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REVIEW

Enzyme-functionalized mesoporous silica for bioanalytical applications

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Abstract The unique properties of mesoporous silica materials (MPs) have attracted substantial interest for use as enzyme-immobilization matrices. These features include high surface area, chemical, thermal, and mechanical stability, highly uniform pore distribution and tunable pore size, high adsorption capacity, and an ordered porous network for free diffusion of substrates and reaction products. Research demonstrated that enzymes encapsulated or entrapped in MPs retain their biocatalytic activity and are more stable than enzymes in solution. This review discusses recent advances in the study and use of mesoporous silica for enzyme immobilization and application in biosensor technology. Different types of MPs, their morphological and structural characteristics, and strategies used for their functionalization with enzymes are discussed. Finally, prospective and potential benefits of these materials for bioanalytical applications and biosensor technology are also presented.

Keywords Mesoporous silica · Enzyme · Immobilization · Biosensors

Introduction

The development and discovery of new materials with suitable properties for enzyme immobilization is an emerging area of research. Efficient immobilization of enzymes on solid supports is of crucial importance in different areas, such as biotechnology, biocatalysis, protein-delivery systems and biological sensors [1]. The main challenge is to design functional biocompatible materials and interfacial structures which allow stable attachment of enzymes while maintaining their activity and function as close as possible to their native state. In order to increase the efficiency of enzyme immobilization and biocatalytic processes, it is highly desirable to develop materials that have a large surface area, hydrophilic character, high porosity, increased stability with changes in the microenvironment, and high mechanical strength. Ideally, the immobilization method should ensure enzyme stability over long periods of time, avoid leaching, be reusable, and allow free diffusion of substrates and reaction products.

Recent advances in nanotechnology have made it possible to create materials possessing many of the properties of the "ideal" immobilization matrix. Among these, nanostructured mesoporous silicas (MPs) are excellent candidates for enzyme immobilization [1, 2] and bioanalytical applications. MPs are a class of inorganic material with uniform, well-ordered porous structure and an extremely high surface area. Since their discovery in 1992 by the Mobil research group [3], different types of MPs molecular sieves have been developed. Synthesis of mesoporous thin films [4–10], spheres [11–13], curved solids [6, 14], tubes [15, 16], rods and fibers [6, 17], membranes [18], and other monoliths [19] has been reported recently. These shapes are a good fit to host enzymes, because of their relatively large pore diameters (2–40 nm), matching the size of the enzymes, narrow pore size distribution, large pore volumes (ca. 1.5 $cm^3\ g^{-1}$) and a high surface area (up to 1500 $m^2\ g^{-1}$). In addition, their inorganic silicate network is chemically and thermally stable, has low toxicity and high mechanical strength, and can be easily functionalized for future covalent attachment of proteins. These materials have been shown to provide enhanced binding efficiency and increased catalytic activity and stability; they also enable operation in harsh conditions (i.e. high temperature [2],

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denaturing agents [20]) and in organic solvents [21], with excellent recycling characteristics [22] compared with the free enzyme. Most MPs reported in the literature have rather small pores (usually less than 7 nm) which restricts their application to the immobilization of small proteins, for example cytochrome *c* (molecular weight of 13,000 Da), lysozyme (13,930 Da), and α-chymotrypsin (21,600 Da) [23, 24]. There are only anecdotal reports of synthesis of larger pore sizes [25–28].

The stabilizing properties of MPs are attributed to nanosized channels which act as small cages surrounding the enzyme, consequently providing a protective chemical microenvironment similar to the environment near enzymes in biological cells [23, 29, 30]. Moreover, a well defined network of the MPs, and interconnected mesoporous channels [31, 32] protect the enzymes from leaching while allowing free diffusion of the substrate and product molecules to/from the catalytic active sites [33]. Channel geometry is, therefore, a key property determining the enzymatic reaction rate [29, 34, 35]. Enzymes can be immobilized inside the matrix of MPs by using different approaches, including physical adsorption via hydrogen bonding, electrostatic and hydrophobic interactions between the enzyme and the silica support [36, 37], encapsulation [36, 37] and chemical binding [29]. Various strategies for preventing leaching and stabilizing the enzyme–MPs aggregate have also been reported and will be discussed in this review.

These MPs–enzyme hybrids have potential for implementation in practical applications in different areas of biotechnology and bioanalytical assays. However, most research has so far been oriented toward the synthesis, characterization, and functionalization of different types of MPs. Relatively little effort has been dedicated to studying their suitability for practical applications. This review mainly focuses on the most recent technological advances in the biofunctionalization of MPs with proteins, and discusses prospective and potential benefits for future developments of this technology to build enzyme biosensors. Special emphasis is given to the various types of MPs, mechanisms and methods used to attach the enzyme, and various factors affecting the binding efficiency, stability, and activity of the immobilized biocatalyst in harsh conditions.

Mesoporous silica: synthesis, types, structure, and control of morphology

Table 1 summarizes the most commonly used MPs with selected examples of protein immobilization. An important challenge in the preparation of these materials is to tune their morphological and structural properties, including control of pore diameter, to match the size of the specific enzyme, regulation of the presence of functional groups, and achieving a high surface area. In addition, pore interconnectivity is highly desirable to allow mass transport and molecular diffusion of reaction substrates and products. Extensive research has been dedicated to the modeling of their pore size in order to accommodate different biomolecules with different molecular sizes. Hosts of research papers have reported the possibility of tuning the pore diameter using various synthetic methods and experimental conditions, such as the use of cationic surfactants with different alkyl chain length, choice of temperatures, adding organic material during the crystallization step, and post-synthesis hydrothermal processes [38–42]. Depending on the fabrication method, functionalization can be achieved post-synthesis or in situ, during synthesis of the mesoporous material [33, 43–55].

