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Immobilized Proteins

Proteins in Mesoporous Silicates

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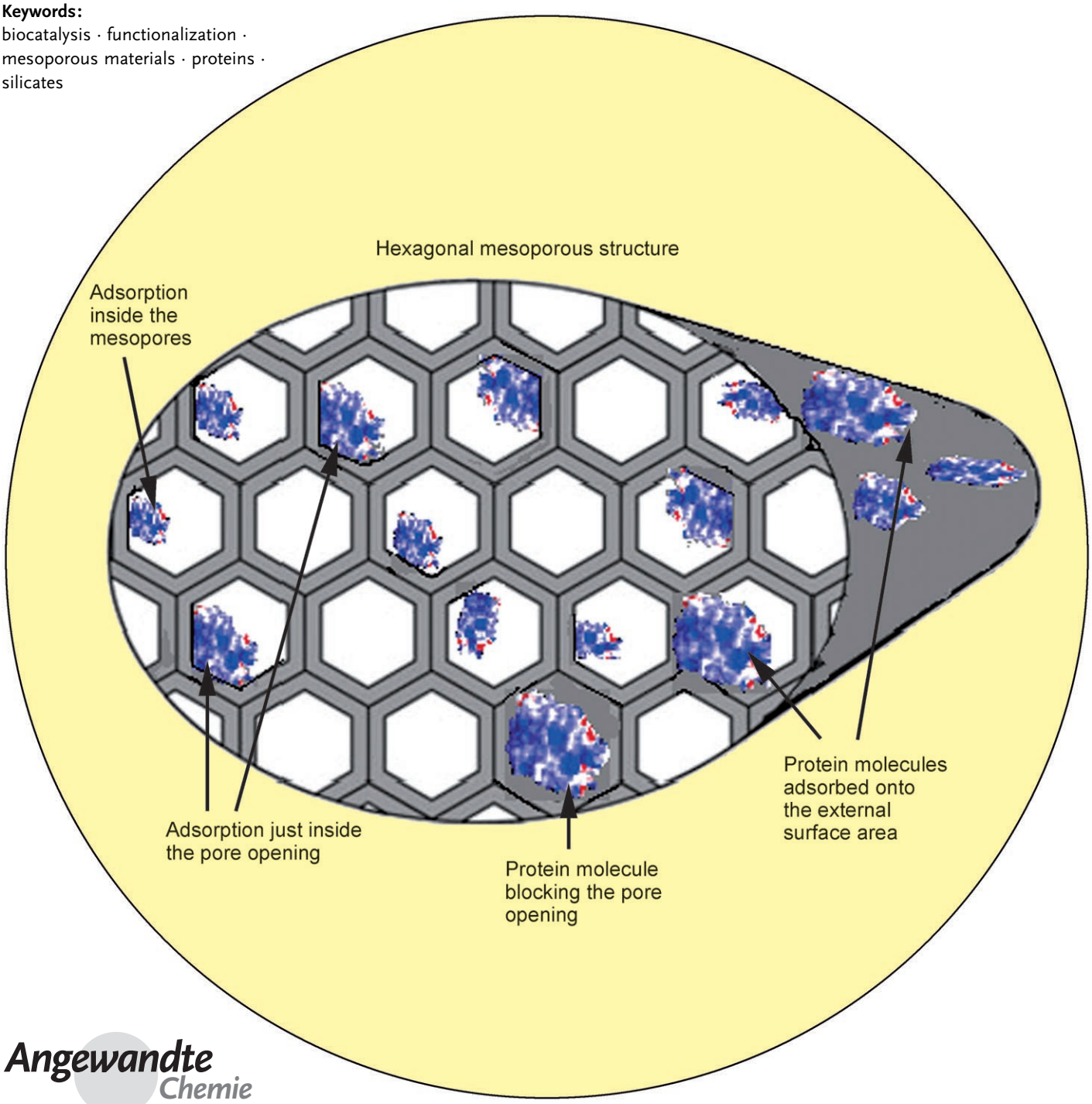

Mesoporous silicates (MPS) have an ordered pore structure with dimensions comparable to many biological molecules. They have been extensively explored as supports for proteins and enzymes in biocatalytic applications. Since their initial discovery, novel syntheses methods have led to precise control over pore size and structure, particle size, chemical composition, and stability, thus allowing the adsorption of a wide variety of biological macromolecules, such as heme proteins, lipases, antibody fragments, and proteases, into their structures. This Review discusses the application of ordered, large-pore, functionalized mesoporous silicates to immobilize proteins for biocatalysis.

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1. Introduction

The successful application of biological entities, such as enzymes, antibodies, and other proteins as well as whole cells, in biotechnology depends on the ability to successfully stabilize the biological component in what is often an unnatural environment while retaining its function and activity. This stabilization is frequently achieved by entrapment or immobilization in organic and inorganic structures and has proved beneficial for protein digestion[1] and separation,[2] biosensors,[3] biocatalysis,[4] drug delivery,[5–7] and tissue engineering.[8]

Generally speaking, enzymes operate under mild conditions and have much higher catalytic turnover rates than synthetic catalysts whilst catalyzing reactions with high enantiospecificity and selectivity. Unfortunately, they are not always ideal for industrial applications.[4,9] In organic solvents or at high temperatures, many enzymes are unstable and often show low activity. Denaturation of the enzyme, which destroys its catalytic activity, can be induced by organic solvents, reagents, low or high pH values, or by mechanical treatment or heat. In commercial use, the process of extracting the enzyme from the reaction mixture can cause denaturation and loss of activity. However, these disadvantages can be overcome by immobilization of the proteins on a solid structure, allowing them to be recycled and increasing their stability.[10–17] This approach provides scope to enhance the ability of enzymes to catalyze specific reactions under mild conditions. A good example is the production of 6-aminopenicillanic acid, a precursor to many semisynthetic penicillins, by immobilized penicillin acylase in an aqueous environment under mild conditions. By contrast, the chemical route to 6-aminopenicillanic acid requires anhydrous conditions, low temperatures, organic solvents, and the environmental pollutant phosphorus pentachloride.[10]

