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PERSPECTIVE

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Perspective of Recent Progress in Immobilization of Enzymes

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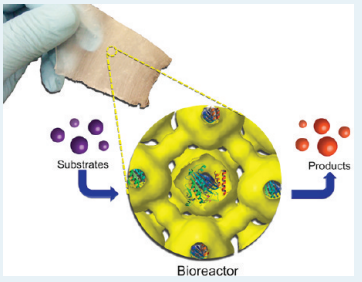
ABSTRACT: The commercial application of biocatalysts depends on the development of effective methods of immobilization. The immobilization of enzymes greatly increases the stability of enzymes and eases the burden of enzyme cost and thus, is widely pursued for efficient, selective, and environmentally friendly catalysis. This brief perspective focuses on recent development in the area of enzyme immobilization in porous materials. Recent work regarding the immobilization of enzymes in inorganic mesoporous materials as well as the modifications to those materials is summarized in this paper. The configuration of supported enzyme as membranes and fibers may facilitate their application in areas that require a biocatalytic process. Enzymes immobilized in or on fibrous membranes provide high surface area for high throughput biocatalysis. These membrane bioreactors also allow for biotransformations to be carried out within a continuous flow process while maintaining enzyme stability under operating conditions as a result from the enzyme immobilization. A summary of efforts to prepare immobilized enzymes in fibers and electrospun fibers will also be discussed.



KEYWORDS: enzyme immobilization, enzymes, CLEAs, mesoporous, electrospinning, biocatalysis

INTRODUCTION

Professor Victor Lin had a significant impact on the development of mesoporous materials for biomedical and biofuel production. In memory of his lasting contributions we have prepared a short review of recent progress in the area of enzyme immobilization in mesoporous materials, as well as the configuration of supported biocatalysts as membranes and nanofibers. The interest in biocatalysts for chemical production continues to grow because they generally have high stereo-, chemo-, and regioselectivity. They also offer an efficient and environmentally friendly catalyst without the need of high pressures, temperatures, and harsh chemical environments. However, even with these advantages, the practical use of enzymes is limited. While enzymes exhibit efficient catalytic activity under mild conditions, that is, in ambient temperatures and aqueous media, the stability and activity is also limited to operation under those conditions. The fragile nature, high cost, and high loadings required for commercial production limits the use of free enzymes. Enzyme immobilization is utilized to surmount the stability, recovery, and recyclability disadvantages of using enzymes in solution, making them industrially and commercially viable. There are too many strategies for immobilizing enzymes on organic and inorganic supports to summarize here. However, an approach that continues to be more widely explored is the immobilization of enzyme in ordered mesoporous materials. Up until the discovery of ordered mesoporous silica by Mobil in 1992,[1] the immobilization of enzymes on inorganic supports was limited to oxide surfaces or encapsulation in sol gels. We recognized mesoporous silicas (3−10 nm) were in the size range of many different enzymes. We were able to demonstrate that small enzymes like trypsin, papain, and cytochrome c were readily adsorbed in MCM-41 type materials.[2,3] In the last 15 years there have been hundreds of papers that have followed this general approach. The progress in this area has been summarized in several reviews.[4−7] In the present paper, the focus will be on advances in the past few years as well as perspective on opportunities for future development.

To best describe the recent progress in enzyme immobilization in mesoporous materials, it is necessary to organize the work by the method of immobilization. The approaches to immobilization of enzymes can be categorized into three types: noncovalent adsorption, encapsulation, or covalent attachment, each with their own advantages and disadvantages. Regardless of method, the essential function of immobilization is for the optimization of catalytic activities of immobilized enzyme. This is usually realized through improved thermal and environmental stability, mainly the prevention of denaturation resulting in deactivation. Immobilization also serves to insolubilize the enzymes making it easier for recovery with potential for reuse.

The adsorption of enzymes onto a support is one of the most basic methods of enzyme immobilization. It involves physical surface interactions between the support matrix and the enzyme and can be driven by combined hydrogen bonding, electrostatic forces, and hydrophobic effects. There is a dependence on the isoelectric point for enzyme and support. Often, these physical interactions alone are not enough to keep the enzyme from desorbing from the support during catalysis. Encapsulation is the physical confinement of the guest enzyme into a host support matrix. This immobilization method is useful as leaching and excessive denaturing is reduced. The matching of support material and enzyme size must be carefully considered. Enzymes with sizes equal to or larger than that of the host system will have lower loadings and simply adsorb on the external surface while those with sizes much smaller than the host material will also

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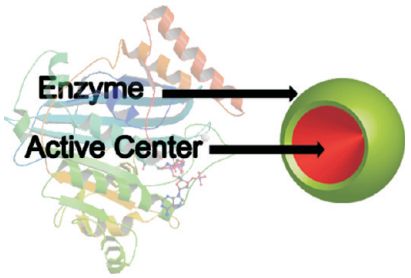

Figure 1. Representative illustration of an enzyme (green) with active site (red).

freely leach from within the host. Covalent attachment involves binding amino acid residues ($-NH_2$, $-CO_2$, $-SH$) of the enzyme to the support matrix. This method is popular for high surface area support matrixes with large pore diameters where substrate and product can freely diffuse without the worry of enzyme leaching. Unfortunately, the covalent attachment of enzymes to support generally lowers activity of the enzyme. While these immobilization strategies have their distinct advantages and disadvantages, they can be combined or modified to overcome their limitations to various degrees. This paper will attempt to summarize recent efforts made in enzyme immobilization for stable biocatalysis in mesoporous support. Additionally, the practical applications of these immobilized enzymes may require the configuration as membranes or fiber mats. Therefore, we will also explore enzymes supported in membranes and electrospun fibers.

■ ADSORPTION

When adsorbing enzymes onto inorganic supports, many factors must be taken into consideration. As the driving forces of adsorption are physical interactions, appropriate support matrix properties are crucial to successful adsorption. A variety of inorganic supports have been used for the immobilization of enzymes.[8,9] However, ordered mesoporous silicates (OMS) have received the most attention. These materials are appealing for enzyme immobilization because they possess uniform pore sizes, large surface areas, and are easily functionalized while providing a thermally and chemically stable support. The immobilization of enzymes in these supports has shown great promise in providing an efficient and compatible support matrix for biocatalysis. The first reported immobilization of enzymes on mesoporous supports was primarily based on simple adsorption from solution.[2] Since then, many advances have been made from the simple adsorption of enzymes into mesoporous materials, with a focus on modifying the silica based molecular sieves SBA-15 and MCM-41.