The first and the most well known type of MPs is the MCM-41 [3]. This is a unidimensional system with a narrow pore-size distribution and hexagonal arrangement where access of the enzymes may be restricted to the end of the channels [23]. Other MCM molecular sieves with different morphologies are also available, including a cubic (e.g., MCM-48) and a laminar structure (e.g., MCM-50). The structures of three representative MCM sieves are presented in Fig. 1. Typically, MPs from the MCM family have relatively small pore sizes (of about 2–10 nm).

Table 1 Characteristics of the most common types of MPs and selected examples of the immobilization of proteins

MPs type	MPs pore size	Proteins size	Observations	Ref.
MCM-41	1D hexagonal ~2–10 nm	Cytochrome *c*, papain, trypsin	Enhanced stability; prevented enzyme leaching by silanation of the pore opening; size and pH-dependent	[23]
MCM-48	2D cubic with interconnecting pore systems; 3.2–3.5 nm	Cytochrome *c*	Increased hydrothermal stability and overall catalytic activity	[52]
SBA-15	Tunable 5–30 nm; 2D hexagonal	Larger proteins (ex: porcine pancreatic lipase)	Small proteins such as cytochrome *c* immobilized by silanation of the pore	[53–55]
MCF-meso cellular foam	~10 nm spherical	Lipase B (6.9×5.0×8.7 nm)	Enzyme loading was pressure-driven; enhanced thermal stability (80 °C)	[56]

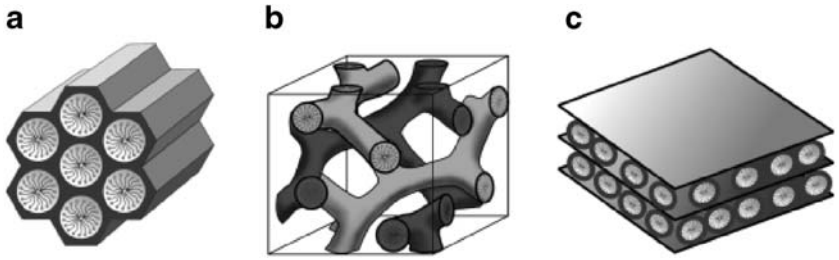

Fig. 1 Structure of hexagonal MCM-41 (**a**), cubic MCM-48 (**b**), and lamellar MCM-50 (**c**). Reproduced with permission from Ref. [57]

Preparation, characterization, structural, and morphological properties of silica-based mesoporous materials, with focus on those from the MCM class, can be found in Refs. [57–59].

More recently, MPs with larger pore sizes have been prepared. A representative example is the Santa Barbara Amorphous-15 (SBA-15), a 2D hexagonal MPs structure with tunable pore dimensions in the range 5–30 nm and high hydrothermal stability [53]. Han et al. reported two siliceous mesocellular foams, MCF and FDU-12, with large and tunable pore size of about 10 nm (sizes ranging between 10 and 50 nm) and a 3D interconnected pore structure [56]. Preparation and functionalization of several families of mesoporous materials have been reviewed by Vinu et al. [53] and Hartmann [1]. Another important aspect of the synthesis of MPs is their overall morphology and the shape of the particles [60–62]. Typically, the MP material as synthesized is a shapeless powder, consisting of particles of various dimensions. Obviously, diffusion of molecules towards the encapsulated enzymes will be quite different for small particles (short diffusion path) and for large particles (long diffusion path). The problem of "shaping" of MPs, sometimes called morphogenesis has been studied in Refs. [60, 61]. Many syntheses produce particles of various shapes, see, e.g., the Origami type of synthesis [62]. While,