Immobilization on a solid support can enhance enzyme stability as well as ease of separation and recovery for reuse while maintaining activity and selectivity. A range of supports have been described for proteins, for example, sol gels,[18–19] hydrogels,[6,20] organic microparticles,[21] and nonporous[22] and porous[23] inorganic supports. Each has associated advantages and disadvantages for use in protein biocatalysis. Sol-gel matrices are porous and thermally stable, and they prevent leaching of the enzyme, owing to effective entrapment. However, they have brittle structures, and enzymes are encapsulated during the sol-gel synthesis, which can lead to denaturation of the protein. Diffusion of bulky substrates can also be restricted,[18] although recently sol-gel matrices have been made more porous by the addition of surfactants and used to successfully entrap lipase and alcohol dehydrogenase.[24,25] Controlled-pore glass has also been successfully used but is an expensive technique.[23] Nonporous supports normally have low surface areas, which result in low protein loadings.[22] Inorganic porous materials in general have larger surface areas than nonporous materials. However, mass-transfer difficulties for enzymes and their substrates can arise, owing to uncontrolled pore size distributions. Hydrogels and organic microparticles, for example, poly(lactic acid-*co*-glycolic acid) and Eudragit (cationic methacrylate polyester) have not been used extensively for biocatalysis, as the enzyme is often released owing to swelling of the support matrix and/or degradation of the support. Moreover, low enzymatic activity arising from denaturation and restricted mass transfer has hindered the use of these materials as supports.[4,6,21,26]

Mesoporous silicates (MPS), synthesized using a surfactant templating method, have ordered porous structures with narrow pore size distributions and thick walls, enhancing their stability. The large regular repeating mesoporous structures of mesoporous silicates offer the possibility of adsorbing or entrapping large biomolecules within their pores as well as on the external surface area. The distinct order of the channels as well as the ease with which pore size, structure, and wall composition can be varied offer advantages over sol-gel or nonporous formulations. Adsorption of the enzymes usually

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occurs after synthesis, and the ordered porous structure provides a sheltered environment in which reactions with selected substrates can proceed. Since the first report by Diaz and Balkus[27] in 1996, this research area has grown rapidly.[13,17] However, MPS have not, as yet, proved as useful as originally hoped in biocatalysis. Leaching has been one of the major problems, resulting in loss of activity upon reuse, while immobilization of large enzymes has been hampered by the small pore openings in many of the mesoporous materials developed. Furthermore, the synthesis of the materials can be time-consuming and expensive.

2. Mesoporous Silicates

2.1 Historical Background

In 1992 Kresge et al.[28] synthesized mesoporous solids, designated as MCM-X (Mobil Composition of Matter), with regular arrays of uniform channels. The dimensions of these channels could be tailored through choice of surfactant, auxiliary chemicals, and reaction conditions by the proposed liquid-crystal templating mechanism.[29] This mechanism is based on the formation of liquid crystals in the reaction mixture. Liquid crystals form in mixtures of polar solvents (water) and surfactants with a nonpolar tail group. The surfactant molecules cluster together as micelles, as their hydrophobic tails tend to congregate and their hydrophilic heads provide protection in water. These micelles will only form above the critical micelle concentration at a particular temperature. It has been suggested that the introduction of the inorganic species mediates the assembly of the liquid crystals (Figure 1). Overall, the structure is defined by the organization of the surfactant molecules into micellar liquid crystals that serve as templates for the formation of the mesoporous silicates. These liquid-crystal structures are highly sensitive to solution conditions. The structure and phase behavior of the composite inorganic–organic assembly depend on the nature of the inorganic species and its electrostatic and steric interactions with the organic species.[29] Cooperative interactions among inorganic and organic species can lead to a variety of structures that would not be found in surfactant or inorganic systems alone.[30] A variety of surfactants (neutral block copolymers, cationic surfactants, gemini surfactants) and additives (trimethylbenzene, alcohols, salt) can be used to develop different mesoporous

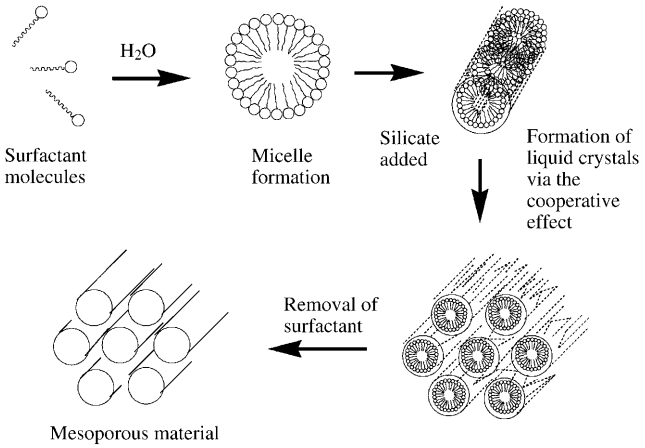

Figure 1. Proposed mechanistic pathway for the formation of mesoporous materials.

structures, for example, hexagonal, cubic, or lamellar, with different pore sizes.[31–38] The adsorption characteristics of each material depend on its individual properties, for example, degree of crystallinity, pore diameter, surface area, pore volume, surface chemistry, shape and particle size. The physicochemical properties of each structure depend, in turn, on the surfactant used, the synthesis conditions (pH value, temperature, concentration ratios, stirring), salt content, cosolvents, organic additives, and the silica source.

Inorganic–organic hybrid mesoporous materials offer the opportunity of tuning the chemical composition of the surfaces to possess desired properties. There are three general methods of generating functionalized materials: direct functionalization, postsynthesis functionalization, and incorporation of organic moieties into pore walls using bridged silsesquioxanes as the source of silica.[37] A wide range of organic functional groups have been incorporated into mesoporous materials by these methods, for example, thiol, amine, chloro, and carboxylic acid groups; *N*-(trimethoxysilylpropyl)ethylene diamine triacetic acid units; oxirane rings; as well as cyano, alkane, and aromatic functional groups.[37,39–46] Extensive reviews on the development of mesoporous silica structures,[36] inorganic–organic hybrid materials,[37] and mesoporous thin films[38] that explore thoroughly the development of MPS since the discovery of MCM-41 in 1992 have been published, and thus these aspects are not discussed in detail herein. To determine the phys-

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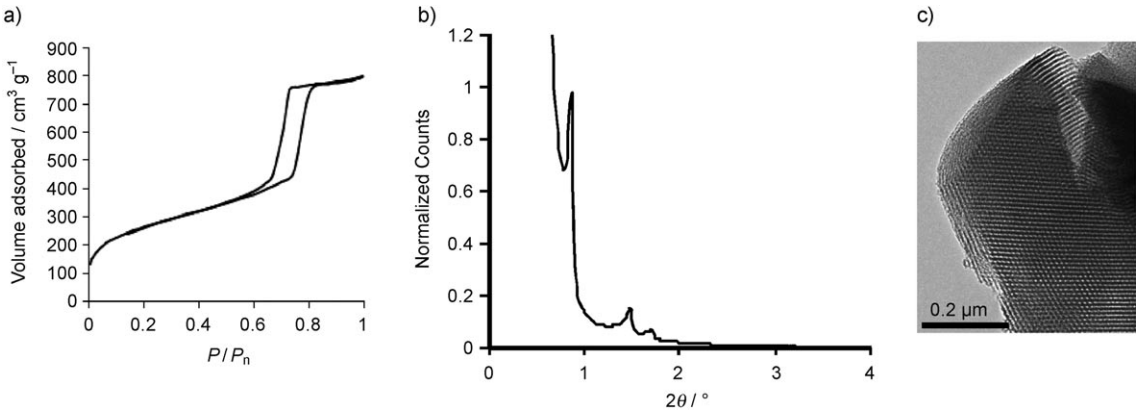

