Bioanalysis based on nanoporous materials

Zhihui Dai, Huangxian Ju

Nanoporous materials possess nanometer-sized pore distribution and are widely used in biosensing. The unique properties of nanoporous materials include large surface area, good chemical, thermal and mechanical stability, very uniform pore distribution with tunable pore size, high adsorption capacity, and an ordered porous network for free diffusion of substrates and reaction products.

Usage of nanoporous materials can significantly improve the analytical performance of biosensors in biomedical diagnosis and monitoring of food and environmental quality.

This article reviews some major advances in bioanalysis based on nanoporous materials, including biosensing based on zeolite, mesoporous silica, mesoporous carbon, mesoporous metal and metal oxide. These nanoporous materials have shown promising applications in electrochemical biosensing, electrocatalysis, proteomics analysis and biorecognition.

Keywords: Bioanalysis; Biorecognition; Biosensing; Electrocatalysis; Electrochemical biosensor; Mesoporous material; Nanoporous material; Pore; Proteomics analysis; Zeolite

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1. Introduction

According to the definition by International Union of Pure and Applied Chemistry (IUPAC), porous materials are divided into three classes: microporous (<2 nm), mesoporous (2–50 nm) and macroporous (>50 nm). When the pore dimension is in the nanometer (nm) range, such materials can be denoted ''nanoporous'' [1]. Recently, nanoporous materials have increased applications in many areas including bioengineering [2], catalysis [3], and biosensing [4], due to their large surface area, tailored pore-size distribution, controllable pore structure, and versatile composition. Bioanalysis especially has potential applications in biomedical diagnosis and monitoring of food and environmental quality. The composition of these nanoporous materials includes silica, carbon, metal, and metal oxides [5,6].

The fabrication of mesoporous materials is mainly concerned with building monodispersed, meso-sized pore spaces and arranging them in a long-range ordered array [7], generally using two kinds of template:

(1) supramolecular aggregates (e.g., surfactant micelle arrays); and,
(2) mesoporous solids (e.g., silicates and carbons) [8].

The corresponding synthesis methods are commonly described as soft templating and hard templating (nanocasting) (Fig. 1), respectively. Two classes of ordered mesostructures are thus obtained and integrated as the components of mesoporous materials, which are continuous framework structures with cylindrical or spherical mesopores and their counter-replica structures, which can also be regarded as nanowire and nanosphere arrays [8].

Nanoporous materials possess several advantages for bioanalytical applications:

(1) the large surface area and the uniform pores can provide more catalytic sites *via* its catalytic mesostructure or the loading of a large amount of catalyst, hence making possible high-sensitivity detection [9];
(2) large amounts of hydroxyl left on the nanoporous metal oxides after removing the template make possible the introduction of functional groups into the porous walls, which can endow modified nanoporous materials with many new functions [10];

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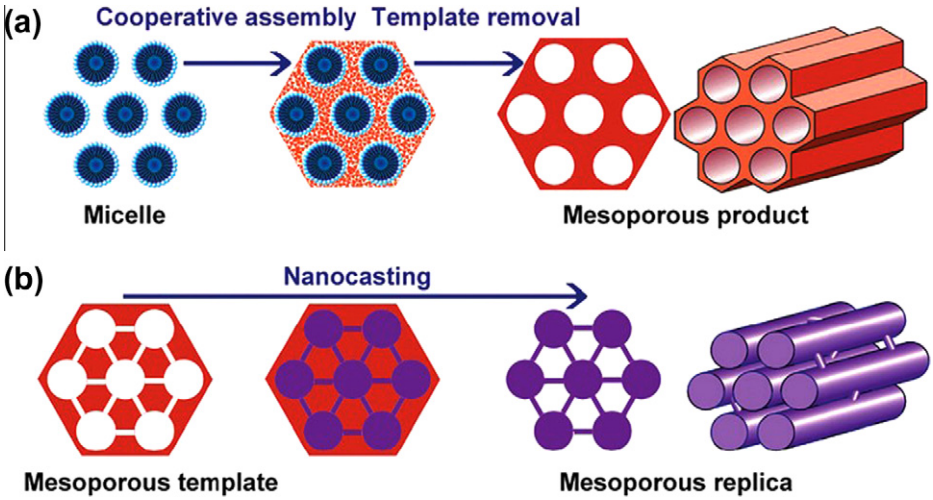

Figure 1. Two representative synthesis routes for ordered mesoporous materials: (a) soft-templating method; and, (b) hard-templating (nanocasting) method. (Reprinted from [8], with permission from Royal Society of Chemistry Publications).

(3) the great porosity and uniform structure facilitate the fast transport of the target analytes to active sites in the nanopores [6];
(4) the inorganic matrices of most nanoporous materials are stable due to their highly cross-linked structure, so they can resist biodegradation under extreme conditions; and,
(5) structure, pore size, hydrophilic/hydrophobic character, water insolubility, charge distribution, pH environment, conductivity, and catalytic ability can be tailored to satisfy practical applications by using different kinds of templates or changing the composition of the materials.

In this review, we highlight recent advances in the bioanalytical applications of mesoporous materials, including zeolite, mesoporous silica, mesoporous carbon, and mesoporous metal and metal oxide.

2. Bioanalysis based on nanoporous zeolites

Zeolites are characterized by a regular structure or a three-dimensional arrangement at the microporous or mesoporous level. Zeolites are naturally-occurring old materials, but they can also be synthesized in the laboratory in various compositions and structures [11]. They are inorganic solids with large surface areas and well-defined internal structures of uniform cages, cavities or channels of monodisperse dimensions due to the crystallization of aluminosilicates in the presence of cation-directing agents. Fig. 2 shows framework representations of zeolites [12]. Their main properties are ion exchange and size selectivity at the molecular level, which are the origin of most of their applications [13].