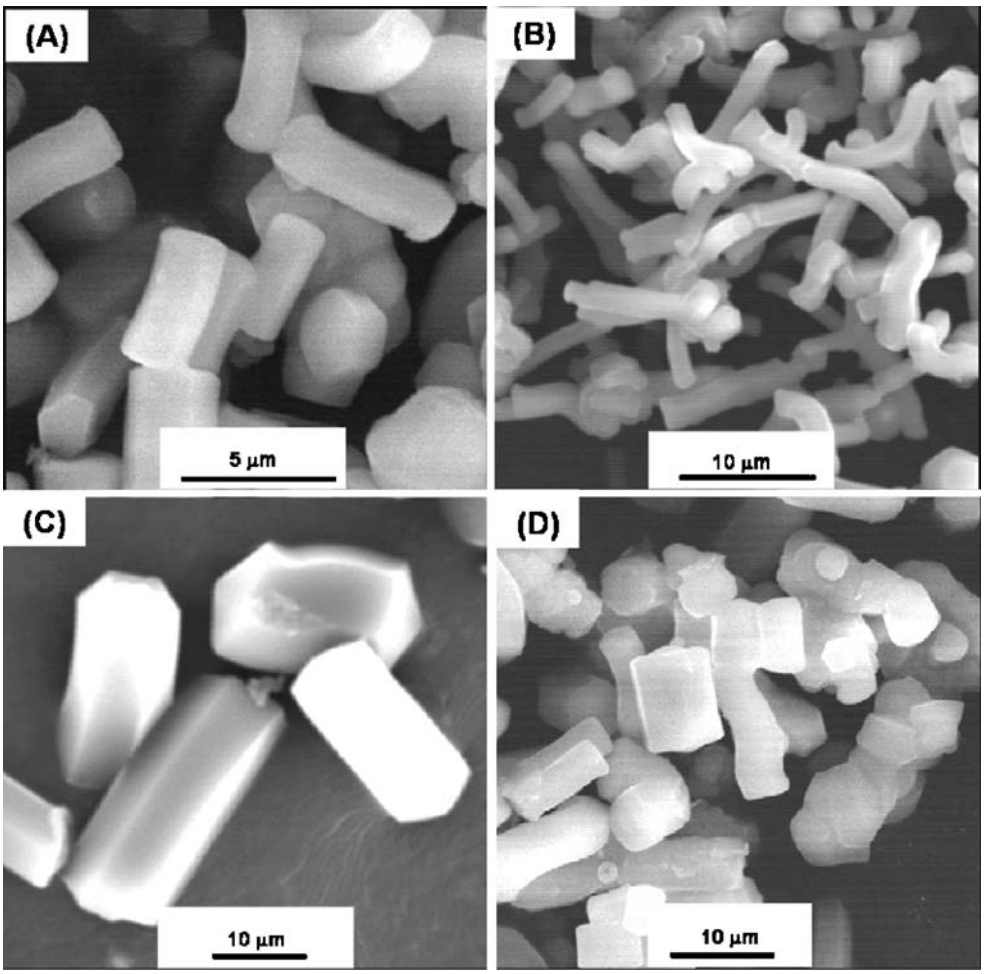

Fig. 2 Fibers of MP silica of different aspect ratios. Reproduced with permission from Ref. [63]. Silica precursor concentration, acidity, and temperature were changed in images A–D

in principle, it is possible to separate some shapes, it is definitely easy and more reliable to synthesize particles with similar morphology. Several works have recently reported synthesis of such monodispersed shapes [63–65]. The reported particles are mostly fibers in which nanochannels run along the axis of the fibers. By changing precursor concentration, acidity, and temperature one can obtain fibers (nanochannels) with different aspect ratios. An example of such syntheses is shown in Fig. 2. These shapes may serve as good hosts for encapsulation of enzymes.

Fabrication of enzyme–mesoporous silica systems

Modeling the interactions between the enzymes and MPs matrices

Obtaining stable and active enzyme–MPs aggregates with high loading efficiency and fast reaction rates requires precise control and careful optimization of several properties: the size of the MPs pores, available surface area, solution pH, isoelectric point, pore size distribution, ionic strength, and the surface characteristics of the MPs and the proteins [24]. The choice of the MPs is dictated by the type and the structural characteristics of the enzyme. A generic approach for the systematic study of MPs-based enzyme systems, the experimental conditions, and the expected performance is presented in Fig. 3. The effect of the key factors contributing to the success of the immobilization is described in this section.

Pore diameter, pore volume, and size matching

Access of enzymes into the MPs pores is restricted by pore dimensions. If pores are too small, enzyme attachment is carried out exclusively on the external surface of the silica and, subsequently, the binding efficiency is low. On the other hand, if the pores are too large, enzyme leaching might occur. In the latter situation, it is possible to graft different organic functionalities such as amines and carboxylic acids on to the siliceous surface to allow further covalent binding of the protein to achieve stable attachment [29, 43]. Research has shown that using MPs with sizes close to that of the enzyme facilitates stable protein adsorption with high activity [65–68]. Early studies on the immobilization of small proteins in MPs [23] indicated that the adsorption efficiency is correlated with the size match between the diameter of the protein and the pore-openings

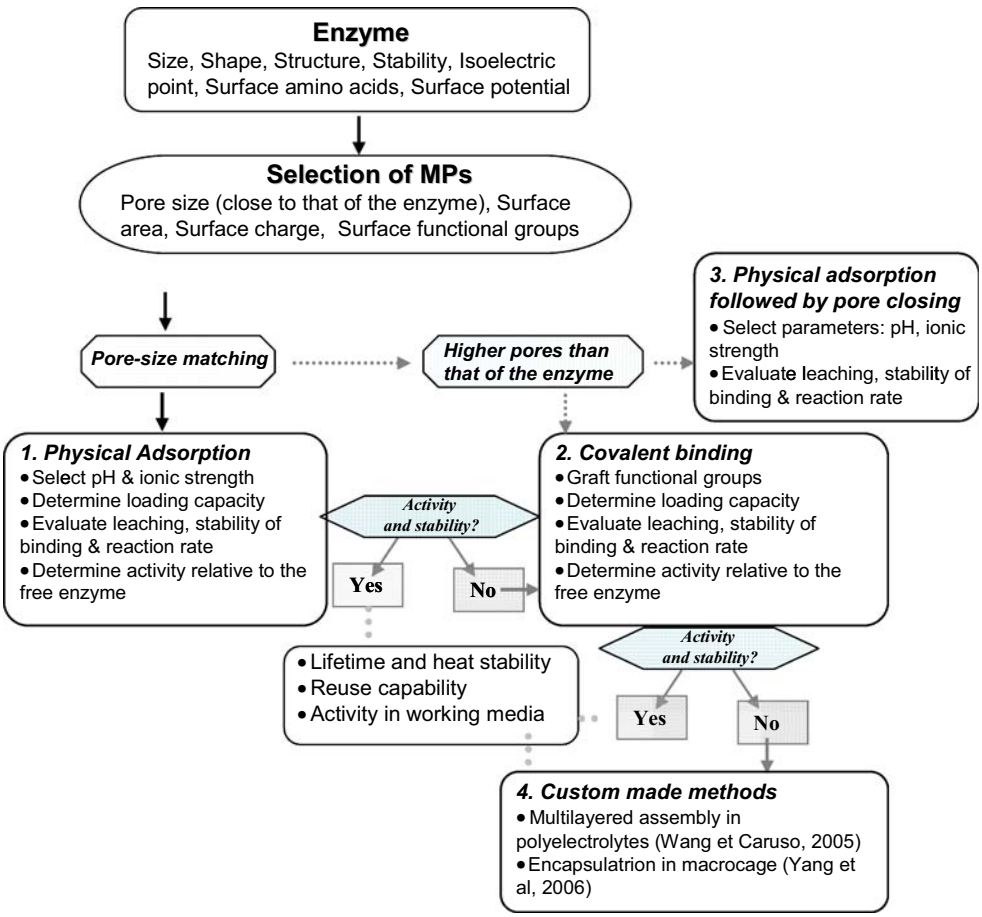

Fig. 3 Systematic approach for selection of MPs, the immobilization method, and study of enzyme–MPs system