Figure 1 illustrates an enzyme with active site that would require specific orientation in the mesopore to be accessible. There is little control over the orientation unless certain protein residues interact more favorably with the pore walls resulting in a preferred orientation. The pore size might also force a certain orientation if the enzyme is not spherical and if there is not a tight fit. An important factor when adsorbing enzymes in OMS materials is the pore opening diameter. It is obvious that enzymes that are larger than the pore opening cannot fit through the pore openings in their active state as shown in Figure 2B. However, it has also been shown that materials with pore openings much larger than the enzyme size had an adverse effect on enzyme activity.[10] Enzyme loading efficiency and retention is clearly dependent on the size matching between enzyme and host matrix pore diameter. With pore diameters smaller than enzyme diameter, adsorption is restricted to the external surface of the OMS, and the interior high surface area is not accessible. Such is the case with glucose oxidase (4−6 nm) and MCM-41.[11] The smaller MCM-41 pore diameter (3.36 nm) prevents glucose oxidase (4−6 nm size) adsorption in the inner pore but also can hinder substrate and product diffusion for any enzyme that might adsorb in the inner surface. In contrast, materials with pore diameters much larger than the adsorbed enzyme are highly susceptible to leaching during operation (Figure 2D). By matching pore diameters with enzyme sizes, leaching may be reduced and additional stability is observed (Figure 2C). When adsorbed onto OMS materials, enzymes have been shown to maintain high levels of activity even after long periods of storage.[12] For example, horseradish peroxidase adsorbed on SBA-15 demonstrated high thermal and environmental stability.[13] The molecular sieve mesopores give the enzyme additional stability by limiting exposure to environmental factors and restricting excessive denaturing to within the confined inner pore volume. When exposed to high temperatures (70 °C) for 30 min, the adsorbed horseradish peroxidase enzyme did not lose any activity while free enzyme retained only 17% of original activity. The adsorbed horseradish peroxidase enzyme also showed a high stability, retaining 80% of activity when exposed to 5 M of urea, a known denaturing agent. There may also be a loss in activity if the outermost enzyme inhibits access to enzymes deep in the pores. Supports like SBA-15 and MCM-41 have one-dimensional (1-D) pores which could limit enzyme loading if an adsorbed enzyme blocks the end of the pore. There have been surprisingly few studies dealing with three-dimension pore architectures which would reduce diffusional and orientation issues.

■ ELECTROSTATIC INTERACTION

For an efficient loading of enzymes, the electrostatic interaction between OMS support and enzyme must also be considered. This is usually controlled by the enzyme solution pH when mixing with support. Typically, enzyme loadings are much higher when electrostatic forces are maximized by having the pH of solution below the isoelectric point of the enzyme and above that of the support material. In this situation the enzyme has a net positive charge and the support material a net negative charge resulting in attractive electrostatic forces.[14] At a pH either above or below the isoelectric point of enzyme and support, a similar charge will lead to repulsive forces and a much lower enzyme loading.[13] For example, this was observed when β-glucosidase was adsorbed on SBA-15.[12] The enzyme loading was highest at pH 3.5, between the isoelectric points of β-glucosidase (pI $\sim$ 4.5) and SBA-15 (pI $\sim$ 2). At higher pH the enzyme is either neutral or negatively charged which prevents high levels of adsorption. Not only was the optimum pH important for loading, it was found that catalysis at higher pHs would result in leaching of the enzyme in to the supernatant. In contrast no leaching was observed at pHs between both isoelectric points.

■ SURFACE MODIFICATION

The effects of pore size and enzyme-support material interactions have been demonstrated to have significant roles in the loading efficiency of enzymes and their activity. Surface modification of the support could further enhance interactions between the pore walls and the enzyme, greatly affecting stability, reactivity,

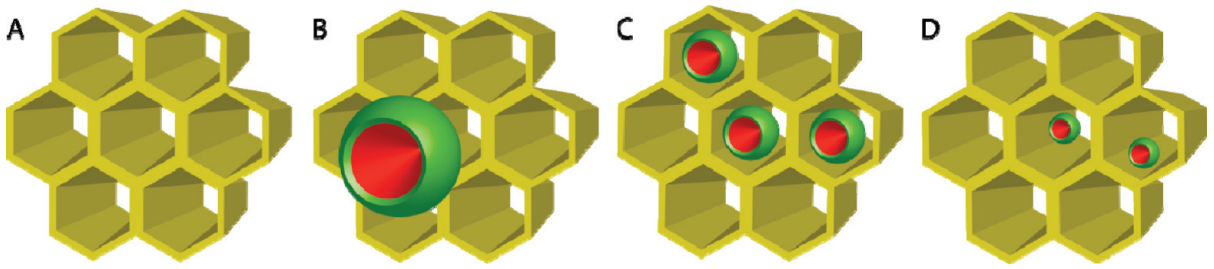

Figure 2. (A) Representative image of ordered mesoporous silicate (OMS). (B) Depicts the situation where an enzyme is too large to fit inside of the pores and is mainly adsorbed in the outer surface of the OMS. (C) Portrays enzyme adsorption in the inner pore volume when pore diameter and enzyme size matching is achieved. (D) Depicts the opposite of case B, where the pore opening is much larger than the enzyme and is susceptible to leaching.

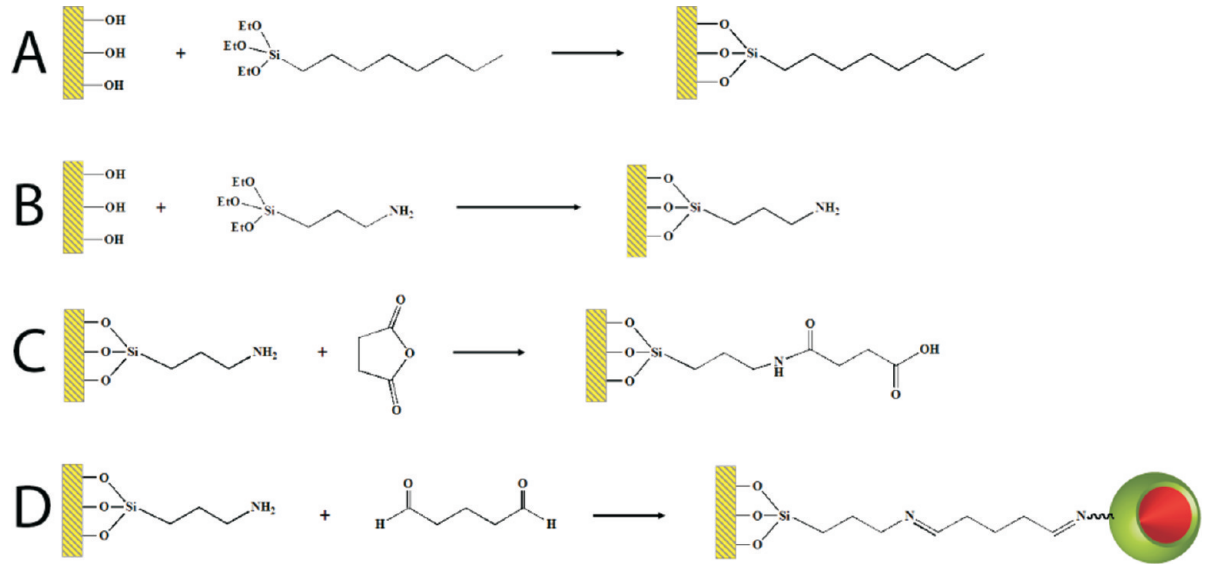

Figure 3. (A–D) Represents the functionalization of OMS material surface silanols with various functional groups. Panel A is the hydrophobic functionalization by attachment of octyltrimethoxysilane. Panel B represents the hydrophilic functionalization with the addition of 3-aminotrimethoxysilane (APTES). Panel C represents the subsequent treatment of APTES functionalized OMS with succinic anhydride for carboxylate functionalization. Panel D represents the cross-linking of APTES functionalized OMS with external anime groups found on enzymes.

and recyclability of enzyme reactors. OMS materials provide a unique opportunity as they contain surface silanol groups that can be functionalized with a variety of organic linkers, including amine, carboxylate, phenyl, and alkyl groups. These functional groups can act to strengthen van der Waals interactions or serve as anchoring points for covalent attachment of enzymes.