Figure 2. Typical analysis profile of an ordered MPS material. a) Nitrogen isotherm, b) XRD spectrum, and c) TEM image for a well-ordered hexagonal mesoporous silicate with a pore size of about 6 nm.

icochemical properties of a particular mesoporous structure, however, the structure must be carefully characterized.

2.2. Characterization of Mesoporous Structures

Upon synthesis of an MPS, the material requires a battery of analyses to elucidate and understand its character. The characterization employs many techniques, some of which are described below.

1) Nitrogen adsorption analysis[47] provides information on the surface area, pore diameter, pore size distribution, and pore volume. While nitrogen adsorption analysis is an exceptional tool for the characterization of mesoporous materials, each theory (BET,[48] BJH,[49] NLDFT,[50] t plots, and α_s plots[51]) has limitations, especially as many of the mesoporous materials have micropores and macropores as well as mesopores. Some examples of these limitations include: a) Surface areas calculated by the BET theory depend strongly on the chosen data range.[52] b) Pore sizes vary depending on whether the adsorption or desorption branch of the isotherm is used; for example, BJH calculations on the desorption branch underestimate the size of pore openings of materials such as SBA-15.[53] c) NLDFT theory cannot be used for more disordered materials.[54] d) t plots and α_s plots cannot give values for the external surface areas of large-pore materials, as the nitrogen adsorption isotherm extends towards infinity at high pressures.

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2. Low-angle X-ray diffraction provides information on the three-dimensional structure, the order, and the unit cell parameter a_0. In general, mesoporous materials tend to have little long-range order, that is, they are amorphous. However, low-angle X-ray diffraction shows a distinct X-ray pattern if the material consists of ordered mesoporous channels. The spectrum can indicate whether a hexagonal, cubic, lamellar, or disordered structure is present.[55] Together with the pore diameter obtained from nitrogen adsorption analysis, the thickness of the pore walls of the mesoporous material can be estimated.
3. Transmission electron microscopy (TEM) provides information on the short- and long-range order of mesoporous materials as well as an indication of the width of channels and pore openings. However, care must be taken when taking measurements of pore channels from TEM images, as it is the *d* spacings which are observed, not the pore widths. Direct measurements of the pore openings from TEM images are inconsistent, owing to different orientations of the particle face. For a hexagonally ordered mesoporous material, measurements of the *d* spacings between the 100 planes and the 110 planes have been shown to be within 7% of the spacings deduced by XRD.[56]

Materials with distinctive nitrogen isotherms (characteristic hysteresis loops), low-angle X-ray diffraction patterns, and well-ordered TEM images for ordered mesoporous structures with pore sizes of 10 nm or less have been described (Figure 2). It appears very difficult to retain this order with larger mesopores. Bimodal porous materials have recently been generated to circumvent this problem. These materials consist of macroporous cages with a mesoporous wall structure and are synthesized using a variety of surfactants and templates.[57]

Other characterization techniques[12,13] that may be used for mesoporous silicates include scanning electron microscopy; Fourier transform infrared (FTIR), resonance Raman, and solid-state NMR spectroscopy; thermogravimetric analysis (TGA); ammonia temperature-programmed desorption; and CHN elemental analysis. These techniques provide information on the particle size and morphology, the extent

of surfactant removal, loading of functional groups, and density of silanols and other surface groups.

3. Immobilization of Proteins in MPS

Immobilization of proteins was first described in 1916, when invertase immobilized on charcoal was found to retain the same activity as the native enzyme.[58] The aim of immobilization is to maintain the catalytic activity of the enzyme whilst improving its stability and ease of recovery and reuse. Numerous methods of immobilizing enzymes on a wide variety of supports have been studied.[4,11–26] Table 1 lists some characteristics of proteins and mesoporous structures that have been used to generate biocatalysts. A logical approach to the selection of a mesoporous material for the immobilization of a particular protein involves matching the physicochemical properties of the mesoporous support and the protein surface (Figure 3).[59] Smart immobilization involves the adsorption of the protein with its active site oriented away from the mesoporous surface, with little leaching yet sufficient mobility to retain catalytic activity. In the following sections, the relevance of the factors listed in Figure 3 will be illustrated by discussion of the published work on proteins immobilized on MPS and will indicate the key characteristics that should be examined when looking for a mesoporous support for a particular protein.

3.1. Relative Size of the Mesopores and the Protein

If the protein is smaller than the pore opening, it will have access to the large internal surface area and mesoporous volume of the mesoporous support. If not, it will adsorb only

Table 1: List of proteins and mesoporous materials used for their immobilization.