of the MPs. Vinu et al. [35, 66] systematically studied the adsorption of cytochrome *c* (cyt *c*, molecular dimensions 2.6×3.2×3.0 nm) on to MPs with different pore diameters: MCM-41, A1MCM-41, SBA-15, and A1SBA-15. They found that adsorption capability increases as pore volume and/or pore diameter increases. The amount of adsorbed protein occupied ~30% of the total pore volume.

Effect of pH, ionic strength, and surface charges

pH and ionic strength strongly affect the binding efficiency and stability of the protein–MPs system. This is because the forces driving adsorption of proteins on to the MPs materials are mainly hydrophobic and electrostatic interactions. The optimum pH is selected according to the isoeletric point (pI) of the protein. The silica surface is mainly negatively charged at a pH > 2. Deere et al. [69] confirmed that the surface charges of the protein and MPs must be complementary in order to achieve high adsorption efficiency. Vinu et al. [35] showed that the maximum adsorption of cyt *c* occurred at a pH near the pI of the protein. At this pH, the ionic forces between the protein and the silica are small and, thus, the hydrophobic interactions become more important. In this case, the surface properties of the silica play a critical role in binding efficiency. For instance, higher protein adsorption occurred on MPs templated with cationic surfactants compared with those with nonionic surfactants [21]. Deere et al. [69] investigated the adsorption of cyt *c* on cyano-functionalized MPs, and the function of the ionic strength of the buffer solution, and showed that the amount of adsorbed protein strongly decreases as the ionic strength increases.

Methods of enzyme immobilization on MPs materials

Physical adsorption

The simplest approach used to immobilize enzymes on to MPs is by direct adsorption of the protein within the mesoporous channels. The method is very simple to perform, is non-destructive and can be carried out in mild conditions, which are favorable for maintaining biological activity. The experimental procedure consists of simple mixing (or stirring) of the MPs with enzyme solution, followed by centrifugation and washing to remove excess unbound enzyme. The first information about the efficiency of the immobilization is obtained by UV–visible analysis of the supernatant for residual protein content.

Balkus' group [23, 55] studied the immobilization of cyt *c* on to MCM-48, SBA-15 and a layered niobium oxide (Nb-TMS4) and demonstrated that protein loading is influenced by the structure and the available surface area of each material. The greatest protein loading was obtained with MCM-48, which has a 3D structure and the highest surface area. In another study, the highest adsorption (536 mg g^{-1}) of lysozyme was obtained by using a cellular foam structure, while the fastest adsorption was obtained with mesoporous hollow spheres, with an adsorption rate of more than 97% protein within 5 min [59]. Urabe et al. [67] reported that encapsulation of another small protein, hemoglobin (Hb, molecular dimensions 6.5×5.4×5.5 nm) on to three different folded-sheet mesoporous type material (FSMs) with diameters of 2.5, 4.0, and 7.5 nm, was highly dependent on pore size, the higher binding being obtained for the FSM with a 7.5 nm diameter. The results of these studies also indicated that binding occurred mostly inside the pores and not on the outer silica surface. The protein structure and the peroxidase like activity of Hb were preserved even in harsh conditions (heat treatment at 85 °C) [67]. This resistance to heat was attributed to the protective effect of the MPs matrix, which prevents the protein undergoing extensive conformational changes inside the pores. This process is schematically presented in Fig. 4.

The physical binding occurs by physical forces and ionic and hydrophobic interactions, as described previously, which are relatively weak and could result in protein

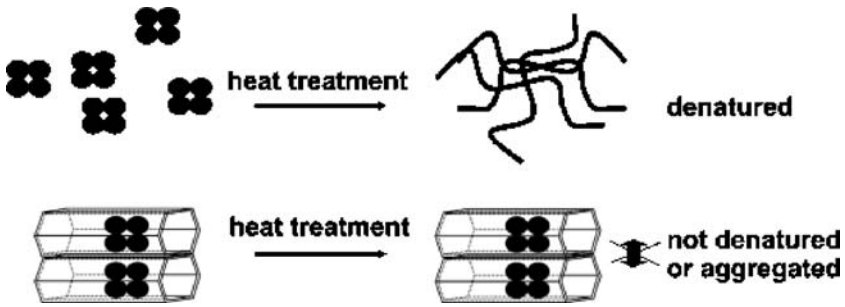

Fig. 4 Schematic diagram suggesting the behavior of free and immobilized Hb in FSM after heat treatment. Reproduced with permission from Ref. [67]

leaching. Adsorption of proteins has been enhanced by modification of the MPs with transition metals, such as Nb [55, 70, 74], which favor protein binding via the metal oxide surface. In another study, adsorption of cyt *c* on Al-substituted MPs was higher than on the analogous pure silica material in the pH range 3.0–9.6 and lower at pH 10.6 [35]. At low pH, the positively charged amino acid residues interact electrostatically with the negatively charged Al and silanol groups, resulting in a stronger binding. Han et al. [56] used a "pressure-driven method" instead of the conventional stirring of MPs particles in enzyme solution to obtain an enhanced enzyme loading. The procedure consists in applying a high pressure of ~3000–5000 psi to force protein adsorption inside the MPs channels. The method was applied for the immobilization of *Candida antartica* lipase B (CALB) on hydrophobic mesoporous foam, MCF [56]. The resulting biocatalyst showed higher enzyme loading, small enzyme leaching and high thermal stability compared with that of the commercial immobilized enzyme, Novozyme 435. The method, however, might not be generally applicable, because some proteins could be denatured by the high pressure. Using an emulsion confinement route, Sun et al. synthesized nanoscale silica particles with ordered large mesopores (13 nm), with ultrafast adsorption (10 min vs 20 h) and higher binding capacity for lysozyme compared with the conventional SBA-15 [71].

