Biocatalytic Membrane Nanosystems (BMNs)

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PIs: **Vadim V. Guliants[1], Neville G. Pinto[1], Jerry Lin[2] and Carol A. Caperelli[1]**
University of Cincinnati[1] and Arizona State University[2]

1 INTRODUCTION, STATEMENT OF THE PROBLEM AND PROJECT SCOPE

Recent advances in molecular engineering of ordered nanoporous (<100 nm) materials with tunable surface chemistries and their integration into macroscopic architectures offer new, unprecedented opportunities to design highly efficient biomimetic systems for applications ranging from biosensing and degradation of nerve agents to bioseparations and industrial biocatalysis [1]. Among such engineered materials, new nanoporous silicas containing ordered nanoscale voids [2,3] are particularly promising hosts for enhancing the biocatalytic efficiency of current immobilized, encapsulated and microbial enzymatic processes, which suffer from a series of drawbacks. These drawbacks include mass-transfer limitations and unpredictable behavior of immobilized enzymes, the difficulty of handling living cells in a large-scale process and concerns about the release of genetically engineered organisms into the environment [1]. Significantly, present immobilized and encapsulated enzyme systems are limited to reactions that do not require co-enzymes and cofactors, severely narrowing the range of potential applications.

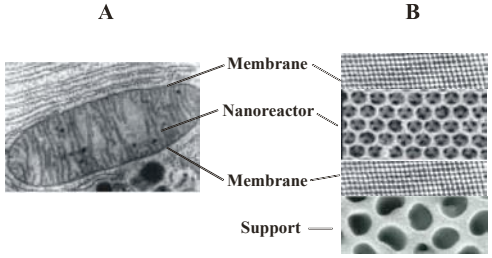

Figure 1. Natural and biomimetic biocatalytic systems: (A) mitochondrion (~5 μm in size) and (B) proposed BMNs.

In order to develop new highly efficient biomimetic systems for multienzyme biocatalysis, the co-PIs are (1) investigating the effects of confined nanoscale environment and surface chemistry of nanoporous silica on biocatalytic functions of encapsulated enzymes in order to (2) integrate this confined environment into the macroscopic architecture of new Biocatalytic Membrane Nanosystems (BMNs). The BMNs will be nano-engineered membrane reactors containing encapsulated enzymes that will offer significant efficiency advantages over present biocatalytic systems (Figure 1B). The fundamental relationships between the pore structure and surface chemistry of nanoporous silica and biocatalytic properties of encapsulated enzymes are being investigated for the major classes of enzymes, respectively, *with and without the requirement of cofactors*. Two types of enzyme-catalyzed reactions represent particularly promising applications: *kinetic resolution of achiral molecules and oxidation of non-activated C-H bonds to afford chiral pharmaceutical intermediates.*

Lipase-catalyzed esterification/de-esterification has been employed for kinetic resolution of achiral molecules. Although lipases *do not require cofactors*, they are activated for biocatalysis upon interaction with a hydrophobic interface or surface, which is systematically investigated in the proposed NIRT research. On the other hand, regio- and stereospecific oxidation of non-activated carbons in organic molecules remains a critical challenge for production of large classes of drugs used in the treatment of the heart disease, AIDS and cancer that are top causes of mortality and morbidity in the world. The P450 family of *oxygenases*, *which require cofactors* is quite adept at catalyzing these chemically difficult transformations.

Thus, these two enzyme systems serve as models for the development of BMNs. Once the "proof of principle" is established, other applications will be pursued.

We are investigating two promising scenarios in the proposed NIRT research: (1) fine-tuning interfacial activation, reactivity and stereo-/regioselectivity of lipases in nanoscale voids via grafting of surface functional groups; (2) fine-tuning reactivity and stereo-/regioselectivity of oxygenases by surface grafting or enlargement of cofactors to retain them in the nanoscale voids of the BMNs.

2 RESULTS TO DATE

Our studies to date focused on understanding the lipase-surface interactions and interfacial activation in ordered mesoporous silica hosts [4,5]. Ordered mesoporous SBA-15 silicas are a promising class of host materials for confining our enzymes to the nanoreactor layer of the BMN device. We explored the effect of pore size and surface curvature on the lipase-surface interactions, i.e. immobilization, and catalytic activity employing dense nonporous silica spheres (330 nm diameter) and ordered mesoporous SBA-15 as model hosts. The effect of the functional groups (-NR_3^+, -NH_2, and *n*-alkyl, etc.) attached to the surface of SBA-15 silica was also investigated.