Protein	pI	Size [nm]	Mesoporous support	Pore sizes[b] [nm]
cytochrome c[5,64,73,123]	10.7	2.6×3.2×3.3	MCM-41, COS, CNS, MPS-F127, MAS-9, Al-MCM-41, Al-MCM-48, PMO	2.5–13
microperoxidase-11[118]	4.7	3.3×1.75	PMO, MCM-41, SBA-15	2.7–5.5
lysozyme[70,71,74,84,89,124,125]	11.3	1.5×2.5×4.3	SBA-15, CMK, CKT, Al-MCM-41, Al-SBA-15, MCM-48, MCM-41, PMO	3.2–9.1, 24
trypsin[1,41,89,112,114,126,127]	8–10.5	ca. 3.8	CNS, COS, MCM-41, MCM-48, SBA-15, FDU-12, MCF, (bimodal MPS)	2.8–18, (100–1000)
organophosphorus hydrolase[45,91,92]	8.3	9.2×5.6×4.0	MPS, FMS	30
α-chymotrypsin[57,63,116]	8.6	4.0×4.0×5.0	MCM-41, (bimodal HMMS)	4, (13,37)
horseradish peroxidase[27,62,73]	ca. 7.2	4.0×4.4×6.8	MCM-41, SBA-15, FSM-16, CNS	2.7–13
chloroperoxidase[65,90,93,94,106]	4.0	ca. 6.5	SBA-16, MCF, SBA-15, MCM-48, PMO-PA	3–14
manganese peroxidase[66]	–	–	FSM-16	3–9
papain[27,117]	8.75	3.7×3.7×5.0	MCM-41, MCM-48	4, 6.2
pepsin[73]	<1	33 kDa[a]	CNS	13
bovine serum albumin[69–71,73]	4.9	5.0×7.0×7.0	CNS, MCM-41, SBA-15, Al-MCM-41	2.5–24
subtilisin[62,73]	10.4	30 kDa[a]	FSM-16, CNS	2.7–13
myoglobin[71,127]	7.1	ca. 17.6	SBA-15, FDU-12, MCF	3.8–24
metmyoglobin[128]	–	ca. 17.6	FSM-16, FSM-22	2.7, 4
hemoglobin[109,110]	6.8–7.0	5.3×5.4×6.5	SBA-15, FSM	3–10
conalbumin[2,127]	6.0	5.0×5.6×9.5	SBA-15, MCF, FDU-12	5.9–18
ovalbumin[2]	4.9	4.0×5.0×7.0	SBA-15, MCF	5.9, 16
trypsin inhibitor protein[2]	5.2	14 kDa[a]	SBA-15, MCF	5.9, 16
α-amylase[101]	–	ca. 7–10	MCM-41, SBA-15, MCF	2.6–33.5
cytochrome P450 CYP2C9[81]	–	ca. 9.0	MCM-41, Al-MCM-41	4–5
cytochrome P450 CYP2B4[81]	–	ca. 5.2	MCM-41, Al-MCM-41	4–5
penicillin acylase[98,99,104,107]	7.0	7.0×5.0×5.5	MCM-41, Al-MCM-41, SBA-15, KIT-6	3.3, 6–9
glucose oxidase[85,92,100]	4.6	7.0×5.5×8.0	MCF, CMK, MSCF, FMS, (bimodal MSU-F-C)	17–34, 3.9, (5.6, 21)
Candida antarctica lipase B[97,113,119]	6.0	3.0×4.0×5.0	HMS, MS-3030, MCF, FDU-12	3.6, 20–50, 10–15
porcine pancreatic lipase[61,75,82]	–	4.6×2.6×1.1	MCM-41, SBA-15	4.5–6.6
Mucor javanicus lipase[102,116]	ca. 6.2	–	SBA-15, (bimodal HMMS)	6.7, (13, 37)
Pseudomonas cepacia lipase[122]	–	ca. 5	MCM-41, MCM-48, SBA-15	1.6–6.2
newlase F lipase[129]	–	–	SBA-15	7.5
α-L-arabinofuranosidase[87]	3.5	3.9×9.7×14.4	(bimodal UCM-7)	(3.6, 28)
fumarase[126]	–	ca. 7.8	SBA-15, (bimodal MPS)	(100,1000)
porcine liver esterase[126]	–	ca. 7.3	SBA-15, (bimodal MPS)	(100, 1000)
glucose isomerase[92,103]	4.0	173 kDa[a]	SBA-type, FMS	30
acetylcholinesterase[130]	–	4.6±5	MCM-41, FSM-16	4–7
invertase[105]	–	–	MCF	12–14
glucoamylase[105]	–	–	MCF	12–14
polyphenol oxidase[131]	–	–	mesoporous carbon	2–3.5
thermolysin[132]	4.5	34.6 kDa[a]	SBA-15, MSU, MCF	9–32
aldolase I antibody 84G3[67]	–	8	MCF	15–20
scFv antibody fragment[3]	8.73	5.0×4.0×4.0	SBA-15, CNS, MSE, MCM-41	6–24

[a] No geometric dimensions given. [b] Figures in brackets represent the pore sizes for bimodal materials.

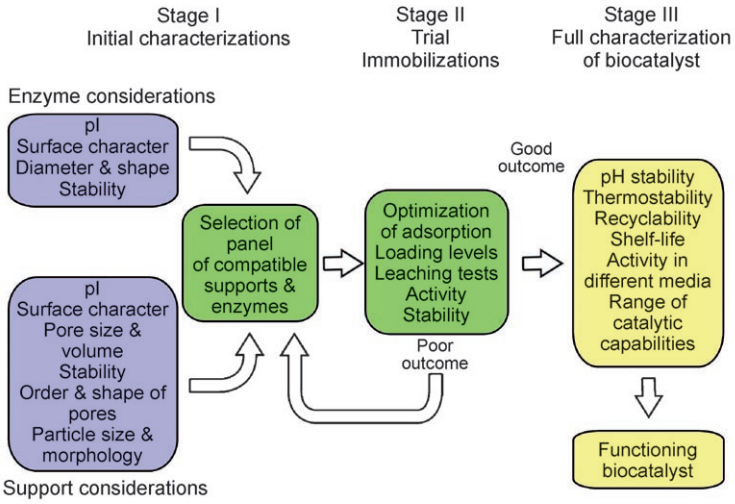

Figure 3. Influential factors in the generation of a novel immobilized protein biocatalyst.[59]

on the outside of the material and will not benefit from the shelter of the mesopores. Diaz and Balkus[27] deduced that horseradish peroxidase (average spherical diameter ca. 4.6 nm) was not successfully immobilized as it was too large to fit into the pores of the MCM-41 material (pore diameter ca. 4 nm). Similarly, 8 μmol cytochrome c was adsorbed per 1 g MCM-41 when the pore size was 4.5 nm, but less than 1 μmol g^{-1} was adsorbed under the same conditions when the pore size was reduced to 2.8 nm.[60] This result emphasizes that matching the protein's hydrodynamic diameter and pore diameter is a critical factor in attaining high loadings. However, the loading is further influenced by the available pore volume and surface area. Pore size restrictions have limited the use of MPS for the immobilization of antibodies for biosensors, although Hu et al.[3] immobilized an antibody fragment in MPS that exhibited good binding properties for a potent neurotoxin from shellfish. Direct proof of entry of protein molecules into the pores of mesoporous structures has yet to be reported, for example, with indisputable images of enzymes or proteins located inside the pores.[61] Entry into the pores has, however, been inferred from higher loadings and enhanced stability and activity when the pore entrance was large enough to allow the protein to enter the channel.