These ordered mesoporous materials were first described in 1992 when researchers of the Mobil Oil Corporation had the idea to use surfactant micelles instead of small cation-directing agents to induce silica polymerization around these larger templates (Fig. 3) [14]. This gave rise to a novel class of solids made of well-defined, uniform channels regularly arranged in the space in various configurations (hexagonal, cubic, lamellar, wormlike [15]), combining both sieving properties (with much larger pores than zeolites) and a surface chemistry very similar to that of non-ordered silica gels. Due to their attractive, unique characteristics, these two families of nanostructured solids have attracted strong interest from the bioanalytical community [16].

Charge-transfer-mediator guests can be immobilized in zeolite hosts. When they are confined at an electrode surface, the mediator-immobilized zeolites can be exploited for electrocatalytic purposes.

Selectivity in analysis is highly desirable. Combining zeolites as size/charge filters or analytes/reactant preconcentrators with biorecognition elements for preparation of biosensors offers exciting analytical prospects. There are two basic processes of preparation:
(1) the first relies on the exchange of a small mediator cation; and,
(2) the second implies encapsulation of bigger species in the zeolite cages.

The first method usually gives rise to high current response but poor long-term stability due to electrocatalyst leaching, whereas the second ensures durable immobilization of active species but suffers from rather low electrochemical response due to the insulating character of zeolites [17].

The bioanalytical applications based on zeolites may be classified into three categories (i.e. indirect amperometric

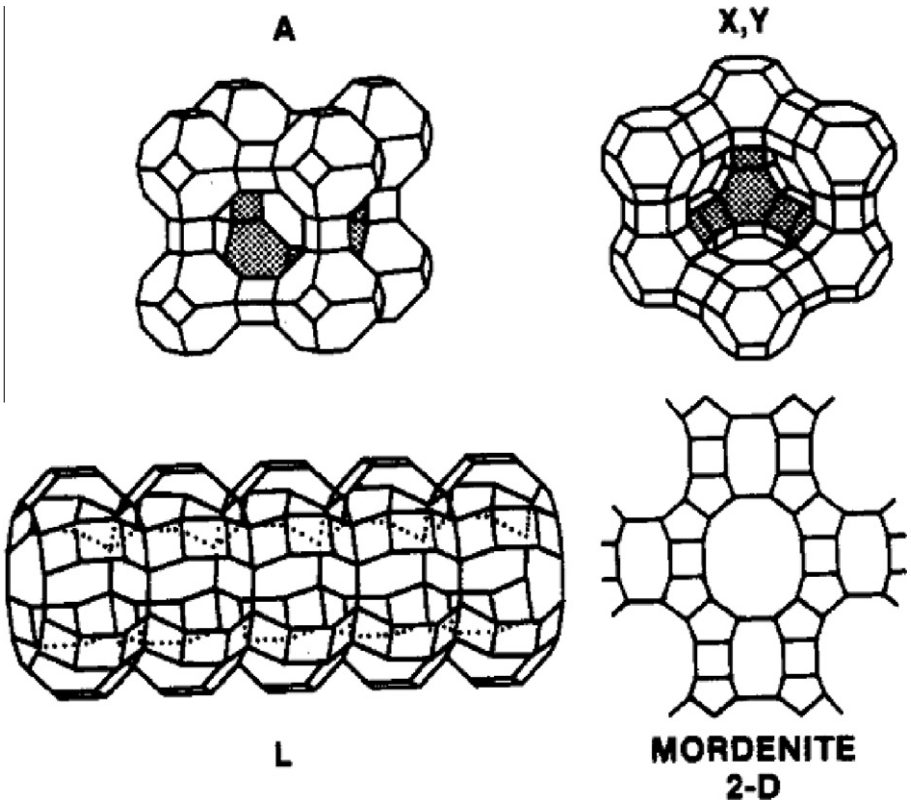

Figure 2. Three-dimensional representations of the framework structure of zeolite types A, X, Y (faujasite) and L, and a two-dimensional representation of the framework structure of mordenite. (Reprinted from [10], with permission from ACS Publications).

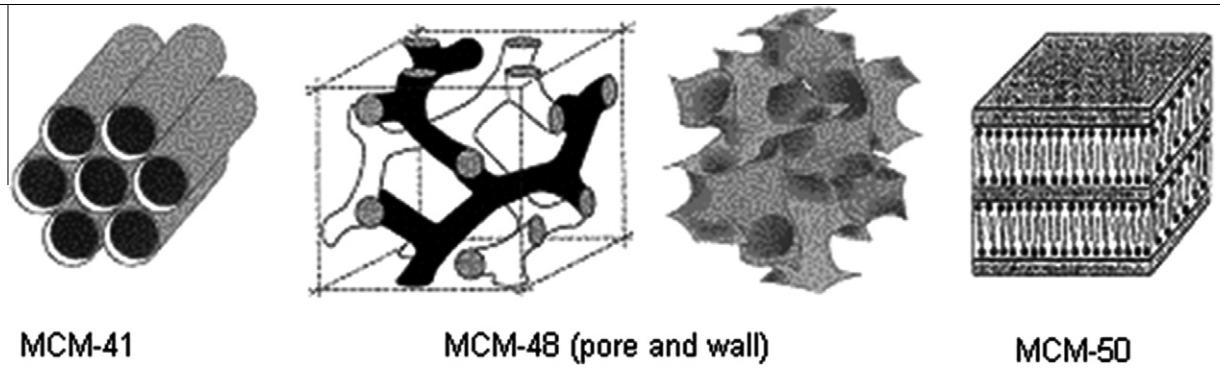

Figure 3. Structural models of 2D hexagonal p6 mm (MCM-41), 3D cubic Ia3d (MCM-48) and layer mesophase (MCM-50).

detection, amperometric biosensor, and preconcentration and protein analysis).