If protein leaching occurs, strategies such as pore-size matching, reduction of pore diameter by silylation, modification of the pore walls with thiol or carboxylic groups, and in-situ polymerization of vinyl groups have been effective in overcoming the problem [54]. Desorption of cyt *c* from molecular sieves (MCM-48, Nb-TMS4, and SBA-15) at or above the pI of the protein was prevented by blocking the pore openings by organosilane functionalization [23, 55]. However, post-synthesis functionalization could result in pore blocking and reduced or random distribution of functional groups on the MPs walls. As an alternative, Wahab et al. [72] functionalized the surface of the mesoporous material with propylamino terminal groups in situ, during the synthesis of the MPs, by including organic groups in the reaction mixture. In other work, Hudson et al. synthesized amino-functionalized periodic mesoporous organosilanes (PMOs) with large pore size (~10 nm) to adsorb larger molecules [73]. The resulting PMOs were investigated as an immobilization matrix for chloroperoxidase (CPO, 6.2. nm in length). The results were compared with those obtained using a post-synthetic functionalization procedure. It was shown that CPO immobilized on PMOs could be reused 20 times without loss of activity. In contrast, the post-synthetic amino functionalization procedure did not yield a stable biocatalyst.

Covalent attachment

An alternative to the physical adsorption method is via covalent attachment procedures. The most common approach involves several steps:

1. grafting of functional groups (amino or carboxyl) on the silica surface [44–49];
2. derivatization with a cross-linking agent such as glutaraldehyde or carbodiimide/succinimide; and
3. enzyme binding.

MPs–enzyme systems obtained by covalent methods are generally characterized by good stability. However, the procedure is more complex, requires a large amount of bioreagent, and could be difficult to reproduce. Comparison of physical adsorption and covalent attachment procedures for immobilization of penicillin acylase on MCM-41 showed that the enzyme immobilized through physical adsorption is characterized by higher activity than that of the covalently linked enzyme (via glutaraldehyde) [75].

Zhang et al. synthesized amino-functionalized MCF with large pores by post-synthesis grafting of tetraethoxysilane and 3-aminopropyltriethoxysilane and used this material for covalent immobilization of glucose oxidase (GOX) [44]. The resulting enzyme–MCF material had high biocatalytic activity and enhanced thermal stability. Wang et al. attributed the enhanced stability of enzymes immobilized on MPs to the concave pore surface, which facilitates multipoint attachment of the enzyme inside the nanochannels [76]. To demonstrate this concept, they covalently immobilized α-chymotrypsin in a highly ordered nanoporous silica support with a pore size of 15.3 nm and a surface area of 560 m^2 g^{-1}. They hypothesized that, in contrast with flat or convex surfaces, a concave surface provides a favorable area for covalent attachment of the enzyme at multiple points, especially when the radius of the curvature matches the size of the biological molecule. This model is presented in Fig. 5. Stronger covalent binding of GOX was also achieved by modifying MPs with gold nanoparticles [77] or magnetite [78].

Enzyme entrapment in macrocages and bimodal MPs structures

In addition to the traditional procedures described above, other innovative approaches have been developed. Wang and Caruso reported encapsulation of several enzymes (catalase, peroxidase, lysozyme) in mesoporous silica spheres by physical adsorption, followed by stabilization through deposition of multilayered polyelectrolyte shells on to the enzyme-loaded silica [36, 37]. The silica spheres have a bimodal mesoporous pore structure (2–3 nm and 10–40 nm) and a surface area of 630 m^2 g^{-1}. Enzyme loadings

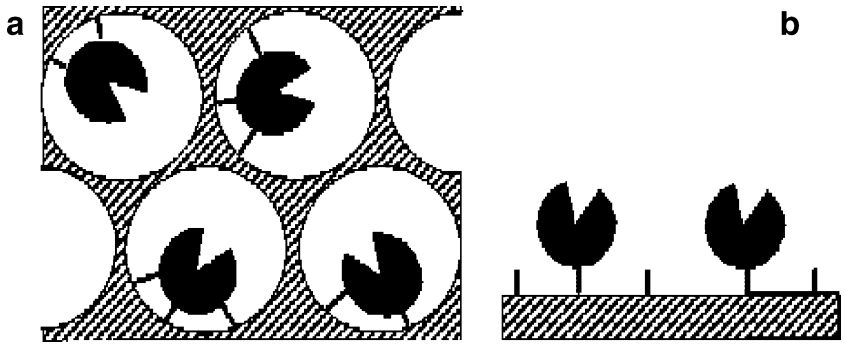

Fig. 5 Covalent attachment of enzymes on to nanoporous silica showing a multi-point attachment of the protein to the concave surface (**A**) and single-point binding to a flat surface (**B**). Reproduced with permission from Ref. [76]

ranging between 20 and 40% were obtained for small enzymes with high pI, and loadings were ~8% for larger enzymes. Using this approach, enzymes had enhanced stability with increasing number of polyelectrolyte layers, reusability up to 25 times, and retention of activity after exposure to degrading substances such as proteases. However, reaction rates were reduced because of the kinetic limitations imposed by the presence of the polyelectrolyte multilayers.