Conflicting reports exist regarding whether the pore size should be significantly larger than the protein to allow for diffusion of protein and substrate down the pore or if pore and protein should be of similar size to increase stability and protection of the protein from the external environment. Takahashi et al.[62] reported that for enhanced activity in organic solvents and enhanced hydrothermal stability, pores for the immobilization of horseradish peroxidase should be large enough to accommodate the protein but should size match; that is, they suggest that if the pore is too big, the enzyme will not be as well protected as it might be when the enzyme just fits inside the pores. Others have also advocated that a snug fit inside the pores was desirable.[63–66] Smaller pore entrances can also improve overall catalytic activity by reducing leaching; for example, MCF materials with smaller pore entrances exhibited better stability, activity, and recycling properties than materials with larger entrances for an immobilized antibody in organic solvents.[67] However, there must be room for the substrate to have access to the protein, as limitations on the substrate diffusion rate can reduce the overall activity of the immobilized enzyme.[64,68] The hydrophobicity or hydrophilicity and size of the substrate will have a very strong influence on the catalytic activity of the immobilized enzyme.[46,64,66] If the substrate is hydrophobic, diffusion through the channels of a hydrophilic mesoporous silica structure may be slow. Larger pore sizes may compensate for this mismatch, with higher diffusion rates than for smaller pores. On the other hand, a hydrophilic substrate might diffuse easily through the pores, and the smaller pores could protect the specific activity of the immobilized enzyme.

External surface areas can also strongly influence protein loadings.[7b,69–71] From thin layer ellipsometry studies, Deere et al.[72] deduced that 6–10 layers of cytochrome c could form on the external surface of mesoporous materials. Pore-volume analysis before and after enzyme adsorption does not give absolute evidence for entrance into the pores, as pore blockages at the entrance to the mesopores can reduce pore volumes and surface areas even when the protein molecules have not fully entered the pore.[73–77] However, constant mesopore volume is evidence for adsorption only on the external surface of the mesoporous material.[78–80] XRD analysis before and after adsorption of a protein can indicate retention of the mesoporous structure and also entry of the protein into the mesopores by a reduction in the intensity of the reflection peaks.[74,75,78,81–83]

3.2. Particle Size and Morphology

It has been shown that the reduction in pore volume of mesoporous supports after protein immobilization is often much higher than the estimated volume of protein adsorbed because of pore blocking.[73,78] Materials with shorter mesoporous channels (i.e. smaller particles) but the same overall mesopore volume can exhibit a higher loading capacity (ca. 37 vs. 5 μmol lysozyme per 1 g SBA-15) owing to reduced pore blocking.[83] Hence, the particle size distribution and the morphology of mesoporous materials should be characterized when comparing adsorbents. These attributes will have a strong influence on adsorption isotherm characteristics, adsorption kinetics, and specific activities of proteins on mesoporous materials.[83–88]

3.3. Stability of the Mesoporous Material

To reuse an enzyme immobilized on a mesoporous support, the material itself must be inherently stable under the appropriate working conditions. If it degrades, it may release the enzyme and thus lose activity upon reuse. If it is to be used as a sequester/release material, it must retain its

structure.[89] SBA-type materials have been reported to be hydrothermally more stable than MCM-type, owing to their thicker walls.[31,74] However, some conflicting reports exist, chiefly owing to different synthesis conditions. Ji et al.[69] found that Al-MCM-41 was unstable only at pH values greater than pH 9, supporting observations by Katiyar et al.[70] However, Hernandez et al.[81] reported that MCM-41 decomposed at pH 7.4. Different groups have employed synthetic strategies to improve the hydrothermal stability of MCM-41-type materials, for example, using zeolitic clusters as the silica source with a variety of surfactants.[64] A periodic mesoporous organosilicate with amino bridging groups (PMO PA) is inherently unstable in the range pH 4–10, but upon changing surfactant and synthesis conditions, the PMO PA materials developed from the same organosilica precursors were more stable and allowed immobilized chloroperoxidase to be recycled without loss of activity.[90]

3.4. Isoelectric Points of MPS and Protein

The isoelectric point of a particle or enzyme refers to the pH value at which it has an overall neutral charge. Above or below this pH value, the protein will have an overall negative or positive charge, respectively, with the charges being predominantly located on the surface of the molecule. Emphasis has often been placed on the isoelectric points (pI) of enzymes, with the implication that adsorption will occur on a support if the pH value of the immobilization buffer is below the pI of the enzyme and above that of the support.[27] If the solution pH value is below the pI of the enzyme, the enzyme will have a net positive surface charge, which will favor interaction with a silica support that has a negative charge on its surface at pH > 3 and vice versa.[60,73] Cytochrome c and trypsin have similar isoelectric points and yet exhibit very different surface potentials at pH 7 (Figure 4).

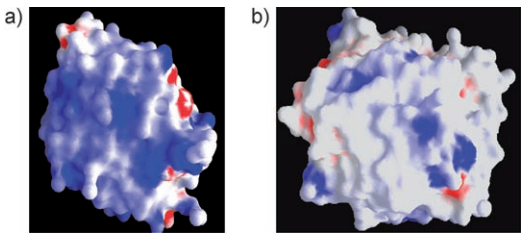

Figure 4. Electrostatic surface potential (red negative, blue positive) calculations in GRASP[96] of a) cytochrome c (PDB code: 1HRC, pI = 10.7[59]) and b) trypsin (PDB code: 1S0Q, pI = 10.5).

While compatible size and isoelectric points appear necessary for enzyme immobilization,[3,91,92] they do not always guarantee adsorption. For example, subtilisin is small enough to fit inside the pores of a cyano-modified MPS (CNS), and the interaction is electrostatically favorable at pH 6.5, but low loadings were obtained.[73]

The surface charge density and distribution on the enzyme has an influence on the interaction between the two surfaces. They are not described adequately by just the isoelectric point and should be determined to enable a better understanding of the surface characteristics of the support material. Often, the isoelectric point of mesoporous silica is assumed to be approximately 2,[89,93,94] as reported for amorphous silica.[95] The isoelectric point of mesoporous materials was first measured by Deere et al.,[73] who reported values of 3.6 and 3.7 for MCM-41 and CNS, respectively. Those of commercially available silica gel and a mesoporous material made with F127 were somewhat lower (2.9 and 2.8, respectively). Functionalization of a pure mesoporous silicate with organic functional groups will alter its pI and its consequent electrostatic interactions with proteins.

