smaller than other substrates, indicating the preference of acylation process where causes the initial rapid burst of *p*-nitrophenol production with larger values of decomposition rate from enzyme–substrate (ES) to enzyme and substrate (E + S), or formation rate from enzyme–substrate to enzyme and product (E + P). Although C_8 may be predicted the best catalysis because of the preference of deacylation process with the largest k_{cat}, it is difficult to form acyl-enzyme product (ES) because of the decomposition property with larger K_m value. Meanwhile, the C_2 and C_4 show good catalytic activity in the plots of catalytic efficiency (Figure 3). The reaction between the long-chain substrates and the catalyst suffers from steric hindrance. The catalytic efficiency constants (V_{max}/K_m) and specificity constants (k_{cat}/K_m)[8] of the shortest chain are 20 times larger than the longest chain. This implies that longer alkyl chains become too large to optimally fit into the required triad spacing.

In conclusion, the biomimetic catalysis for *p*-nitrophenyl alkanoates with multifunctionalized nanoporous silica was demonstrated. The catalytic rate is inversely proportional to the alkyl chain lengths and exhibits a maximum rate for the shorter chain length. The data fits the Michaelis–Menten parameters for enzyme kinetics.

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Published on the web (Advance View) August 21, 2004; DOI 10.1246/cl.2004.1204