Using a so-called "ship-in-a-bottle" approach, Lee et al. [79] immobilized enzymes in hierarchically ordered mesocellular mesoporous silica materials (HMMS) consisting of a mixed structure of mesocellular silica foam (MCF) and one-dimensional SBA-15 channels (Fig. 6). Following adsorption, enzymes were crosslinked with glutaraldehyde, resulting in the formation of crosslinked enzyme aggregates in HMMS. These bioassemblies showed no decrease in biocatalytic activity for over a month, under conditions of continuous shaking. Without glutaraldehyde treatment, the enzyme continuously leached from the silica matrix. Yang et al. [22] used "macroporous cages" of MPs connected by uniform mesoporous channels to encapsulate several enzymes (fumarase, trypsin, lipase, and porcine liver esterase) using a so-called" fish-in-net" procedure. As opposed to the "fish-in-a-bottle" method, here the enzyme served as template for the formation of the macroporous cage. Entrapment was achieved by incorporating the enzyme during hydrolysis and condensation of the precursors in the silica network. The resulting bioaggregate was an ordered mesostructure containing a xerogel with the encapsulated enzyme in a "cage" connected by uniform mesoporous channels. This model is schematically presented in Fig. 7. When water is introduced in the channels, it creates an aqueous environment in the interior of the cage, similar to that of the native enzymes in their natural environment. This approach provided good biocatalytic activity, long-term stability, and excellent recycling characteristics.

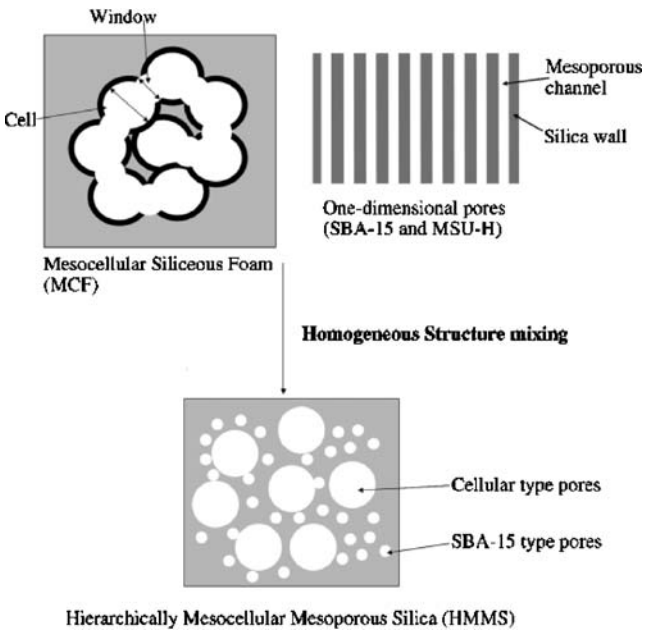

Fig. 6 Schematic diagram showing the bimodal structure of HMMS: mesocellular silica foam (MCF) pores and one-dimensional SBA-15 pores. Reproduced with permission from Ref. [79]

Benefits and potential applications of mesoporous silica in bioanalysis. Comparison with other supports

Potential bioanalytical applications of MPs include mainly biosensors, different types of optical bioassay, and bioreactors. Enzyme entrapped within MPs pores can be deposited on to a variety of transducer surfaces and act as a biomolecular recognition element to catalyze specific reactions. The main advantages of these materials in bioanalysis, compared with commonly used supports (e.g. various types of polymer, sol–gels, metals, carbon), is the likelihood of providing enhanced stability of the encapsulated biomaterial over long periods of time. However, they also have several limitations that hinder their full exploita-