2.1. Indirect amperometric detection

Due to the ion-exchange properties and size selectivity at the molecular level, zeolite particles doped with ion-exchangeable redox probes exhibit voltammetric behavior dramatically affected by the size of the electrolyte cation. Indeed, using a size-excluded cation (i.e. a cation bigger than the zeolite-pore aperture) does not enable the redox probe to be exchanged to diffuse to the electrode surface so it does not result in measurement of significant electroactivity. This observation is at the origin of the indirect amperometric detection at zeolite-modified electrodes [18]. Cu^{2+}-doped zeolite-modified electrodes polarized at a potential value likely to reduce Cu^{2+} ions do not give rise to any current response in a supporting electrolyte made of a large cation, because the large cation is not allowed to enter the zeolite-pore aperture. However, when a sample solution containing a small cation is injected onto the electrode surface, a nice amperometric response can be observed.

Detailed study on the factors affecting such indirect amperometric detection of non-electroactive cations reports that the sensitivity of this method is mainly governed by diffusion of both the electron-transfer co-factor and the cationic analyte [19]. A reliable, durable approach for *in situ* monitoring of superoxide anion released from living cells has been proposed by the direct electron transfer of zeolite-stabilized biomimetic superoxide dismutase, $Mn_3(PO_4)_2$, in which Mn^{2+} is ion-exchanged into zeolite (ZSM-5) microstructures to obtain the detectable signal and high selectivity [20].

2.2. Amperometric biosensors

The first example of a zeolite-modified enzyme electrode was published in 1995. Kotte et al. prepared a screen-printed thick-film sensor made of a polyurethane hydrogel-immobilizing tyrosinase, coated on a mediator modified carbon electrode containing zeolite particles [21]. The role of the zeolite was clearly defined as a host for the positively charged mediator which was fixed on the Y-type (faujasite) zeolite by ion exchange. This electrode showed good sensitivity for the detection of eight phenolic compounds (35 tested), including phenol, m-cresol, p-cresol, 2,4-xylenol, p-ethylphenol, p-chlorophenol, cathechol, and p-methoxyphenol, but long-term stability was not achieved.

Dai et al. [5] reported an H_2O_2 biosensor based on immobilization of cytochrome c on NaY zeolite without the aid of an electron mediator. NaY (Y-type zeolite sodium salt) provided a good matrix for protein immobilization and biosensor preparation. The controllable nanozeolite film provided a biocompatible surface for study of the interaction between enzymes and target molecules. The zeolite matrix had a large surface area and unique surface property to adsorb enzymes with high performance. Zhou et al. [22] reported a sensitive tyrosinase biosensor, which was assembled by β-zeolite (sodalite) and polydiallyldimethylammonium. The amperometric response of the enzyme electrode was acquired using the trace phenol as a target molecule.

An iron ion-doped zeolite-multi-walled carbon nanotube (CNT)-modified glassy-carbon electrode (GCE) was reported for simultaneous and sensitive determination of ascorbic acid (AA), dopamine (DA), uric acid (UA) and tryptophan [23]. This sensor could be used for analysis of human serum and urine samples.

Rohani et al. [24] developed a method for the catalytic oxidation of AA at the copper(II) zeolite-modified electrode. Cu(II), loaded in zeolite, could oxidize AA catalytically. The modified electrode offered the advantages of easy fabrication, fast response time, high sensitivity, low background current and low limit of detection (LOD).

2.3. Preconcentration and protein analysis

This application involves the ion-exchange properties of zeolites, which can be advantageously coupled due to their size and charge selectivity, and the subsequent analytical detection of the accumulated species. It has been applied to the determination of proteins and peptides.

Cao et al. [25] developed an approach to enrichment of proteins in human hepatocellular carcinoma cells using zeolite. This approach showed promising potential in early detection and diagnosis of disease.

Zhang et al. [26] used zeolite to enrich the low-abundance peptides based on their strong adsorption ability and high dispersibility. The peptides absorbed on the zeolites were analyzed for protein identification. Taking advantage of zeolites being inorganic materials that possess a neutral or an anionic framework with water and cations in the void system, Bergé-Lefranc et al. [27] reported the preconcentration of creatinine from a physiological medium by adsorption onto zeolites and used diffuse reflectance UV spectroscopy for the first time to quantify the creatinine concentrations.

3. Bioanalysis based on mesoporous materials

3.1. Bioanalysis based on mesoporous silica

The unique properties of mesoporous silica materials (MPSi) have attracted substantial interest for use as enzyme-immobilization matrices. MPSi are a class of inorganic materials with uniform, well-ordered porous structure, large surface area, chemical, thermal, and mechanical stability, very uniform pore distribution and tunable pore size, high adsorption capacity, and an ordered porous network for free diffusion of substrates and reaction products. Starting with their discovery in the early 1990s [14], MPSi have been explored as an immobilization matrix for a variety of enzymes and other biologically active agents [28]. 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. The enzymes encapsulated or entrapped in MPSi can retain their biocatalytic activity and are more stable than enzymes in solution. These materials have been shown to provide enhanced binding efficiency and increased catalytic activity and stability. They enabled operation in harsh conditions with excellent recycling characteristics [2] compared with the free enzyme. The immobilization also enabled recovery or prevented loss of ''expensive'' enzymes.

Enzymes entrapped within MPSi pores can be deposited onto a variety of transducer surfaces and act as biomolecular recognition elements to catalyze specific reactions. The main advantages of these materials in bioanalysis are the likelihood of providing enhanced stability of the encapsulated biomaterial over long periods of time. Most of the work in this field concerns immobilization of relatively small proteins.