3.5. Surface Functional Groups of MPS and Protein

By examining the functional groups on the surface of the enzyme, a suitable complementary group on the surface of the support could be found to provide a strong interaction for immobilization. Mesoporous silicates have been functionalized with a large variety of functional groups.[37] The method and extent of functionalization can influence the final loading and activity of the immobilized protein.[90,91] Residual solvent molecules from postsynthesis functionalization can also reduce protein activity. Moreover, high levels of functionalization can cause pore blocking or reduction in pore diameters. Functionalization after immobilization of the protein can enhance the hydrophobicity and/or amphiphilicity of the support for effective substrate diffusion through the pores.[82,97] However, the solvent used for this reaction can reduce the activity of the immobilized protein.[41]

Covalent coupling with surface amine functional groups on the support using glutaraldehyde, which can bind to enzyme amine groups, can increase the stability of the immobilized enzyme, significantly reduce the amount of leaching, and allow the immobilized enzyme to be reused. A range of enzymes, including penicillin G acylase,[98b,99] glucose oxidase,[100] α-amylase,[101] *Mucor javaniscus* lipase,[102] glucose isomerase,[103] trypsin,[41] invertase,[105] and glucoamylase,[105] has been covalently immobilized on MPS in this manner. Aminopropyl ethyl carbodiimide was used to covalently bind chloroperoxidase[65,106] and α-L-arabinofuranosidase[87] by coupling amine groups on the support to the carboxy groups on the enzyme. Penicillin acylase was also covalently immobilized on MPS through epoxy groups on the support, which can react with amine groups on the enzyme.[104] The increased rigidity in a covalently bound enzyme can, however, cause a reduction in activity, for example, in penicillin acylase[98b] and glucose isomerase.[103]

The effect of metal-ion incorporation into MPS on the ability to adsorb proteins and enzymes has also been investigated. Aluminum-substituted mesoporous silicates have been used to adsorb different enzymes, for example, cytochrome c,[64] bovine serum albumin,[69] lysozyme,[74] and cytochrome P450.[81] Aburto et al.[65] impregnated an SBA-16 material with Cs^+, which increased the loading of chloroperoxidase. Xue et al.[107] functionalized MCM-48 materials with Co^{2+}, increasing the number of weakly acidic sites on the

material surface and thus increasing the loading of penicillin G acylase.

3.6. Optimal Conditions for Immobilization

Optimal immobilization conditions are those that give the highest loadings and the highest specific activity. Several groups have studied the effect of pH value and initial concentration on the loading of proteins.[27,89,108] Without a full isotherm, it is difficult to draw any conclusions about loading capacities, as loadings are influenced by initial concentrations as well as the immobilization pH value.[78] To illustrate the general capacity range of mesoporous silicates for proteins, examples of loading capacities are listed in Table 2. It is important to emphasize that a particular support may not appear to provide a high loading capacity for a particular enzyme simply because the immobilization factors have not been optimized. For example, Liu et al.[109] obtained a loading of 3.9 μmol g^{-1} hemoglobin on SBA-15 with a pore size of 10.6 nm, while Urabe et al.[110] achieved a slightly lower loading of 3.3 μmol g^{-1} on MPS that had a pore size of 7.5 nm. While it appears that the material with the larger pore size can accommodate more protein, the loadings are not comparable, as different initial hemoglobin concentrations (0.258 mg mL^{-1} at pH 7.2[109] and 5 mg mL^{-1} at pH 6.8[110]) with different MPS concentrations (5 and 10 mg mL^{-1}, respectively) were used. From the data provided, it is not possible to ascertain if SBA-15 can adsorb more hemoglobin.

Table 2: Examples of loading capacities of MPS for proteins.

Protein	MPS	Loading [μmol g^{-1}]
cytochrome c[78]	SBA-15	41.5
porcine pancreatic lipase[61]	SBA-15	3.7
Pseudomonas cepacia lipase[111]	MCM-41	0.14
chloroperoxidase[90]	PMO	0.95
lysozyme[83]	SBA-15	37
glucose oxidase[100]	MCF	6.36

The time required to reach equilibrium varies for each protein and each support, with the rate of adsorption depending on the relative size of the pores and proteins,[110] the channel length, and the reconfiguration of the protein once it has been adsorbed.[70,78,86] When long adsorption periods are used, care should be taken to ensure that a decrease in free concentration is due to adsorption of the protein and not to denaturation or precipitation of the protein from solution.

The pH value used for the adsorption process is very important in obtaining both the maximum loading and the maximum activity.[74,76,78,93,94,98,102,112,113] The maximum loading and maximum specific activity do not always coincide, (see Section 3.8). The pH value can influence the conformation in which the protein is adsorbed and the strength of the interaction between the protein and the surface, which strongly influence the resultant activity.[98,112] Vinu et al.[74] noted that the buffer and pH value used for adsorption may cause protein aggregation, especially at high pH values, and that this may have an effect on the adsorption loadings. Fitting adsorption curves to an L- or S-type curve can indicate the monolayer capacity (the amount of protein necessary to cover the available surface area of the particle with a single layer of protein) and the strength of an interaction,[74,86] but the appropriateness of using the Langmuir model for protein/support isotherms is not usually discussed. The assumptions associated with this model are not preserved in many protein immobilization cases—adsorption sites inside and outside the mesopores differ in binding energy, multilayer adsorption can occur before the monolayer is complete, and the adsorbed protein molecules interact with each other.

Others have studied the effect of temperature, pressure and enzyme purity on adsorption loadings. Lei et al.[86] reported that the adsorption rate was independent of the initial concentration but that increasing the temperature led to a faster adsorption rate and higher loading for lysozyme. One of the steps thought to influence the adsorption rate of a protein onto a mesoporous material is the movement of adsorbed protein molecules further down through the channels (reconfiguration), thus reducing pore blocking near the entrances and allowing more protein molecules to enter the channels. This process requires breaking the initial interaction between the protein and the support surface, which is an endothermic event that occurs more easily at higher temperatures. Recently Han et al.[97b] found that high pressures increased the loading of hydrophilic *Candida antarctica* lipase B (CALB) molecules into large-pore hydrophobically functionalized materials, generating higher loadings with less leaching than when immobilized by conventional stirring. They noted that while CALB was stable to such high pressures, other proteins might not be. Protein purity can also influence loading values. Adsorption isotherms with pure and impure trypsin on MCM-41 showed that typically five times more trypsin was adsorbed when pure trypsin was used (ca. 5 μmol g^{-1}). Similar results were obtained with CNS.[114,115]

3.7. Nature of the Interactions between Protein and Support

It is difficult to deduce the exact nature of the interactions between the protein and support from the loadings observed under one set of conditions. It is usually clear whether covalent binding or physical adsorption is involved, but several experiments can be carried out to deduce whether the interaction between a protein physically adsorbed to a support is hydrophilic, hydrophobic, or electrostatic in nature. Such experiments involve using desorbents, for example, poly(ethylene glycol), methanol, and ammonium sulfate, or altering the ionic strength of the immobilization buffer.[73,92,114] For example, cytochrome c does not always appear to adsorb in the same manner with different materials. Diaz and Balkus[27] implied that the interactions of cytochrome c with MCM-41 were hydrophilic in nature. Studies by Deere et al.,[73] using desorbents and varying ionic strengths, implied that electrostatic interactions were domi-

nant with cytochrome c and CNS but that hydrophobic interactions dominated with silica gel. In a separate study, cytochrome c was found to interact electrostatically with SBA-15 but in a hydrophobic/hydrophilic manner with a PMO with bridging ethylene groups (MSE).[59]