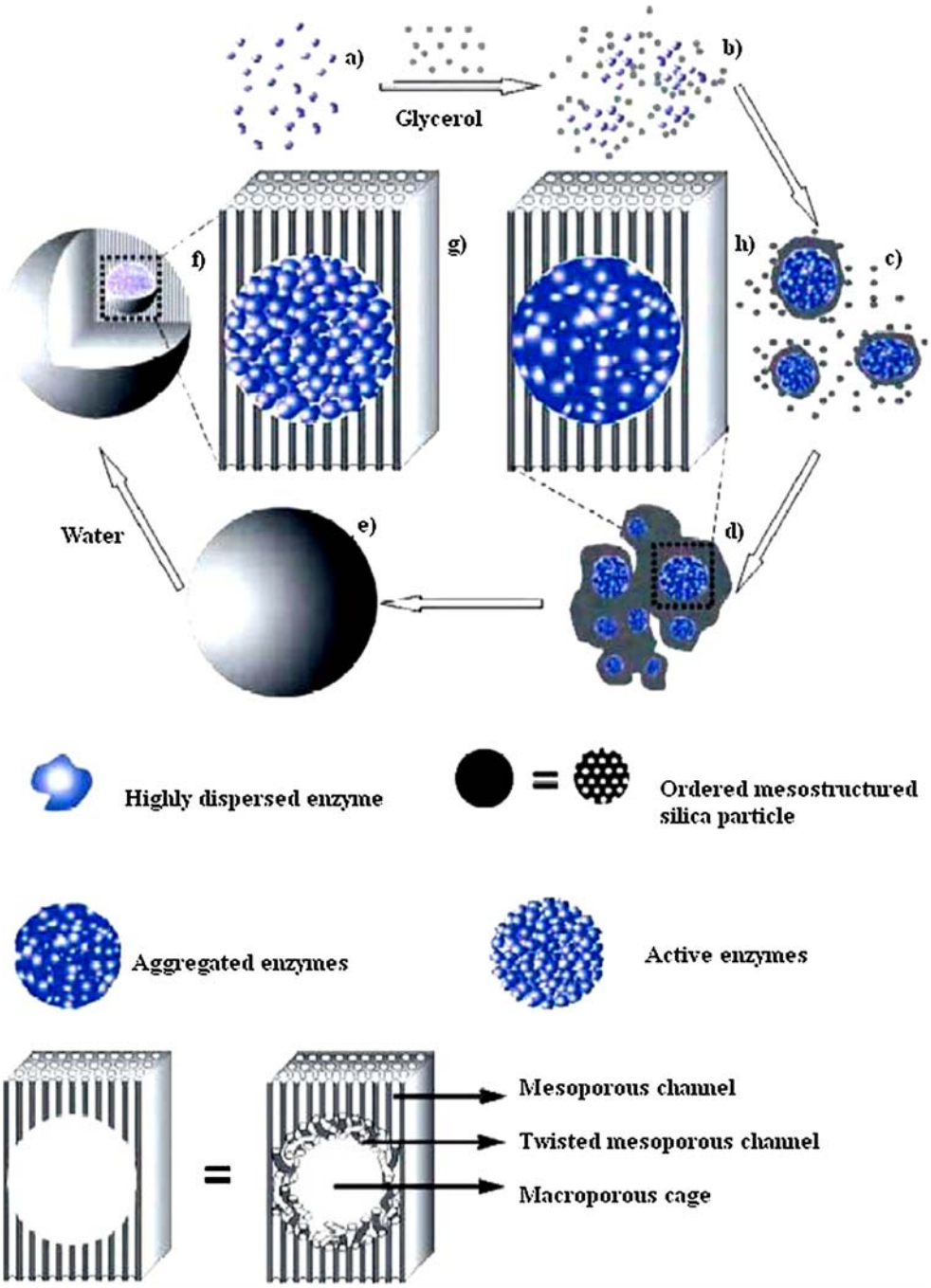

Fig. 7 "Fish-in-net" procedure for enzyme immobilization: **a**, enzymes in a buffer solution; **b**, preformed precursors with ordered mesostructured silica particles mixed with the enzymes; **c**, assembly of enzymes with ordered mesostructured silica particles; **d**, inorganic polymerization and condensation; **e**, formation of silica spheres encapsulating enzymes in macroporous cages; **f**, silica sphere with the encapsulated enzymes under biocatalytic conditions, with a chemical environment for the enzymes in the macroporous cages similar to that of native enzymes in solution. It is probable that the enzymes are dispersed; **g**, magnification of the enzyme-filled macroporous cages from **f**; **h**, magnification of the enzyme-filled macroporous cages from **d**, where the enzymes are aggregated in the macroporous cages. Reproduced with permission from Ref. [22]

tion, for example diffusion barriers, requirements for size matching, and possible enzyme leaching. Table 2 summarizes the advantages and limitations of these materials for enzyme immobilization in comparison with other supports.

The use of MPs as immobilization supports provides a way of stabilizing enzymes for use in industrial bioprocesses and applications which require operation in harsh conditions or non-aqueous environments. For example, Takahashi et al. [68] reported that immobilized horseradish peroxidase (HRP, average molecular diameter 4.5 nm) had high activity in an organic solvent (toluene) when the enzyme was adsorbed in MPs with an average pore size just exceeding the molecular diameter of the enzyme. The immobilized enzyme had high thermal stability, with 80% of the enzyme remaining active after exposure to 70 °C for 120 min. The highest enzymatic activity was obtained with FSM-16 and MCM-41, with pore diameters of ~5 nm. It was found that the surface characteristics and the matching size are critical properties for enzyme stabilization in an organic solvent and at high temperatures (70 °C).

Table 2 Advantages and limitations of MPs and comparison with commonly used immobilization materials

Electrode material/ procedure	Advantages	Limitations	Ref.
MPs	Enhanced operational and storage stability, operation in harsh conditions (organic solvents, high temperatures); chemical, thermal, and mechanical stability; high surface area	Diffusion barriers; access restricted to pore dimensions; most MPs have small pore-size; synthesis of MPs with tunable porosity and morphology is a challenge; possible leaching; sensitive to pH and ionic strength changes	[2, 20–28]
Nanomaterials (e.g. carbon nanotubes metal nanoparticles)	High electrochemical active surface area; high conductivity; suitable for development of miniaturized devices	Generally require further functionalization; agglomeration and aggregation of nanoparticles is difficult to control	[80–83]
Solid supports (e.g. metal, glassy carbon) through covalent binding	Stable; absence of diffusion barriers; short response time	High amount of enzyme; possible denaturation of the biomolecules; poor reproducibility;	[84–87]
Polymers/entrapment	Simple; many biocompatible polymers available; suitable for a large variety of bioreceptors	Diffusion barriers	[88]
Sol–gel/entrapment	Chemical, thermal, photochemical and structural stability; one-step procedure at ambient or low temperature	Diffusion barriers; problems of reproducibility and control of pore size	[88–91]

These stabilizing effects are attributed to confinement of the protein in the rigid silica nanochannels, mainly through electrostatic interactions with the charged silica surface. These interactions affect the folding/unfolding and the rotational mobility of the protein [50, 51]. Typically, a protein confined within the MPs pores loses its ability to unfold and destabilize its native conformation, providing resistance to denaturing agents, as illustrated in Fig. 4. This property can be beneficial in designing robust biological sensors. The mechanical properties of the MPs could facilitate application in flow-through systems and in biocatalytic microreactors. For example, MPs silica with encapsulated enzymes could be used to pack a micro-capillary tube and construct a "micro-bioreactor" for a variety of biotechnological applications.