One of the most common functionalization methods for OMS involves organosilanes, such as of 3-aminopropyltriethoxysilane (APTES) (Figure 3B). The amine functionalization of OMS has two possible effects. First, at lower pH, the amine is protonated promoting the adsorption of hydrophilic enzymes. For example, penicillin acylase has been immobilized in APTES functionalized SBA-15 in this manner and demonstrated a 26% higher enzyme loading.[15] Similarly, immobilization of laccase on propyl- and butyl-amine functionalized SBA-15 maintained high activity, attributed to higher enzyme loadings.[16] Second, APTES can be used to decrease the OMS pore size after adsorption, eliminating leaching while allowing higher loadings. Manyar et al. immobilized porcine pepsin in SBA-15 and post treated resulting in a pore size decrease from 6.0 to 5.4 nm. This pore diameter decrease reduced pepsin leaching from 20% to 7.8%.

The functionalization of OMS materials with hydrophobic groups such as in Figure 3A, is commonly done for the immobilization of enzymes such as lipases. Access to the active sites in the internal structure of some lipases (*Rhizomucor miehei, Humicola lanuginose*) is limited by a hydrophobic lid structure. By increasing the hydrophobicity of support materials, a favored active conformation of the enzyme is induced. It has been reported that octyl functionalized SBA-15 can adsorb lipase in a monolayer fashion with high activities in an ethanol cosolvent.[17] While the increased hydrophobicity increases activation of immobilized lipase, it hinders the adsorption in aqueous solution. However, when a small amount of ethanol was included during adsorption, the monolayer capacity of enzyme in SBA-15 material doubled while activity improved 5-fold. A recent study by Sang et al describes how surface of propyl funtionalized SBA-15 affects enzyme confirmation and activity of immobilized enzymes.[18] When lysozyme was adsorbed into funtionalized SBA-15, a dramatic decrease in catalytic activity was observed when compared to enzyme adsorbed into similar pore sized non funtionalized SBA-15. From this they concluded that the hydrophobic nature of the support matrix is the dominant factor in adsorbed enzyme conformation.

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While many studies of enzyme immobilization in inorganic materials have been mainly focused on the functionalization of OMS, other nanoporous materials have also emerged with promising applications toward enzyme immobilization for catalysis. Mesoporous carbons,[19−23] mesoporous and macroporous zeolites,[24,25] periodic mesoporous organosilicas (PMO), mesocellular foams, and metal organic frameworks[26] (MOFs) have many of the desirable properties found in OMS materials. The PMO materials are potentially interesting because of the ability to control the surface properties, including optical and electrical. PMOs are inorganic−organic hybrid materials that have also been studied for enzyme immobilization.[27] Similar to functionalized OMS, PMOs have terminating bridging and surface organic functional groups in the framework structure that provide the same activation and stabilization effects. Lysozyme has been shown to exhibit remarkably high adsorption on a variety of PMOs synthesized with hydrophobic precursors. Li et al. showed the strong adsorption of lysozyme onto PMOs synthesized with 1,4-diethylenebenzene in the pore walls.[28] Work by Park et al. further demonstrated that surface modification could be performed so that hydrophobic interactions are the driving force for lysozyme adsorption.[29] Lysozyme was adsorbed onto PMOs synthesized with bis[3-(trimethoxysilyl)propyl] amine (BTMS-amine), 1,4-bis(triethoxysilyl)benzene (BTES-benzene), 4-bis(triethoxysilyl) biphenyl (BTES-biphenyl) precursors. BTMS-benzene and BTES-biphenyl adsorbed higher amounts of lysozyme with little leaching observed, while BTMS-amine adsorbed almost no enzyme.

COVALENT ATTACHMENT

Another effective immobilization technique is the covalent attachment of enzymes to a mesoporous support. The major drawback to covalent attachment is lowered activity of the enzyme after attachment. This, however, is offset by the enhanced thermal and environmental stability during catalysis. Thus a covalently immobilized enzyme in a bioreactor can withstand prolonged periods of use and recovery. Covalent attachment of enzymes to OMS materials typically begins with the functionalization of the silanol surface with amines (APTES). After the enzyme is adsorbed on to the functionalized surface, glutaraldehyde is introduced which covalently links the OMS surface amines with enzyme surface amine residues, typically lysine. Figure 3 shows the common surface functionalization techniques used on OMS materials. For example, the covalent attachment of penicillin acylase onto APTES functionalized mesoporous silica KIT-6 (7.2 nm) yielded a high loading and specific activity.[30] The specific activity of penicillin acylase immobilized on KIT-6 reaches to 3522 IU/g of dry support with only an 11% specific activity loss after five recycled uses. The high specific activity was seen for large pore systems where more enzymes could be attached to the interior high surface area pores. This is a vast improvement over the noncovalently attached enzymes in larger pore diameter materials discussed earlier. The larger pores allow not only for higher enzyme loading, but provide a greater surface for substrate and product diffusion resulting in higher specific activity. In another report, *Candida rugosa* lipase was successfully adsorbed onto SBA-15 and stabilized in a chitosan "mesh."[31] Glutaraldehyde was used to covalently attach the amine groups of chitosan to form a large and stable network that covered both the external and the internal surfaces of the mesoporous material. Lipase was simultaneously covalently attached to the chitosan network during the glutaraldehyde treatment. The cross-linked lipase exhibited approximately 4.5 times higher activity than free enzyme compared to the 56% activity of the lipase adsorbed in SBA-15. This can be the result of the hydrophilic environment of unfunctionalized SBA-15 and amine residues of chitosan which aid in formation of an active conformation that exposes the hydrophobic core, which is known to contain the active center, to substrates. The operational stability of these bioreactors was greatly improved as they retained 80% of their activity after six cycles of reuse.

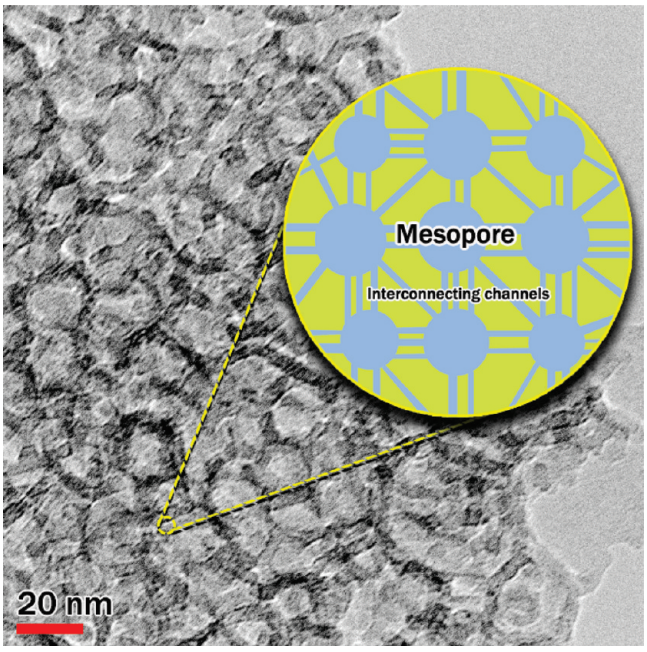

Figure 4. TEM of mesocellular foam with an inset representing the internal mesopore structure with interconnecting channels.