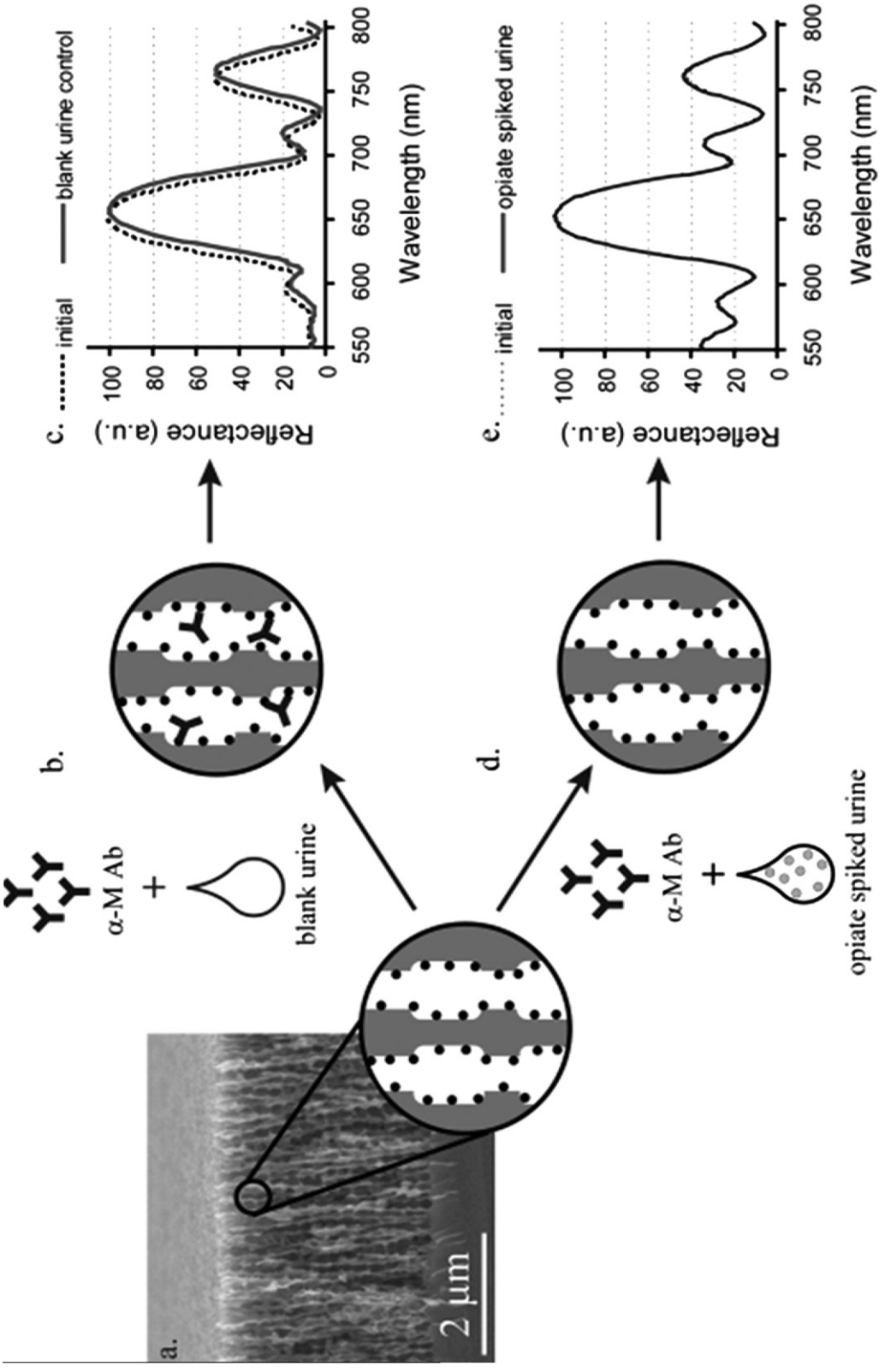

Figure 4. Principle of the PSi sensor technique (Reprinted from [33] with permission from ACS Publications).

Dai et al. [7,29–31] developed very stable electrochemical biosensors based on hemoglobin (Hb), horseradish peroxidase (HRP), myoglobin (Mb) and glucose oxidase (GOx) by immobilizing the enzymes in mesoporous silica. The direct electron transfer of these redox proteins inside the MPSi matrix was successful. This group also co-immobilized two enzymes, tyrosinase and HRP, in mesoporous composite material no. 41 (MCM-41), and used this approach to fabricate a biosensor for trace-phenol detection [32]. The co-immobilization of the two enzymes greatly improved the sensitivity of the sensor (14 μA/μmol/L/cm^2) compared with immobilization of tyrosinase alone (1.7 μA/μmol/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 and a low LOD.

Recently, some works have focused on the application of MPSi in immunosensor construction and DNA analysis. Lin et al. [33] reported a label-free immunosensor for simultaneous determination of tumor markers. They first prepared functional 2-D hexagonal P6 m (SBA-15) with amino-modified internal pore walls, and then prepared label-free probe of carcino-embryonic antigen (CEA) with ferrocenecarboxylic acid (FCA) and monoclonal antibody of CEA encased in the mesopores and label-free probe of α-fetoprotein (AFP) with carcino-embryonic antigen monoclonal antibody of AFP and HRP entrapped in the mesopores, using o-phenylenediamine (OPD) and H_2O_2 as the electro-chemical substrates. By observing the electrical responses of FCA and OPD, the concentration of relevant antigen was detected.

MPSi are very promising materials that satisfy the consensus criteria of an ideal diagnostic device, including inexpensive fabrication, label-free optical analysis and potential for multi-component analysis with an array of probe molecules [34,35]. DeLouise et al. [36] evaluated the performance of a label-free MPSi immunosensor assay in a clinical study. The MPSi-sensor substrate was modified with an opiate-analogue connected with lysine groups present on a bovine serum albumin (BSA)-blocked surface; after drying, 15-μL urine specimens were added to the sensor directly followed by a fixed aliquot of antibody and incubated in a humidity chamber (Fig. 4). This study validated the analytical screening capability of label-free MPSi immunosensors in authentic patient samples and supported its potential for future development in various clinical assays.

Also, MPSi have been widely used in DNA sensing. When the probe DNA immobilized in the pores binds its complementary DNA, this hybridization increased the effective index of the MPSi. The low LOD of 42 nM was reported [37].