Higher loadings of cytochrome c on both SBA-15 and MSE were obtained at pH values near the pI of the protein. Close to the pI, the net charge of each protein molecule is near zero, and lateral repulsion between cytochrome c molecules is reduced; thus, higher loadings are obtained.[59] At pH values far below the pI, lateral repulsion between the protein molecules is more dominant than the electrostatic attraction to the surface of the mesoporous support, thus reducing the loadings. Similar observations were made with the adsorption of lysozyme on MCM-41 and SBA-15.[71,74,78] When immobilizing lysozyme on an ethylene-bridged PMO, the highest loadings were observed close to the pI of the enzymes, allowing hydrophobic interactions between the nonpolar side chains of the enzyme and the hydrophobic ethylene groups on the support to dominate.[84] Bovine serum albumin and myoglobin exhibited similar behavior when adsorbed on SBA-15.[71] Desorption studies by Goradia et al.[114] implied that hydrophobic/hydrophilic interactions were dominant for trypsin immobilized on CNS.

3.8. Maintaining Enzymatic Activity after Immobilization

The activity of the immobilized enzyme can be greatly affected by the strength and nature of the interaction with the mesoporous surface.[41,99] It is difficult to strike a balance between an interaction sufficiently strong to prevent leaching but weak enough to allow the enzyme enough flexibility to maintain its catalytic activity. If an enzyme leaches from a support during an assay, it is difficult to deduce whether or not the enzyme has maintained its activity while still immobilized or if it is the leached enzyme that is catalyzing the reaction. If the reaction proceeds after removal of the immobilized enzyme, the enzyme has likely leached from the support.[94] In a manner analogous to cross-linking the enzyme or protein to the support, leaching can be reduced by cross-linking the enzyme molecules to form cross-linked enzyme aggregates (CLEAs) after they have been adsorbed on a mesoporous surface or inside a mesoporous cavity. For α-chymotrypsin and *Mucor javaniscus* lipase, cross-linking resulted in a material in which activity was retained and which displayed enhanced stability to shaking and trypsin digestion.[57,116] Papain was similarly cross-linked with glutaraldehyde after adsorption to MCM-48, and its activity decreased by 30% compared to free protein. It showed enhanced stability to temperature and pH and was recycled without further loss in activity for 12 days.[117] However, such cross-linking can result in lower specific protein activities.[57]

Deere et al.[73,108] reported that cytochrome c exhibited higher activity when immobilized on mesoporous materials than when free in solution, possibly owing to the presence of high-spin Fe^{3+} when immobilized. The highest activity was observed when the loadings of cytochrome c were low. Access to the protein molecules was most likely inhibited at higher loadings, owing to pore blocking or multilayer adsorption. Higher activity at lower loadings was also reported for the immobilization of glucose oxidase,[85,92] microperoxidase-11,[118] and CALB.[119] Overall, the activity of the immobilized protein varies with the individual protein, mesoporous support, substrate, and method of immobilization. For example, organophosphorus hydrolase (OPH) immobilized in carboxy-functionalized mesoporous silicate was twice as active as the free enzyme.[91,92] Chloroperoxidase immobilized on MCF exhibited an activity half of that of the free enzyme,[93] while covalently immobilized chloroperoxidase on SBA-16 was more active than the enzyme immobilized by physical adsorption.[65] Chong and Zhao[98] found that most of the functionalized SBA-15 materials used to immobilize penicillin acylase (PA) reduced the activity of the enzyme; only on vinyl-functionalized SBA-15 was PA twice as active as the free enzyme. Porcine pancreatic lipase immobilized on SBA-15 exhibited over twice the activity of free lipase when the immobilized enzyme was silanized to introduce more hydrophobic functional groups, thus improving the access of insoluble substrates to the lipase.[82]

The enantioselectivity of some proteins and antibodies, for example, α-chymotrypsin[63] and aldolase I antibody,[67] has been shown to be unaffected by immobilization on mesoporous materials. Thus, each protein–mesoporous support system is different, and the relative activity of the biocatalyst needs to be established for each entity.

3.9. Activity of Immobilized Proteins in Organic Media

Native proteins often exhibit low activity in organic solvents,[120] but activity can be improved by immobilization on inorganic supports. Interestingly, this situation can result in unusual applications in organic synthesis.[4,9,121] An advantage to using immobilized enzymes in organic solvents is that there may be less leaching, provided the interaction between the enzyme and support is hydrophilic or electrostatic in nature.[102] Takahashi et al.[62] demonstrated that horseradish peroxidase immobilized in mesoporous MCM- and FSM-type materials showed enhanced activity in organic solvents. Deere et al.[46] showed that cytochrome c immobilized in mesoporous materials exhibited peroxidative activity in organic solvents, and the activity of cytochrome c immobilized inside the pores of MCM-41 was greater than that of the same enzyme immobilized on the external surface of a smaller-pore MCM-41 material in aqueous and non-aqueous solvents across a range of temperatures. Goradia et al.[112] demonstrated that trypsin immobilized on various MPS was more active and stable than lyophilized trypsin in many organic solvents. Other examples are listed in Table 3. The enantioselectivity of lipases has been shown to be retained upon immobilization on mesoporous materials in the acetylation of secondary[77] and primary[113] alcohols; the activity of the immobilized lipases was, in some cases, greater than that of the free enzyme.[101] These findings indicate that the confined space of mesoporous supports may provide the stability necessary for proteins to retain their activity and selectivity in organic solvents.

Table 3: Range of proteins and their properties upon adsorption on MPS.