Mesocellular foams (MCF) are of particular interest as they have cage-like mesopores (20−40 nm) that are interconnected as shown in Figure 4. With an extremely high surface area and silanol surface groups, MCFs can be functionalized similarly to other OMS. The covalent attachment of enzymes in these larger pores allow for higher loading and greater access to the support material without the loss of activity from leaching. Alkaline serine endopeptidase (ASE) and invertase have been covalently attached to MCFs and shown to be more active than free enzyme and the SBA-15 immobilized enzyme.[32,33] A higher temperature and pH tolerance is observed, and over 80% of initial activity is retained in the covalently attached ASE after 15 cycles of reuse. This high activity is due to the high accessibility of substrate to the active site from the large pore openings in the mesocellular foam structure. The entrapment of enzymes in OMS materials like MCM-41 or SBA-15 generally involves the adsorption in uniform 1-D pores such that the enzyme could leach out unless there is some interaction with the pore wall or the pore opening is partially blocked. The MCF materials have cage like structures (Figure 4) such that it may be possible to prepare "ship-in-a-bottle" type catalysts by cross-linking more than one enzyme such that it becomes too large to exit through the cage windows. The cross-linking of enzyme aggregates can form surprisingly stable catalysts.

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Table 1. Recent Methods Used to Immobilize Enzymes for Catalytic Reactions

types of reactions	relevant enzymes	methods	reference
hydrolysis	β-glucosidase	adsorption	12,96
	cellulase	adsorption CLEA	12,23,48,97
	lysozyme	adsorption	18,28,29,98,99
	urease	adsorption	100
	lipase	adsorption CLEA	17,19,31,41,42,45,46,51,101−109
	alkaline serine endopeptidase	adsorption	32
	penicillin acylase	adsorption CLEA	15,30,45,47,110−112
	pepsin	adsorption	113
	invertase	adsorption	33
	papain	CLEA	83,84
	phytase	CLEA	34
	α-amylase	adsorption CLEA	29,83,115,116
	phospholipase	CLEA	49
	pectinase	CLEA	48
	xylanase	CLEA	48
	α-chymotrypsin	adsorption	101,117
	hydroxynitrile lyase	CLEA	31,32,43
	trypsin	adsorption	118
	alkaline serine protease	CLEA	119
oxidation/reduction	glucose oxidase	adsorption CLEA	11,23,34,59,60,120−123
	monooxygenase	adsorption	10
	horseradish peroxidase	adsorption CLEA	13,43,59,105,120
	galactose oxidase	CLEA	34
	laccase	adsorption CLEA	16,34,39,105
	tyrosinase	CLEA	44
	formaldehyde dehydrogenase	CLEA	34
	alcohol dehydrogenase	CLEA	34
	chloroperoxidase	adsorption CLEA	38,60,124,125
	cytochrome c	adsorption	14,126
	microperoxidase-11	adsorption	27
	hemoglobin	adsorption	58
	glutaminase	adsorption	127
	myoglobin	adsorption	18,128
	superoxide dimutase	adsorption	129
other	carbonic anhydrase	adsorption	57
	hydroxynitrile lyase	CLEA	36,37,50
	nitrile hydratase	CLEA	40,50

CROSS-LINKED ENZYME AGGREGATES (CLEAS)

Many of the large pore supports such as the MCF materials require cross-linking to immobilize the enzyme. The cross-linking of enzyme aggregates itself may or may not require a solid support. CLEA immobilization diverges slightly from conventional immobilization methods. Where typical immobilization would involve the entrapment or binding to a support material, CLEAs are formed when aggregation and precipitation is induced by the addition of salts, organic solvents, nonionic polymers, or acids followed by cross-linking.[34] Each step must be optimized to maximize recovery without adversely affecting activity. First reported by Cao et al.,[35] CLEAs retained activity comparable to cross-linked enzyme crystals without the burden of having to obtain highly purified enzymes and crystallizing them. CLEAs can be highly efficient biocatalysts with enhanced thermal and environmental stability.[36−38] The capacity for prolonged storage and remarkably high reusability of CLEAs has been demonstrated.[39,40] In many cases, CLEAs catalytic activity exceeds that of free enzyme.[38,41] This hyper activation is often seen for CLEAs when reactions occur in organic solvent.

ADDITIVES

The coprecipitation of polyionic polymers with CLEAs has interesting effects on the activity of the CLEAs. Common additives used such as bovine serum albumin (BSA), polyethyleneimine (PEI), and dextran sulfate can affect enzyme activity.[42,43] For example, tyrosinase CLEA activity was vastly improved by the addition of BSA.[44] Without BSA, CLEA activity retained only 31% while CLEAs prepared with BSA exhibited 101% activity. With the addition of BSA, the lysine concentration also increases, providing more cross-linking sites maximizing aggregate recovery and forming CLEAs even in low enzyme concentrations. The BSA also serves to protect the enzyme from extensive cross-linking preserving its activity.[45] Indeed long storage stability and

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operational stability has been observed. For example, CLEAs with BSA were stable up to 3 months in storage and could be easily reused for 10 cycles without activity loss. Furthermore, hyperactivation was observed at higher temperatures (45 and 55 °C). Hyperactivation is not only seen in the case of BSA coaggregates but also when PEI and dextran sulfate are incorporated in the CLEA.[46] Under optimal conditions, penicillin acylase realize a 300% activation in ethylene glycol that remains unchanged for over 800 h.[47]

COMBI-CLEAS

The concept of incorporating multiple noncascading reaction enzymes into one CLEA was explored by Gupta et al. with CLEAs comprising pectinase, xylanase, and cellulase.[48] The stable biocatalysts were successfully coaggregated from the commercial Pectinex Ultra SP-L. All three independent enzymatic activities in the CLEA were observed with improved half-life and were usable four times without any activity loss. Similar previous work demonstrated that combi-CLEAs consisting of lipase α-amylase and phospholipase A_2 all retained activity comparable to the free enzyme.[49] With a series of nontandem catalytic reactions that could efficiently operate in a CLEA setting, it would be natural to assume that it would also be viable for tandem reactions as seen in the case with adsorbing enzymes that catalyze tandem reactions in OMS. It was then shown that aliphatic (S)-α-hydroxycarboxylic amides could be synthesized by combi-CLEAs consisting of hydroxynitrile lyase and nitrile hydratase in tandem.[50] The reaction conditions were optimized to prevent racemization and resulted in yields with high enantioselectivity.