MPSi have also been used for the enrichment of protein substrates and peptide products to improve protein digestion and detection sensitivity, respectively. The nm-scale pores of nanoporous materials have been used as enzyme reactors to improve the detection sensitivity and to expedite the speed of protein digestion by two different approaches. The first approach is initially to adsorb trypsin into cyano-functionalized MPSi, and then to incubate the trypsin-adsorbed MPSi in the protein solution [38]. The second approach is initially to adsorb proteins into MPSi, and then incubate the MPSi enriched with proteins in the trypsin solution [39].

3.2. Bioanalysis based on mesoporous carbon

Although the MPSi possess interesting adsorption properties, they have some limitations due to the amorphous character of the pore walls and the neutral silica framework. Although MPSi are anionic, they do not provide acid sites due to the chemical nature of the surface hydroxyl groups. To provide the proper function on the surface, the surface silanol groups require *in situ* or post-synthetic modification with different organic functionalities. Moreover, these materials suffer from poor thermal and hydrothermal stability that limits their widespread use in many applications.

Recently, much progress has been made in the fabrication of mesoporous materials other than silica, namely, non-siliceous mesoporous materials (e.g., carbon, metals and transition-metal oxides). The fabrication of such materials has led to the production of more advanced nanoscale devices, sensors, catalysts and adsorbents. For example, ordered mesoporous carbon materials are promising in many applications, including adsorption of large molecules, chromatography, and manufacture of electrochemical double-layer capacitors.

Nanoporous metal catalysts (e.g., Pt, Pd, and Au) show excellent electrocatalytic activity toward oxygen reduction and the electrochemical oxidation or reduction of hydrogen peroxide due to the increased active surface area. Mesoporous titania nanoparticles (MTNs) have some attractive properties (e.g., fast mass transport, strong adhesion to substrates and good dispersion in solution). However, successful synthesis of MTNs is very difficult due to the rapid hydrolysis of titanium-containing precursors and the crystallization of titania upon thermal treatment.

Introduction of mesoporosity can dramatically enhance the surface properties of ZnO materials. Mesoporous ZnO material has exceptionally high specific surface area and uniform systems of nanopores, along with drastic enhancement in visible emission and photocurrent under visible light irradiation.

Carbon has been the most popular material used in the field of electrochemistry over the past few decades because of its practical advantages (e.g., low background current, wide potential window, relatively inert electrochemistry, low cost, and high conductivity and activity for a variety of redox reactions) [40]. With recent advancements in nanotechnology, carbon-based nanoscaled materials, including CNTs [41], carbon nanofibers

[42], graphene [43], and highly ordered mesoporous carbon (OMC) [44–46], have been utilized intensively because of their superior characteristics (e.g., extremely large surface area, controlled nanoscale structures, and considerably high conductivity).

Among these materials, OMC has gained significant attention due to its unique property of a large pore volume, which can be utilized to accommodate enzyme molecules with a much higher loading capacity than other forms [44–46].

3.2.1. Enzyme-based biosensors. OMC with good pore-size matching for enzymes has proved to be a promising electrochemical-biosensing platform. The fundamental aspects of an electrochemical biosensor involve enzyme immobilization on an electrode surface and efficient electrical communication between the enzyme and the electrode while retaining enzymatic stability and bioactivity [47]. OMC is a promising option for the construction of enzyme-based electrochemical biosensors, as it has the advantage of good electrical conductivity due to the good degree of graphitization. However, few reports have explored the electrochemical-biosensing applications of these fascinating materials [48].

In 2009, Chen's group [48] prepared two kinds of three-dimensional OMC designated OMC-6 (pore diameter = 6 nm) and OMC-13 (pore diameter = 13 nm) by a nickel-catalyzed template-assisted method, and explored them systematically for the immobilization of Hb and the construction of electrochemical biosensors.

OMC stimulates electron transfer between biomolecules, including enzymes and the conducting substrates. For example, OMC electrodes show excellent electrocatalytic behavior for L-cysteine oxidation [44]. Glucose biosensors with immobilized GOx in mesocellular carbon foam coated on a GCE showed excellent sensitivity and selectivity [45]. A very convenient electrochemical glucose biosensor employed a nanostructured multi-catalyst system comprising Fe_3O_4 magnetic nanoparticles (NPs) and GOx in conductive mesoporous carbon [46].

Recently, there was significant interest in the development of novel carbon materials that can be used in the electrochemical determination of some molecules. Ju et al. [4] developed mesocellular silica–carbon nanocomposite foam (MSCF) for immobilization and biosensing of proteins. The MSCF had a highly ordered mesostructure, good biocompatibility, favorable conductivity and hydrophilicity, a large surface area, and a narrow pore-size distribution. Using GOx as a model, the MSCF was tested for immobilization of redox proteins and the design of electrochemical biosensors. GOx molecules immobilized in the mesopores of the MSCF showed direct electrochemistry with a fast electron-transfer rate (14.0±1.7/s) and good electrochemical performance. The proposed biosensor exhibited a linear response to glucose concentrations with a low LOD at an applied potential of -0.4 V. The biosensor showed good stability and selectivity, and was able to exclude interference from AA and UA species that always coexist with glucose in real samples. The nanocomposite foam provided a good matrix for protein immobilization and biosensor preparation.

OMCs, composed of carbon nanorods with highly ordered arrays and large porosity, retain good electronic properties and chemical stability, large specific surface areas (up to $\sim$2000 m^2/g), and large pore volume (up to $\sim$1.5 cm^3/g) [49], making them more promising in heterogeneous electron transfer than CNTs. OMCs have therefore attracted growing interest in diverse fields from catalyst carriers and absorbents to electronic devices. All the advantages make it possible to design the selectivity of substrates for immobilizing biomolecules, by varying pore diameters, mesoscopic topologies and charges of OMCs.