MPs could also be used as probes for absorbance, fluorescence, and fluorescence quenching in optically based measurement methods. A first example in this direction is the adsorption of photosynthetic light-harvesting membrane proteins (LH2) into a folded sheet silica material (FSM) with a honeycomb-like hexagonal cylindrical structure and two different pore diameters (7.9 and 2.7 nm). The silica–protein resulting from the MSF with a 7.9-nm diameter had increased thermal stability and higher binding efficiency [92]. This study demonstrated that light-harvesting membrane proteins conserve their properties when immobilized in MPs matrices with adequate pore size and structure. Although unexplored, a possible application would be as optical sensors. These could be used as non-invasive methods in biological systems. Exploitation of MPs in combination with immunoassays is also possible.

Biosensing applications of MPs–enzyme systems

Despite the great promise of extended stability and operation in harsh environmental conditions, biosensing applications of these materials are scarcely reported. Most of the work in this field concerns immobilization of relatively small heme proteins, such as hemoglobin, HRP, myoglobin, and cyt *c*. However, because of recent advances in the synthesis of large-pores MP, there is a potential application for large enzymes. We summarize below examples of biosensing applications reported so far in the literature, with their advantages and limitations and stress their potential for future developments in this field. Some studies have reported the direct electron transfer of these redox proteins inside the MPs matrix [93–97], but the underlying mechanism is not yet completely understood. The use of such material would most likely result in enhanced analytical characteristics of the biosensors, particularly with respect to operational stability, life-time, and operation in harsh conditions. The presence of diffusion barriers and possible enzyme leaching are major limitations, resulting in high response times and instability problems (Table 2). Even though several strategies are available to avert leaching, these are generally complex and poorly reproducible, involving multiple processing steps

prior to enzyme immobilization. Furthermore, their successful use in biosensors is strongly dependent on how efficient the enzyme–MPs assembly is attached on to the physical transducer surface and the homogeneity of the silica layer. Another problem is the poor conductivity of the silica.

The conductivity can be improved by adding Au nanoparticles to the silica matrix, as described by Bai et al. [77], or by using mesocellular silica-carbon nanocomposite foams (MSCF) [97]. For example, a hybrid Au–silica material was formed by adsorbing $AuCl_4^-$ on to amino functionalized SBA-15, followed by $NaBH_4$ reduction. The presence of Au nanoparticles offers a biocompatible microenvironment for the enzyme. This system was employed for the fabrication of an amperometric glucose biosensor, in which the gold was also used to increase the conductivity of the silica matrix. The biosensor had a fast response time to glucose of less than 7 s, a broad linear range of 0.02–14 mmol L^{-1}, high sensitivity of 6.1 μA $mmol^{-1}$ L cm^{-2}, and good long-term stability and reproducibility. Following a similar approach, Zhu et al. [78] fabricated magnetically separable magnetite containing mesoporous spheres (Fe_3O_4–MSS) by reducing the Fe^{3+} adsorbed on to the MPs. This material was used for the immobilization of laccase via both physical adsorption and covalent attachment methods. Results showed that the covalently immobilized enzyme retained 70% activity after ten consecutive reuses whereas leaching of the physically adsorbed enzyme was observed. An important advantage of this approach is that the resulting biomagnetic assembly can be easily controlled and separated using an external magnet. For example, it can be attached to the surface of an electrochemical transducer by using a permanent magnet, resulting in a magneto-switchable platform for electrochemical sensors. Integration into automatic systems is also possible. For instance, it can be injected into a flow-injection analysis (FIA) system and retained near the detector by using permanent magnets.

Vamvakaki and Chaniotakis developed highly stable HRP and GOX-based electrochemical biosensors by immobilizing the enzymes in porous silica beads with pore sizes of 10 nm [98, 99]. As expected, results showed that matching the size of the pores with the diameter of the enzymes generated biosensors with extended operational stability (five days under continuous operation). Dai et al. co-immobilized two enzymes, tyrosinase and HRP, in MCM-41, and used this approach to fabricate a biosensor for trace phenol detection [100]. Results showed that co-immobilization of the two enzymes greatly improved the sensitivity of the sensor (14 μA $μmol^{-1}$ L cm^{-2}) compared with immobilization of tyrosinase alone (1.7 μA $μmol^{-1}$ L cm^{-2}). The sensor was characterized by a fast response time of less than 10 s, long-term stability (80 days), good reproducibility, a wide linear range from 2×10^{-7} to 2.3×10^{-4} mol L^{-1} and a detection limit of 4.1×10^{-9} mol L^{-1}. Recently, Lei et al. developed a stable fast responding biosensor for toxicity monitoring in aquatic environmental samples via immobilization of organophosphorus hydrolase in amino-functionalized MPs (diameter in the 10 nm range) using glutaraldehyde linking [101]. The biosensor responded rapidly to low concentrations of paraoxon (4.0×10^{-7} detection limit) in less than 10 sec. Using this system it was possible to monitor paraoxon concentrations under simulated rugged environmental conditions at different pH values, total organic carbon, and suspended solids and could be used for field testing and ecological applications.

Conclusions and future perspectives

Starting with their discovery in the early 1990s, mesoporous silica materials have been explored as immobilization matrix for a variety of enzymes and other biologically active agents. This review summarized the most important MPs used for this purpose, their functionalization with enzymes, and applications in biosensor technology. Research demonstrated that enzymes encapsulated or entrapped in ordered MPs retain their biocatalytic activity and are more stable than the enzymes in solution. Most of the work was oriented toward synthesis of different types of MPs with various morphologies and controllable pore size. These MPs were mainly investigated for the immobilization of small proteins. Recent progress in controlling the pore size and morphology of MPs will facilitate immobilization of a broader spectrum of enzymes, and expand their applications in various areas, for example bioanalysis and biotechnology, bioprocessing, food and environmental control, and biosensing. Future work is expected to study systems and devices based on MPs and address their suitability for practical implementation in these areas. The MPs could be the key to obtaining robust, miniaturized, and portable devices for field applications.

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