CATALYSIS

Table 1 list enzymes and methods of immobilization organized by general reaction type. By far the most widely studied type of biotransformation using OMS type supported enzymes is hydrolysis. Some of this interest is driven by the growing need for alternative fuel sources. Typical biodiesel production involves the transesterification of lipids to alkyl esters and glycerol. Enzymes like lipase appear to be more stable and active in organic solvents when immobilized. For example, porcine pancreas lipase was shown to have specific activity three times higher for the transesterification of glycerin triacetate after immobilization on SBA-15.[51] The production of bioethanol requires the hydrolysis of cellulosic feedstocks to glucose and cellulase, a combination of three different hydrolytic activities, facilitates this process. One of the challenges is to immobilize the enzyme while not limiting access to insoluble substrates. Cellulase encapsulated in SBA-15 retained 70% activity for the degradation of cellulose to glucose when compared to free enzyme.[52] Immobilization techniques such as the CLEAs, allows substrate and product diffusion. For example, cellulase combi-CLEAs precipitated in *n*-propanol show no loss in activity for hydrolysis of carboxymethylcellulose.[48] Another area of growing importance is the manufacture of fine chemicals, particularly for pharmaceuticals. The production of 6-amino penicillanic acid (6-APA), the β-lactam nucleus of penicillins, is dependent on penicillin acylase immobilization. Penicillin acylase was able to retain 73% activity for the production of 6-APA when immobilized on APTES functionalized SBA-15.[15] Penicillin acylase CLEAs demonstrated 300% activation in glycol when coaggregated with PEI additive.

The second major reaction type carried out by enzymes is oxidation/reduction. Many of the redox enzymes like glucose oxidase and peroxidase are being studied for electrode materials in biofuel cells and biosensors. In this case natural occurring fuels like glucose are converted to gluconic acid for the generation of electrons. The challenge is to effectively immobilize the enzyme while maintaining electrical contact. For example, glucose oxidase that was immobilized on MCM-41 exhibited fast direct electron transfer.[11] An electrode was made from immobilized glucose oxidase on MCM-41 that showed a high affinity and selectivity to glucose. Nitrile hydratase is used for the production of acrylamide from acrylonitrile and nicotinamide[53] from 3-cyanopyridine.[54] CLEAs of nitrile hydratase retained low activity, 21%, however demonstrated high recyclability, 35 reuses without activity loss.[40]

The industrial use of enzymes has grown greatly which effects growing environmental and economic concerns related to the catalytic process. The pharmaceutical company Pfizer has recently discovered that the efficiency and selectivity required for the synthesis of drugs can be achieved with the use of enzymes.[55] The elimination of excess waste, harmful solvents, and disposal cost by utilizing biocatalysis provides a great opportunities in green chemistry. While the replacement of traditional catalysts with enzymes is very appealing, they also have direct applications for the removal of toxic chemicals from the environment. Laccases have potential as green catalysts as they can oxidize phenol, a common decomposition product of organic dyes and chemicals.[56] CLEAs of laccase have been prepared that exhibit hyperactivation with high conversions.[39] The use of carbonic anhydride CLEAs immobilized in SBA-15 can be used to fix carbon dioxide to carbonic acid at rates comparable to free enzyme yet are more stable and recyclable.[57] Hemoglobin can be used to oxidize polycyclic aromatic hydrocarbons (PAH), a widespread carcinogen and organic pollutant.[58] It was found that hemoglobin immobilized in mesoporous silica can remove up to 86% of a variety of PAHs.

Perhaps one of the most exciting developments in enzyme immobilization for catalysis is the development of tandem reactors. Tandem reactors consist of multiple enzymes that are immobilized within the same matrix and catalyze cascade reactions, where the product of one enzyme is the substrate for another in the same system. These tandem reactors provide an advantage where diffusion limitations are bypassed by the close proximity of secondary substrates to enzymes. In a study involving the co-encapsulation of glucose oxidase and horseradish peroxidase in phospholipid-templated silica nanocapsules, it was shown that the co-encapsulation and separate encapsulation on the system were just as efficient.[59] However, the co-immobilization of materials allows for a higher loading per volume without the need for excessive mixing for substrate diffusion. The immobilization of glucose oxidase and peroxidase as tandem biocatalysts has also been reported.[60−62] The tandem system was designed in an attempt to circumvent oxidative deactivation of chloroperoxidase by the limiting H_2O_2 production with immobilized glucose oxidase. With the tandem system, it is vital that the enzymatic activity is balanced as too high H_2O_2 generation results in deactivation of chloroperoxidase while too low H_2O_2 concentration will limit overall activity.

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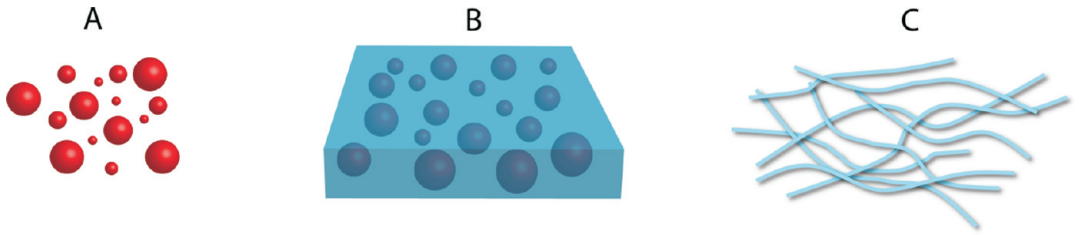

Figure 5. (A) Representative image of porous material with immobilized enzyme. (B) Representative image of immobilized enzyme on porous materials cast into a film membrane. (C) Representative image of unwoven mat of fibers containing immobilized enzymes on porous materials.

MATERIALS AND CATALYSIS OPPORTUNITIES

It is clear that many different types of mesoporous materials have been explored as support materials. Mesoporous silicas and to a lesser extent carbons remain the most popular materials studied. However, there is a vast opportunity to explore different framework compositions that could operate in concert with the biocatalyst to promote new types of reactivity. The mesoporous silicas have been doped with many different catalytically active metal ions. Many of the transition metals will also form templated mesoporous structures that have redox activity. It may be possible to combine the reactivity of the host support with the guest enzyme in a synergistic fashion. For example, an enzyme that produces H_2O_2 like glucose oxidase might be supported in a metal silicate that utilizes peroxide to oxidize olefins or hydrocarbons. We were one of the first to report immobilization of a biocatalyst in a periodic mesoporous organosilica in 2006 but since then there have been relatively few reports of enzyme immobilization in hybrid frameworks. The PMO materials offer the tremendous flexibility in terms of controlling the hydrophilicity/hydrophobicity, acid/base functionality, redox active sites, and chromophores. It may be possible to engineer the pore surface to function as a cofactor and participate in electron transfer reactions. There are also MOF and COF materials with pores in the 2−3 nm range that might be big enough for small proteins. The mesoporous carbons have not been widely studied as supports for enzymes, but the potential electrical conductivity may expand sensor and biofuel cell applications. The combination of microporosity and mesoporosity in the same support might impart additional selectivity. There have been relatively few reports of mesoporous zeolites or zeolites in mesoporous carbon employed as catalyst supports. Is it possible to synthesis a mesoporous phase while encapsulating the enzyme and crystallizing microporous domains at the same time? One might take the mesoporous support to the extreme with metal oxide or chalcogenide nanotubes that can be prepared in many compositions. The pore size of titanium oxide nanotubes can be 3−100 nm depending on the method of preparation. Immobilization in nanotubes might reduce the diffusional limitations of 1-D mesoporous metal oxides. There is great interest in modeling enzymes and proteins in confined spaces. Therefore, it is surprising that the enzymes immobilized in mesoporous materials have not been the subject to any significant computational efforts. Considering the stability enhancement often observed for immobilized enzymes, the role of the support on conformational changes should be relatively easy to model. Mechanistic features of catalytic reactions will be more challenging, but the observed activities of immobilized enzymes merit a better understanding which could be facilitated by a computational study.