Methods: In this study, SBA-15 with different pore sizes (55, 80, 240Å) was synthesized by reported approaches [6] and characterized by SEM, TEM, SAXS and nitrogen porosimetry. Charged polar (-NR_3^+, -NH_2) and uncharged hydrophobic groups (*n*-butyl and *n*-octyl) were attached to the surface of SBA-15 as described in [7]. Subsequently, lipase (Amano Enzyme Inc., research grade) was immobilized in these hosts by adsorption from aqueous solution at pH 4.3 and 7.0. The high purity of as-received enzyme (>95% 26 kDa protein) was confirmed by SDS-PAGE, UV-vis and BCA methods. The lipase concentration in solution before and after immobilization was determined by the BCA method. The catalytic activity of immobilized lipase was investigated in the hydrolysis reaction of *p*-nitrophenol acetate (50-1500 _M) at pH 7 and 23 ^{0}C which produces yellow *p*-nitrophenolate with a strong UV-vis absorption maximum at 400 nm (Figure 2). The hydrolysis reaction was studied as a function of substrate concentration at low substrate conversion (<<10%) in order to determine initial reaction rates. These rates were corrected for nonenzymatic hydrolysis rates measured in the absence of the enzyme. The catalytic properties (K_m, V_{max} and k_{cat}) [4,5] of the immobilized and free lipase were determined

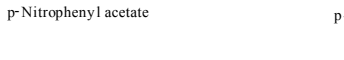

Figure 2. Colorimetric activity assay employed for *Pseudomonas cepacia* lipase.

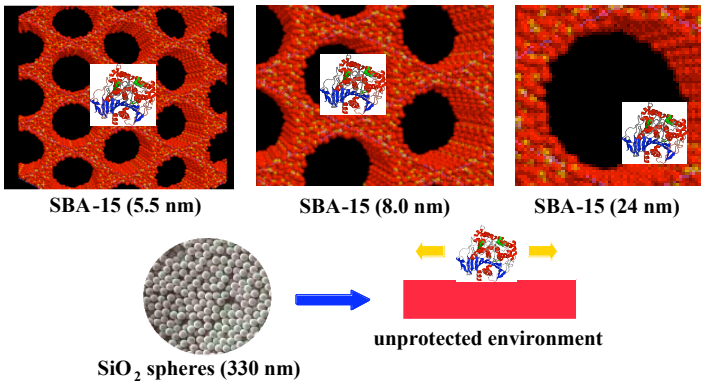

Figure 3. *Pseudomonas cepacia* lipase location in porous hosts.

directly by fitting the initial hydrolysis rates into the Michaelis-Menten equation.

Results and Discussion: The nonporous and porous (SBA-15) silica hosts employed in this study enabled exploring the effects of nanoscale confinement and surface chemistry on biocatalytic activity of immobilized lipase. Figure 3 illustrates the pore size in SBA-15 hosts relative to the size of lipase molecule (~30 x 40 x 50Å). Lipase is expected to be confined close to pore openings in SBA-15 with 55Å pores, while it penetrates much deeper into the pore channels in the case of the 80 and 240Å pore diameter hosts. Accordingly, the loading of immobilized lipase increased with the pore diameter of the SBA-15 silica (Figure 4). Interestingly, the lipase loading on dense silica spheres and the 55Å pore diameter host were very similar for all enzyme concentrations employing confirming very limited access of the 55Å mesopore channels to lipase molecules of comparable size. On the other hand, K_m, the Michaelis-Menten constant, was essentially unaffected by the immobilization process suggesting that the