Protein	Leaching	Activity[a]	Stability[b]	Reuse
cytochrome c[64,73,108]	no	higher	enhanced	–
microperoxidase-11[118]	no	less	–	–
lysozyme[84,86]	no	–	–	–
trypsin[41,112,114,126,127]	no	higher higher[c]	enhanced enhanced[c]	yes
organophosphorus hydrolase[45,91,92]	no	higher	enhanced	–
α-chymotrypsin[57,63,116]	no	less	enhanced	yes
horseradish peroxidase[62,73]	–	higher[c]	enhanced	–
chloroperoxidase[65,90,93,94,106]	no	less[c]	enhanced	yes
manganese peroxide[66]	–	active	enhanced	yes
papain[27,117]	no	less	enhanced	yes
pepsin[73]	–	–	–	–
bovine serum albumin[71,73]	–	–	–	–
subtilisin[62,73]	–	higher[c]	enhanced	–
myoglobin[71,127]	–	–	–	–
metmyoglobin[128]	–	higher[c]	–	–
hemoglobin[109,110]	–	higher[d]	enhanced	yes[c]
conalbumin[2,127]	yes	–	–	–
ovalbumin[2]	yes	–	–	–
trypsin inhibitor protein[2]	yes	–	–	–
α-amylase[101]	no	less	enhanced	–
cytochrome P450 CYP2C9[81]	–	less	–	–
cytochrome P450 CYP2B4[81]	–	less	–	–
penicillin acylase[98,99,104]	no	higher	enhanced	yes
glucose oxidase[85,92,100]	no	higher	enhanced	yes
Candida antarctica lipase B[97,113,119]	no	higher[c]	enhanced	yes
porcine pancreatic lipase[75,82]	no	higher	–	yes
Mucor javanicus lipase[102,116]	no	active	enhanced	–
Pseudomonas cepacia lipase[122]	yes	higher	enhanced	yes
newlase F lipase[129]	–	higher	–	–
α-L-arabinofuranosidase[87]	–	less	enhanced	–
fumarase[126]	no	less	enhanced	yes
porcine liver esterase[126]	no	less	enhanced	yes
glucose isomerase[103]	–	higher	enhanced	–
acetylcholinesterase[130]	yes	less	–	–
invertase[105]	–	active	–	–
glucoamylase[105]	–	less	–	–
polyphenol oxidase[131]	–	less	good	–
thermolysin[132]	–	active	–	–
aldolase antibody 84G3[67]	no	higher[c]	enhanced[c]	yes[c]
scFV antibody fragment[3]	no	–	good	–

[a] Relative to free enzyme in solution. [b] Hydrothermal, denaturant or storage. [c] Organic solvent. [d] electroactivity. – not reported.

3.10. Reusability and Enhanced Stability of the Immobilized Protein

Permanent immobilization of the enzyme on the support is very important in biocatalysis. If the enzyme is too easily leached from the support, it will not be easily recovered from the reaction mixture. The ability of the support to retain the enzyme during the activity assay, and to be reused with minimal loss in activity, is the acid test of successful immobilization. Moreover, immobilization inside the mesopores can also enhance the inherent stability of the protein to heat, extreme pH values, denaturants, and organic solvents. Zheng et al.[122] reported an enhanced thermal stability of immobilized *Pseudomonas cepacia* lipase under the optimal conditions of their reaction assay, that is, at pH 8 in the presence of CTAB surfactant. The immobilized lipase was reused eight times, and the approximately 50% loss in activity was attributed to leaching and/or mechanical losses during the reaction and recovery of the immobilized enzyme. α-Chymotrypsin immobilized on MCM-41 was reused over 100 times in one week.[63] CALB immobilized on octatriethoxysilane-functionalized mesoporous silica exhibited no loss in activity over 15 cycles in organic media.[119] Chloroperoxidase was found to be more stable to hydrogen peroxide when immobilized in the pores of SBA-15[94] and was reused 20 times when immobilized on an amine-functionalized PMO (PMO PA) with little loss in activity.[90]

Silanization to reduce the size of pore openings after protein adsorption is one way to reduce leaching.[27,75] However, it can result in deactivation of the protein. When porcine pancreatic lipase (PPL) immobilized on MCM-41 was silanated after adsorption with vinyltrimethoxysilane, the activity of the PPL was reduced to approximately 40% of that of the free lipase, but the final biocatalyst showed no loss in activity after being reused five times.[75] However, when PPL was immobilized on SBA-15, which has larger pore sizes than MCM-41, silanization did not sufficiently close the pore openings, and leaching occurred. Recently this problem was overcome by polymerizing a methacrylate group onto the silicate after silanization to narrow the pore entrances even further.[61,82]

Immobilization can also enhance storage stability, as mentioned with the cross-linked enzyme aggregates in Section 3.8. Trypsin immobilized on MCM-41 was still active after a week at 25°C, whereas free trypsin had been completely inactivated;[27] trypsin immobilized on CNS also exhibited enhanced stability in aqueous and organic solvents.[112,114] Organophosphorus hydrolase (OPH) immobilized on carboxy-functionalized mesoporous silica was more active after 145 days of storage than free OPH.[91] For immobilized protein biosensors, storage stability is very important. Hu et al.[3] found that the antigen binding capability of an immobilized antibody fragment was unchanged after 42 days of storage.

Provided that the immobilized enzyme does not leach from the mesoporous support, it can be recycled. Even if it shows a reduced activity compared to the free enzyme, it may be a better catalyst overall and have more applications because of its ease of recovery from the reaction mixture and its reusability. Table 3 summarizes some of the properties for published mesoporous biocatalysts. Note that unless the system has been successfully recycled, it is not certain that the enzyme remains immobilized throughout the activity assay. Of the enzymes immobilized on mesoporous materials, only approximately one third have been examined for their ability to be recycled, and activity losses occur in each cycle in many of these cases.

4. Summary and Outlook

Enzymes are large, complex, biological molecules that can be adsorbed on mesoporous materials by a range of electrostatic, hydrophobic/hydrophilic, and covalent interactions, with varying levels of stability, activity, and reusability. The maximum amount of enzyme adsorbed on a mesoporous material varies with pH value, ionic strength, pore diameter, pore volume, surface characteristics, isoelectric point, morphology, particle size, and adsorption conditions. Immobilization can lead to enhanced storage stability of many enzymes, but the surface functionality of the support needs to be such that it allows for sufficient loading of the protein in the correct orientation. Furthermore, to be useful in biocatalysis, the immobilized enzyme must be capable of being reused; that is, the support must retain the enzyme not only during washings with water or immobilization buffers but also when washed with working solutions. A detailed understanding of the conformational properties and active sites of each protein is essential in the design of a successful mesoporous support for an immobilized protein biocatalyst. Recent efforts have focused on the development of functionalized mesoporous silicate structures[37] with ordered pore structures large enough to accommodate proteins with interesting catalytic properties inside their channels. Such efforts reflect the need to move from the use of model proteins such as lysozyme and cytochrome c to more complex, synthetically useful enzymes (Table 3). Considering the factors illustrated in Figure 3 and the availability of MPS with surface functional groups that can be tailored, there is significant potential to develop stable and active biocatalysts.

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