Table 1 shows a very limited set of reaction types that have been studied of late. The activity of immobilized enzymes is often based on well-studied assays and there have been relatively few exploratory studies of reactivity, especially in organic solvents. One of the advantages of mesoporous supports is the enhanced enzyme stability that is generally observed. The constraints of the pores or interactions with the pore walls may prevent denaturing under conditions that would normally denature the free enzyme. So there may be many interesting reactions to explore. There are relatively few examples of multiple enzymes combined in the same support. Cellulase is a good example of how nature combines several enzymes to convert cellulose to sugar. There are many opportunities to design and test cascade reactions with multiple enzymes or enzymes with metal complexes. Although, the emphasis is on catalysis, there are also other areas such as sensors, fuel cells, and medicine that may benefit from the mesoporous supported enzymes.

MEMBRANES AND FIBERS

Enzymes immobilized in mesoporous metal oxide or carbon based supports may be used in a variety of reactors as suspensions of the particles. However, this still requires recovery of the particles, and there may be losses as a result of attrition during agitation. The commercial use of membrane reactors either in flat sheet or fibrous form as shown in Figure 5 has been well established. However, the application of mesoporous membranes or the encapsulation of mesoporous supports in polymer matrixes has barely been explored. Table 2 shows a summary of recent examples of enzymes immobilized in membranes or electrospun fibers.

Lee et al reported a mixed matrix membrane on a graphite electrode based on glucose oxidase immobilized in a mesocellular carbon foam (MSU-F-C) dispersed in a Nafion matrix.[63] The electrode was then used as a glucose sensor. The enzyme activity in the mesoporous carbon foam was reported to be comarable to the glucose oxidase enzyme immobilized in a mesoporous silica foam. The conductivity of the carbon support enables bioelectrocatalytic applications. Yang et al reported the immobilization of glucose oxidase in mesoporous silica having 1-D pores and decorated with 3-aminopropylsilane.[64] The silica particles containing the enzyme were dispersed in calcium alginate fibers (CAF). It is possible to incorporate the enzyme in the alginate fibers but eventually they leach from the support. The mesoporous silica was expected to improve the stability of the composite fibers. A flow-through glucose sensor was fabricated from the composite fibers and shown to be more stable and active than the using just the CAF fibers or mesoporous silica containing glucose oxidase.

Table 2. Enzyme Membranes and Fibers

enzyme	support	reference
glucose oxidase	mesocellular carbon/Nafion	63,64,67,73,
	mesoporous silica/calcium alginate fiber	130,131
	mesoporous silica/alumina membrane	
	electrospun fibers	
formaldehyde dehydrogenase	mesoporous silica/alumina membrane	65
catalase	mesoporous silica/alumina membrane	66,92,132,133
	electrospun polymer fibers	
choline oxidase	mesoporous silica/alumina membrane	68,69
acetylcholines-terase	mesoporous silica/alumina membrane	69
lipase	mesoporous silica/Glass tube	70,72,79−82,
	mesoporous silica/Polymer Membrane	84−88,93,
	electrospun polymer fibers	134−136
cellulase	electrospun polymer fibers	89,89
	concentrically electrospun fibers	
β-glucosidase	electrospun polymer fibers	91
α-chymotrypsin	electrospun polymer fibers	90,137,138
hemoglobin	electrospun fibers	95
myoglobin	electrospun fibers	95

Itoh et al prepared an artificial biomembrane by immobilizing formaldehyde dehydrogenase (FDH) in the mesopores of a silica film coated in the channels of a commercial anodic alumina membrane.[65] The 200 nm pores of the alumina were coated by sucking a TEOS/F127 surfactant solution through the membrane as shown in Figure 6. After calcination at 500 °C the pores were reduced to 100 nm by the mesoporous silica film. FDH was immobilized in the 13 nm pores of the silica and then the reduction of NAD^+ to NADH across the membrane was tested. The activity of the enzyme was maintained even after 10 cycles. Itoh and co-workers have also filled the alumina membranes with mesoporous silica where the1-D channels ∼8 nm in diameter run in the same direction as for the alumina support.[66] In this case, a catalase enzyme was immobilized and tested for the decomposition of hydrogen peroxide. The immobilized catalase remained active after 36 days and 160 cycles while the free enzyme activity declined to 16% activity after 120 cycles. In related work a columnar mesoporous silica with pores ∼12 nm in diameter was fabricated in the pores of the anodic alumina, and glucose oxidase was immobilized in the pores.[67] The conversion efficiency for glucose was reported to be 99%, but after 10 days the efficiency dropped to 57%. The same mesoporous silica in anodic alumina membranes were used to immobilize choline oxidase and acetylcholineesterase.[68,69] Electrochemical sensors for hydrogen peroxide were prepared and shown to be stable even after 80 days.

Endo and co-workers have developed a method for coating mesoporous silica thin films on microreactor walls.[70,71] Borosilicate microcapillary tubes 200 μm in diameter and 20 cm long were filled and with an SBA-16 precursor solution and then heated at 70 °C for 8 h followed by calcinations at 440 °C for 4 h. The resulting 120 nm thick films showed well-ordered three-dimensional (3-D) pores ∼8 nm in diameter as shown in Figure 7.[71] Lipase was immobilized in the SBA-16 film by adsorption from solution for 12 h at 5 °C and then tested for the

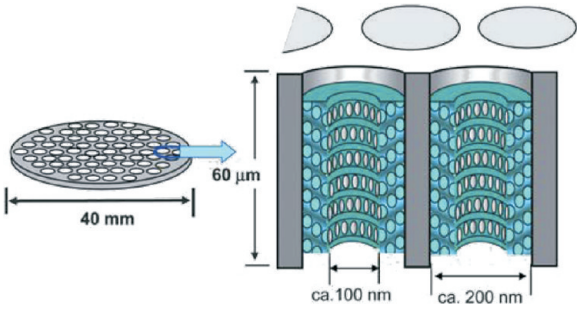

Figure 6. Schematic diagram of an alumina membrane and silica−alumina composite membrane. Reproduced with permission from ref 65. Copyright 2011 Royal Society of Chemistry.

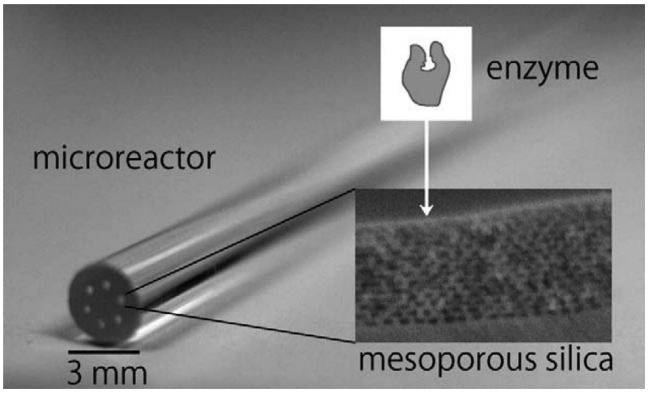

Figure 7. Diagram of a microreactor with mesoporous silica. Reproduced with permission from ref 70. Copyright 2009 Elsevier.

hydrolysis of 4-nitrophenol acetate in a continuous flow.[70] The rate constant derived from a fast flow experiment (12 μL/min) was almost the same as the free enzyme.