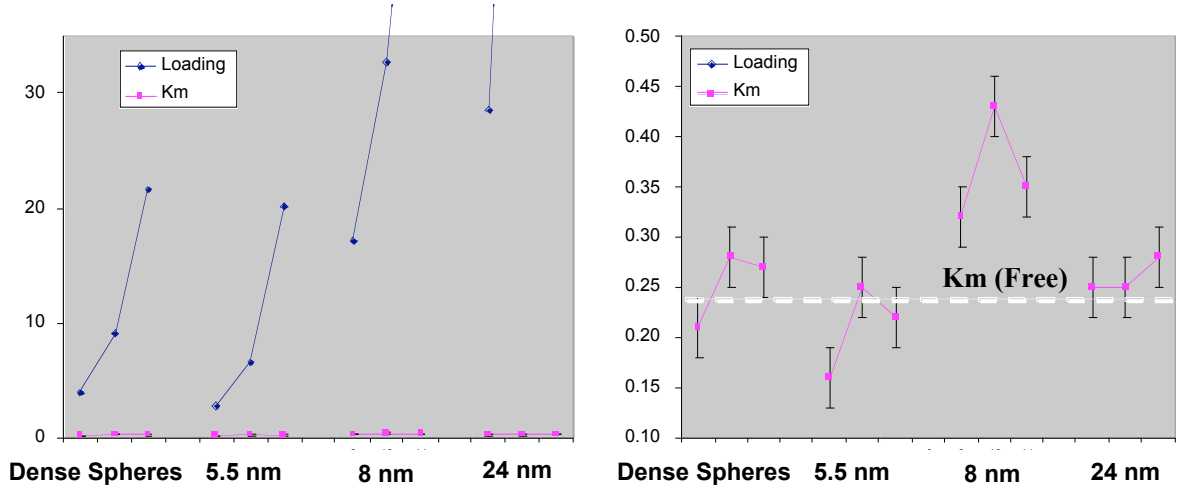

Figure 4. *Pseudomonas cepacia* lipase adsorption (mg/g) and K_m (mM) of lipase immobilized at 1, 4 and 9 mg/ml concentration.

triad active site of this enzyme remained relatively unperturbed by the surface interactions (Figure 4). However, more significant activity (V_{max} and k_{cat}) and specificity (k_{cat}/K_m) differences were observed for lipase immobilized on various silica hosts (Figure 5). Significant loss of catalytic activity and specificity was observed for lipase immobilized on dense silica spheres and the 55Å SBA-15 host as a function of enzyme loading indicating that some enzyme lost catalytic activity upon immobilization. This may be due to significant conformational changes associated with denaturing, active site blockage due to immobilization and mass-transfer limitations in the case of the 55Å SBA-15 host. Much smaller changes were observed for SBA-

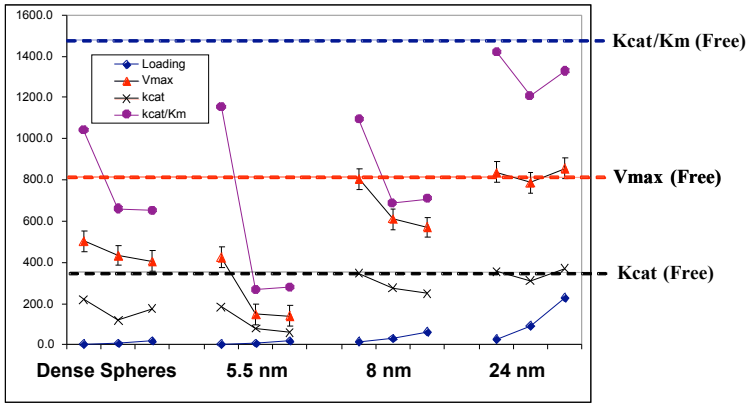

Figure 5. The biocatalytic activity and specificity of *Pseudomonas cepacia* lipase immobilized at 1, 4 and 9 mg/ml concentration.

15 with larger pore diameters suggesting that both surface curvature and pore accessibility play an important role in controlling the activity of this enzyme upon immobilization.

Lipase immobilized in the 240Å pore diameter host displayed the most favorable biocatalytic characteristics and this host was employed further to study the effect of the surface chemistry. Some preliminary results obtained for this host containing the surface hydrophobic alkyl and hydrophilic charged groups are shown in Table 1. These data demonstrated that lipase strongly favored hydrophobic surfaces manifested in both higher loadings and V_{max} parameters, while it displayed significantly decreased affinity and activity after immobilization on hydrophilic surfaces containing positively charged functional groups. These observations strongly suggested that the pore surfaces of the confining membrane layers should be functionalized with charged groups to prevent lipase immobilization and pore blockage, while hydrophobic surfaces of the nanoreactor layer are highly promising for achieving high biocatalytic activity of the BMN devices employing immobilized lipase. Future work will focus on integrating a nanoreactor containing optimized surface functional groups, cytochrome and its co-factor into the BMN architecture and investigating the biocatalytic efficiency of these devices in selective oxidation of pharmaceutical intermediates.

Table 1. Biocatalytic activity of *Pseudomonas cepacia* lipase immobilized in functionalized SBA-15 hosts containing 240Å pores.

Functional group	[Lipase] mg/ml	Lipase loading mg/gm	Vmax U/mg	Km mM
C4- SBA-15	1	67	769 ± 58	0.33 ± 0.05
92% SiOH functionalized	4	234	828 ± 69	0.34 ± 0.03
C8- SBA-15	1	52	759 ± 47	0.32 ± 0.04
22% SiOH functionalized	4	223	814 ± 66	0.34 ± 0.05
NH_2Pr-SBA-15	1	18	611 ± 23	0.22 ± 0.06
	4	21	675 ± 37	0.23 ± 0.04
Me_3N^+Pr-SBA-15	1	14	536 ± 26	0.24 ±0.04
	4	29	599 ± 44	0.27 ± 0.07
Free enzyme		-	815± 60	0.24±0.02
SBA-15	1	32	898±122	0.25±0.02
	4	85	899±120	0.25±0.02

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