The pores or channels of OMCs behave as individual nanocells so that electrochemical reactions are confined to occur inside the pores or near the doors. The electrochemical responses of NADH and H_2O_2 can be detected at OMC-modified electrodes, which exhibit more favorable electron-transfer kinetics than CNT-modified electrodes [50]. Based on the greatly enhanced electrochemical reactivity of NADH and H_2O_2, electrodes modified with Nafion/ADH–OMC and Nafion/GOx–OMC show better electroanalytical performance in detecting ethanol and glucose, respectively, compared with CNT-based enzyme electrodes.

OMCs showing favorable electrochemical activity can be another kind of robust, advanced carbon-electrode material besides CNTs for biosensing applications. An amperometric glucose biosensor based on an OMC-supported platinum NP (Pt/OMC)-modified electrode has been reported [51]. The Pt/OMC nanocomposite-modified electrode also exhibited excellent electrocatalytic activities towards reduction and oxidation of H_2O_2. This feature allows us to use it as a bioplatform on which GOx was immobilized by entrapment in electropolymerized pyrrole film for the construction of the glucose biosensor, which showed good analytical performances in terms of low LOD (0.05 mM), high sensitivity (0.38 μA/mM) and wide linear range (0.05–3.70 mM) [51].

Besides CNTs, OMC can also be employed as a transducer and a support for Meldola's Blue (MB) because OMC possesses many admirable properties (e.g., large surface area, thermal stability, chemical inertness and electrical conductivity) [52]. Jiang et al. [53] fabricated an amperometric ethanol biosensor by integrating alcohol dehydrogenase (ADH) with an MB/OMC-composite-modified GCE (MB/OMC/GCE). They first prepared an MB/OMC-composite-film-modified GCE (MB/OMC/GCE) and systematically investigated its electrochemical behavior in comparison with MB/CNT/GCE with respect to oxidation of NADH, and then presented an ethanol

biosensor by integrating the MB/OMC/GCE with ADH. The MB/OMC/GCE was highly sensitive for nicotinamide adenine dinucleotide (NADH) measurement (9.1±0.25 mA/mM) and gave a low LOD of 0.21±0.02 mM.

3.2.2. Biosensor for small biomolecules. OMCs possess both the good conductivity of carbon materials and the nanostructure, which make it rational to consider them as potential novel materials for investigating the electrochemical behavior of substances [54]. On the electrode, remarkably strong, stable electrocatalytic responses can be obtained toward some important biomolecules compared with a CNT-modified electrode, providing a good model for constructing novel and promising electrochemical sensing platform for detecting various biomolecules [55]. The electrochemical and electrocatalytic properties of the mesoporous carbon can be improved when the electroactive substance is immobilized on this new material. It can be used to modify an electrode that can investigate the electrochemistry of some biomolecules.

Ndamanisha et al. [56] reported a non-enzymatic amperometric glucose sensor based on OMC. The amperometric technique was used to evaluate the electrocatalytic activity of the OMC-modified electrode toward non-enzymatic glucose in alkaline media in presence and absence of chloride ions. The resulting biosensor exhibited excellent performance for glucose determination with high sensitivity and low LOD. They also reported the effects of ferrocene derivative on the properties of an OMC [57]. The OMC containing iron oxide (OMC-Fe) could be prepared with sucrose instead of furfuryl alcohol as a carbon precursor without decreasing the surface area. The electrochemical determination of H_2O_2 at the OMC-Fe modified electrode was more sensitive than that at an OMC electrode. A sensitive, stable H_2O_2 sensor was developed based on an OMC-Fe electrode with a large determination range and a good reproducibility.

A mesoporous carbon nanofiber-modified pyrolytic graphite electrode has been prepared and applied to the simultaneous electrochemical determination of DA, UA and AA [58]. The oxidation overpotentials of these biomolecules decreased significantly. The overlapping of these peaks in a bare pyrolytic graphite electrode was well resolved, and their anodic peak currents increased dramatically in mesoporous carbon nanofiber-modified electrode due to the large amount of edge-plane-like defects, large surface area and especially the mesoporous structure of mesoporous carbon nanofibers. The good sensitivity and reproducibility of the proposed electrode make OMC be used as a good electrochemical sensor for the simultaneous determination of DA, UA and AA in real sample analysis.

3.3. Bioanalysis based on mesoporous metal

3.3.1. Mesoporous platinum. After one-and-a-half decades of extensive research into MPSi, more attention has focused on the study of mesoporous metals in the past few years. Watson et al. [59] studied the application of mesoporous platinum electrodes in biosensing. The materials could be used as amperometric sensors for the detection of H_2O_2 in aqueous solutions over a wide range of concentrations. The H_2O_2 sensors showed efficient mass-transport properties with outstanding qualitative and quantitative results, good reproducibility, high precision, and accuracy of measurement [60].

Mesoporous platinum can also be used as the electrode for a non-enzymatic glucose sensor [61], because the large surface area of the mesoporous platinum electrode can selectively amplify the current from the direct oxidation of glucose without an enzyme-like GOx. Please