Matsuura et al. have reported a new route to enzyme microreactors by dispersion of mesoporous silica particles in a 0.1 mm thick polydimethylsiloxane (PDMS) film followed by heating at 85 °C for 3 h.[72] Lipase (*Phycomyces nitens*) was encapsulated by exposing the film to a solution containing 0.2 mg/mL at a rate of 10 μL/min. The enzyme activity for hydrolysis of a fluorescent triglyceride was measured in the flow cell and found to be greater than the free enzyme. This was attributed to prevention of aggregation and favorable interactions with the silica surface.

Kim et al has recently demonstrated glucose oxidase immobilized on a polyaniline (PANI) nanofiber matrix.[73] Aside from adsorbing on the PANI matrix, glucose oxidase was also precipitated with ammonium sulfate before cross-linking. The precipitation step allowed for higher enzyme loading, and almost no activity loss was seen after 60 days of operation, compared to free enzyme which lost 80% of its relative activity after the same period. The stability improved at higher temperature (50 °C) for up to 4 h with almost no activity loss while free enzyme lost half of its activity. While this particular system was specifically designed for biofuel cell applications, the incorporation of other enzymes could lead to other novel catalysis opportunities.

While the reactor designs described above show promise for microscale applications, the development of commercial processes will require reactors that can be fabricated on a large scale. Though microscale hollow fiber membranes are attractive[74] as

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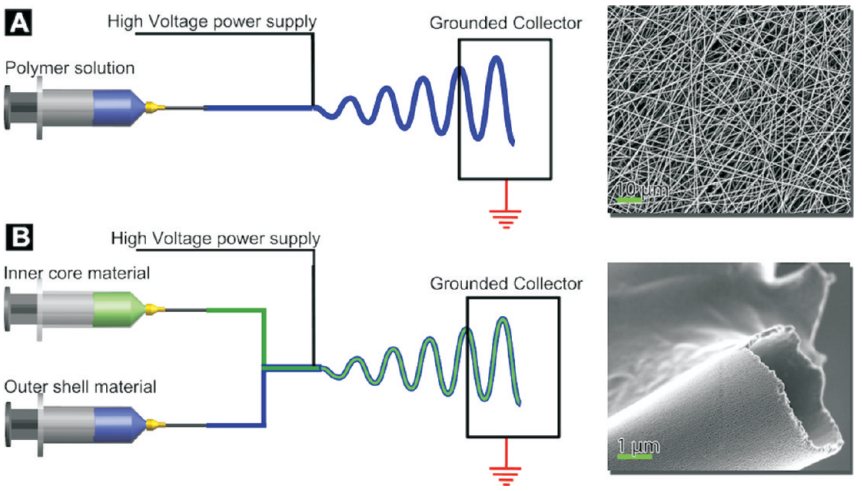

Figure 8. (A) Electrospinning setup with a syringe pump, power supply, and grounded collector. To the right is an SEM image of poly(ethylene oxide) fibers obtained from electrospinning. (B) Concentric electrospinning setup with two independently controlled syringes coaxially aligned to control the flow rate of inner core (green) and outer shell (blue) materials. To the right is an SEM image of the open end of hollow polystyrene fiber obtained from concentric electrospinning.

enzyme supports because of the high surface to volume ratios, nanofibers could dramatically improve on that aspect. There is growing interest in enzyme immobilization on electrospun nanofibers.[75]

Electrospinning is the process in which micrometer to nano-sized fibers can be fabricated through nonmechanical fiber-drawing from a wide range of materials.[76,77] The application of electrostatic forces to a polymer solution fed through a needle causes the polymer to form a Taylor cone and undergo a whipping action.[78] The whipping motion induces stretching of the polymer which increases the surface to volume ratio, expediting the evaporation of the solvent. The resulting fibers can be collected as a nonwoven mat of submicrometer sized fibers as shown in Figure 8A. It is also possible to prepare hollow fibers or core−shell type structures by using concentric needles and feeding two incompatible solutions to the needle tip as shown in Figure 8B.

There are many attractive features of electrospun nanofibers including tunable size and morphology as well as the compositional variance. Figure 9 shows the possible configurations of electrospun nanofibers as enzyme supports. The electrospun fibers may be pure OMS materials, polymers or composites. An enzyme could simply be adsorbed (Figure 9A) or covalently bound to the spun fibers (Figure 9B). An enzyme or an OMS particle containing an enzyme could be directly electrospun (Figure 9C). The electrospun fiber could be dense or porous. The porosity is achieved in polymer fibers by using a mixed solvent system, where one solvent evaporates quickly leaving the pores (Figure 9D). Finally, the core−shell approach allows one to encapsulate almost any material in the core as long as the shell forms a good fiber (Figure 9E).

There are a few examples of immobilization via post spinning modification (Figure 9A and B). For example, Wang et al reported the immobilization of Lipase (*Candida rugosa*) on polysulfone (PSF) composite fibers prepared by electrospinning.[79] The polysulfone was mixed with poly(*N*-vinyl-2-pyrrolidone) or poly(ethylene glycol) to enhance biocompatibility and electrospun to form nonwoven mats with fibers ∼200−500 nm in diameter. The lipase that was adsorbed from solution onto the PSF fibers showed a higher optimal operating temperature and was more stable than the free enzyme. Lipase has also been immobilized on electrospun polyacrylonitrile (PAN) fibers.[80] In this case; the PAN fibers were ∼150−300 nm in diameter with a

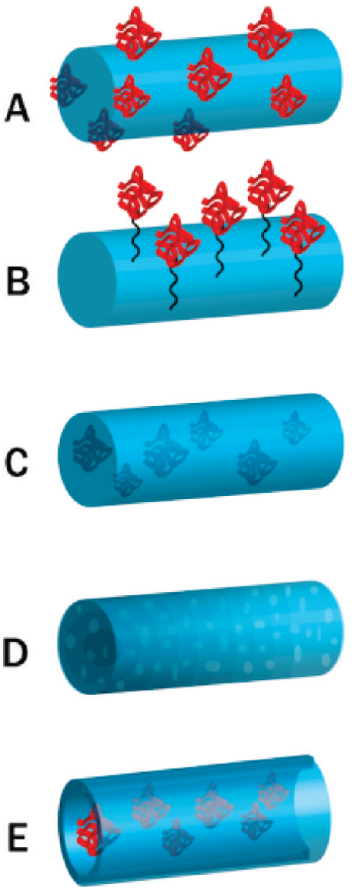

Figure 9. (A) Enzyme adsorption on polymer fiber. (B) Covalent attachment of enzyme to polymer fiber. (C) Enzyme encapsulated within polymer fiber. (D) Porous fiber. (E) Hollow fiber with enzymes adsorbed in the inner core.