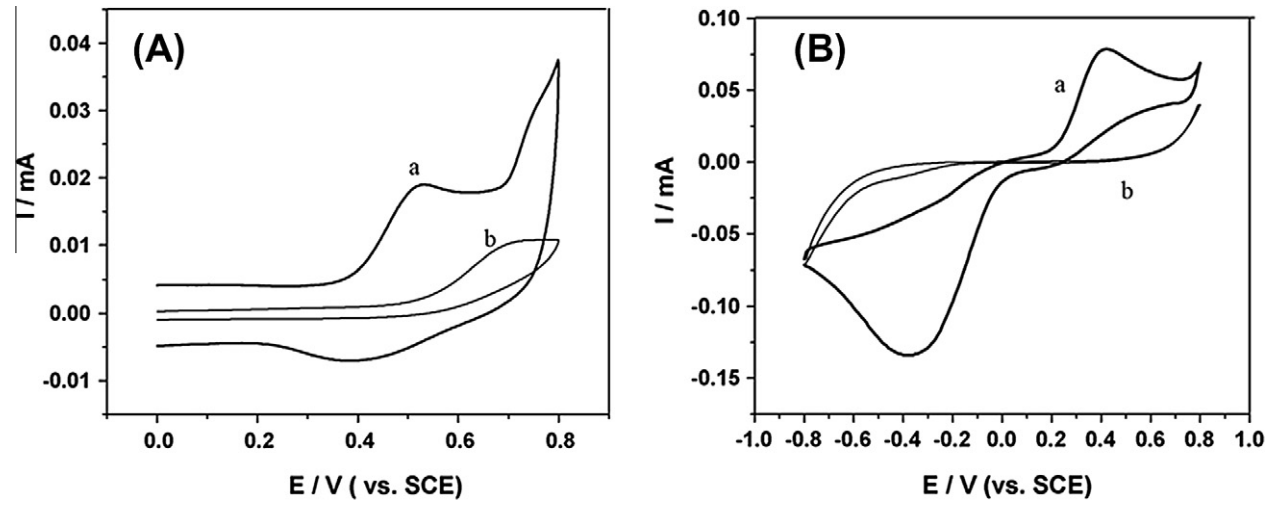

Figure 5. (a) CVs of NADH (1 mM, A) and H_2O_2 (4 mM, B) at NPG/GCE, and (b) gold-sheet electrode in 0.1MPBS (pH 7.2). Scan rate: 50 mV/s. (Reprinted from [58] with permission from ACS Publications).

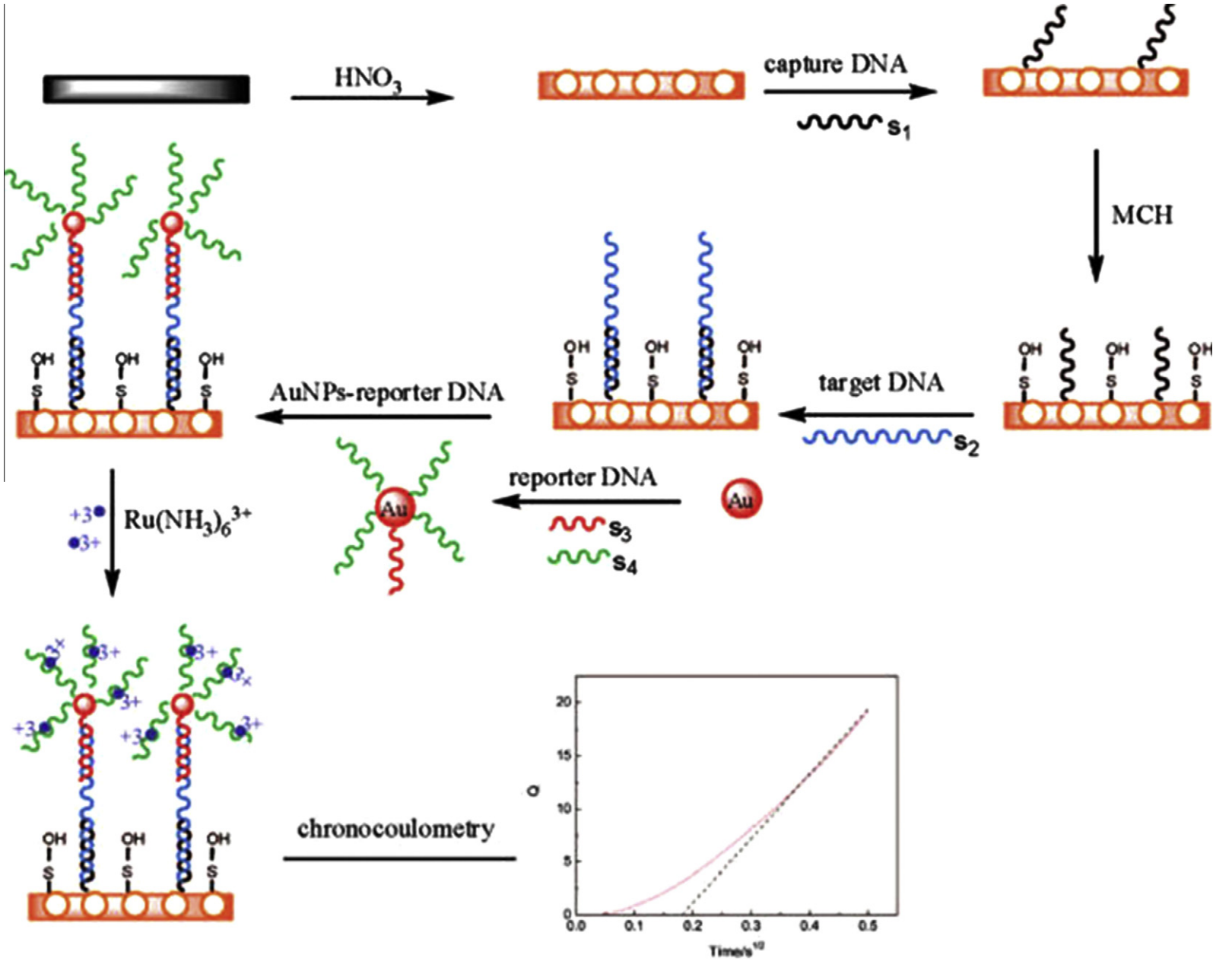

Figure 6. Chronocoulometry determination of DNA hybridization through two steps of amplification. (Reprinted from [61] with permission from ACS Publications).

note that sensitivity as high as 9.6 μA/cm^2/mM was reproducibly obtained in the presence of high concentrations of chloride ions. It was regarded as one of the electrochemical sensors based on noble metals for the non-enzymatic glucose detection, but they almost entirely lose their activity in the presence of chloride ions due to poisoning. Significant enhancement in the catalytic activity of mesoporous platinum in the oxidation of methanol has also been demonstrated [62].