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mesh thickness of ~88 μm. The pendent nitrile groups of the PAN fibers were converted to imidoesters then covalently bonding to the $-NH_2$ groups on lipase. The immobilized lipase was tested for hydrolysis of *p*-nitrophenyl palmitate. Activity retention of 81% was observed, and after 10 cycles the activity retention was 70% compared to the free enzyme. When similar fibers were tested with soybean oil feedstock, 70% conversion was obtained.[81] This can be compared to lipase immobilized in a poly(acrylonitrile-co-maleic acid) hollow fiber membrane where only 34% activity retention was observed.[82] PAN fibers with cellulase attached have been produced through the same amidination activation process.[83] These fibers have an optimal hydrolysis of 63% and were reusable up to five times without significant activity loss. Lipase has also been immobilized on cellulose nanofibers.[84] Electrospun cellulase acetate (CA) fibers 200 nm in diameter were first treated with KOH to convert to regenerated cellulose and then oxidized with $NaIO_4$. Aldehydes were formed in the process and allowed for lipase coupling to the regenerated and oxidized cellulose fibers. Immobilized lipase remained 50% active after 180 min of reaction time while free enzyme was deactivated at that point. These results suggests that the covalent attachment on the electrospun fibers helped retain activity. In addition to covalent attachment to water insoluble polymers, enzymes maybe bound electrostatically. For example, Liu et al reported the modification of electrospun CA fibers by reaction of Cibacron Blue F3GA with the CA hydroxyls.[85] This results in a negatively charged surface under acidic conditions. The lipase is positively charged below the isoelectric point and will attach to the negatively charged fibers. This process can be repeated several times in a layer-by-layer process to build up a coating of electrostatically bound enzyme.

Recently a lipase (*Pseudomonas cepacia*) adsorbed on electrospun PAN fibers were tested for biodiesel production.[86] The nonwoven mesh was placed in a packed bed reactor, and at 94% conversion of rapeseed oil and *n*-butanol, the lipase-pan fibers were 65% more active than commercial Novozym 435. There also was no observed reduction in catalytic activity after 20 days of operation. Sakai et al. also reported the immobilization of lipase (*Rhizopus oryzae*) in electrospun poly(vinyl alcohol) (PVA) fibers as well as in 400 nm organosilica particles dispersed in the PVA fibers.[87] Both electrospun PVA fibers containing lipase were more active for conversion of rapeseed oil and *n*-butanol than the free enzyme, but the lipase encapsulated in the organosilica showed the highest activity. In comparison with Novozym 435 the electrospun fibers exhibited a faster reaction rate at <1% water content. It should be noted that the Novozym 435 is more active when shorter chain alcohols such as methanol are used. Electrospun PVA fibers containing lipase have also been used for transesterification of glycidol to glycidyl *n*-butyrate with improved activity over the free enzyme.[88] Wang et al. electrospun PVA fibers (100−500 nm in diameter) together with lipase (*C. rugosa*). A 30/70 lipase/PVA mixture produced the best fibers, but lipase loadings between 25 and 50% maintained catalytic activity as good as or better than the free enzyme. The PVA fiber encapsulated lipase exhibited a 16 day half-life at 21 °C and 65% RH, which was 8 times better than the crude enzyme. If the lipase were cross-linked, using glutaraldehyde in the water-soluble PVA fibers significantly reduced the catalytic activity. Wu et al also reported that glutaraldehyde cross-linked PVA fibers containing cellulase exhibited reduced activity.[89] Kim et al reported that α-chymotrypsin covalently attached to electrospun poly(styrene-co-maleic anhydride) could serve as an anchor point for adsorption and cross-linking (glutaraldehyde) of additional chymotrypsin.[90] This aggregation at the fiber surface resembles a CLEA and results in enhanced activity. The same strategy was used to immobilize β-glucosidase on the poly(styrene-co-maleic anhydride) fibers.[91] Again the cross-linked aggregate coating exhibited higher activity than just the covalently attached enzyme.

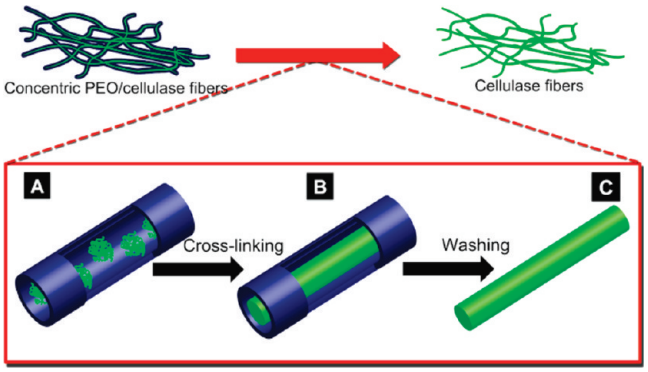

Figure 10. Schematic of the processing of cellulase fibers prepared via concentric electrospinning.

There appears to be only one example where silica fibers were electrospun with an enzyme as in Figure 9C.[92] In this case, horseradish peroxidase was mixed with PVA and glucose (to control porosity) in a silica sol and electrospun at room temperature (RT). After extraction of the glucose, the nonwoven fiber mat was tested for conversion of H_2O_2, and the electrospun fibers showed greater activity than the free enzyme.

Fiber diameter is an important variable, and there are relatively few examples where enzymes are loaded on nanofibers (i.e., <100 nm). Chen et al reported the preparation of poly(methyl methacrylate) fibers in the range of 36−500 nm.[93] Subsequent adsorption of lipase revealed that the highest loading was achieved with the nanofibers. In contrast, the activity did not vary with fiber size.

There may be situations where an enzyme requires contact with insoluble substrates such as in the case of cellulase. In this case, if the enzyme were encapsulated in a polymer of OMS matrix one might expect minimal activity. However, if the core−shell approach (Figure 9E) is employed, an enzyme could be placed in the core and then cross-linked to form an enzyme fiber. For example, cellulase fibers have been fabricated via concentric electrospinning.[94] Poly(ethylene oxide) (PEO) and cellulase were concentrically electrospun as the outer shell and inner core, respectively (Figure 10A). After collection, the fibers were treated with glutaraldehyde to cross-link the cellulase located in the inner core, making it insoluble (Figure 10B). The water-soluble outer shell PEO could then be washed away leaving the cellulase fibers that retained 24% activity of free enzyme (Figure 10C). The lower activity can be explained by the limited contact and diffusion constraints of the solid filter paper substrate employed. It may also be possible to electrospin enzyme fibers without a support matrix. Both hemoglobin and myoglobin have been electrospun and cross-linked with glutaraldehyde.[95]

■ OPPORTUNITIES FOR MESOPOROUS MEMBRANE AND ELECTROSPUN FIBER SUPPORTS

The preparation of mesoporous silica thin films and membranes as well as mixed matrix membranes has been well studied

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over the past 20 years. It is somewhat surprising that there have not been more reports of membrane supported enzymes. Part of the challenge is that OMS materials with 1-D pore structures would require a preferred orientation. Wormhole or 3-D pore architectures would enable access to the enzymes without the need for orienting the film. There are relatively few examples of membrane reactors based on nanoporous supports. Given the many porous materials and compositions described above, there are many opportunities to explore enzyme immobilization in films and membranes. The electrospun fiber supports have also been explored to a limited extent.

CONCLUSIONS

Enzyme immobilization in well-ordered nanoporous materials continues to attract interest. There are clearly many opportunities to explore new materials and configurations as membranes or nonwoven mats. However, the area where the most significant advances may be realized is catalysis, particularly in identifying new types of reactivity.

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DEDICATION

Dedicated to the memory of Professor Victor Lin.

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