3.3.2. Nanoporous gold. Nanoporous gold (NPG) is a good conductor. It has suitable pore-size distribution and large surface area, and can greatly enhance the electrochemical response toward enzymatic substrates NADH and H_2O_2 based on their low coordinated Au atoms. All these advantages make it attractive for the construction of dehydrogenase- and oxidase-based biosensors [63], which may have improved sensitivity and anti-interference ability.

NPG can be prepared simply by dealloying Ag from Au/Ag alloy. The nanoporous structure makes NPG more active than gold sheet because both oxidation and reduction peaks of the NPG/GCE are negatively shifted as compared with those of the gold-sheet electrode. In addition, the peak currents of NADH and H_2O_2 at the NPG/GCE are much larger than those at the gold-sheet electrode, as shown in Fig. 5. The high density of edge-plane-like defect sites and the large specific surface area of NPG are responsible for the electrocatalytic behavior of the NPG/GCE, which permits effective low-potential amperometric biosensing of ethanol or glucose via the incorporation of ADH or GOx within the three-dimensional matrix of NPG. The ADH-modified and GOx-modified NPG-based biosensors show good analytical performance for biosensing ethanol and glucose, due to the clean, reproducible and uniformly distributed microstructure of NPG. The stabilization effect of NPG on the incorporated enzymes also makes the constructed biosensors very stable. After 1 month storage at 4°C, the ADH-based and GOD-based biosensors lose only 5.0% and 4.2%, respectively, of the original current response. All these results make NPG another ''popular'' material for constructing electrochemical biosensors.

In recent years, the development of highly sensitive, selective DNA sensors to bring down the LOD to pico-molar and femto-molar levels is of ever increasing interest, since genoassays are suitable for various applications, including clinical diagnosis, environmental control and forensic analysis [64]. For the preparation of DNA biosensors, the immobilization of biomolecule probes on a desired substrate is a very important process, since the sensitivity, LOD and reproducibility are all significantly affected by the steps.

Gold substrates have attracted special attention as an electrode material for the construction of DNA electrochemical biosensors due to their strong interaction with thiolated-DNA *via* Au–thiol binding. Thiolated-DNA can be monolayered on gold by self-assembly, which provides stable, structurally well-defined electrochemical interfaces [65].

Recently, Zhang et al. [66] developed a sensitive electrochemical DNA sensor based on an NPG electrode prepared by dealloying Ag from Au/Ag alloy and multifunctional encoded AuNP. The active surface area of the NPG electrode was 9.2 times larger than that of a bare flat as characterized by CVs. A DNA biosensor was fabricated by immobilizing capture-probe DNA on the NPG electrode and hybridization with target DNA, which further hybridized with the reporter DNA loaded on the AuNP. The AuNP contained two kinds of bio bar-code DNA, one complementary to the target DNA, while the other was not, reducing the cross reaction between the targets and reporter DNA on the same AuNP. Electrochemical signals of $[Ru(NH_3)_6]^{3+}$ bound to the reporter DNA *via* electrostatic interactions were measured by chronocoulometry. The scheme is shown in Fig. 6. Taking advantage of dual-amplification effects of the NPG electrode and multifunctional encoded AuNP, this DNA biosensor could detect the DNA target quantitatively, in the range 8.0×10^{-17}–1.6×10^{-12} M, with an LOD as low as 28 aM, and it exhibited excellent selectivity even for single-mismatched DNA detection.

Besides DNA detection, NPG can also be used in constructing an amperometric immunosensor. A label-free amperometric immunosensor for fast, sensitive assay of human serum chorionic gonadotropin (hCG) using a GS/NPG/anti-hCG/BSA film-modified electrode has been reported [67]. The synergy between NPG foil and GSs yields a highly conductive composite that can easily be modified with biomolecules to serve as a platform for clinical diagnosis. The proposed immunosensor can determine hCG in the range 0.5–40 ng/mL with an LOD of 0.034 ng/mL, exhibiting excellent selectivity.

3.4. Bioanalysis based on mesoporous metal oxide

Metal-oxide materials are of tremendous importance in all areas of technological development and are required for the construction of modern electronic devices due to their electrical and band-gap properties. Table 1 lists the band gaps of several semiconductors. The creation of nanoporosity in semiconducting materials opens up a wide range of applications, including biosensors, catalysis and nanodevices.

Table 1. Band gaps of several semiconductors

Semiconductor	Band gap/eV	Semiconductor	Band gap/eV
Silicon	1.11	SiO_2	8.90
TiO_2	3.20	ZrO_2	5.00
ZnO	3.20	Nb_2O_5	3.40

3.4.1. TiO_2. Among the non-siliceous inorganic materials, TiO_2 has particular importance in many applications (e.g., solar cells, lithium-ion batteries, and electrochemical devices) [68]. Because of its great biocompatibility, it has also been widely used as an additive in paint, toothpaste and cosmetics. Recently, it has been proved that modification of TiO_2 on the electrode can enhance the enzyme catalytic performance for promising biosensor applications [69]. The electrochemical behavior of TiO_2 depends on not only the crystal structure and surface properties, but also the textural properties, which include specific surface area, pore volume, and pore dimension and distribution. Redox proteins or enzymes can be immobilized into various kinds of TiO_2 films without denaturation due to their good biocompatibility, and the direct electrochemistry of the proteins in TiO_2 films can be realized [70].

Li et al. developed a simple, effective method to fabricate a loose porous nanostructured TiO_2 with a large specific surface area and uniform nanopore distribution by using a multiwall nanotube (MWNT)-assisted hydrothermal method [71]. The TiO_2 is an attractive matrix to immobilize proteins and exhibits facile, direct electrochemistry of GOx without any electron mediator. The good electron transfer of GOx at the porous TiO_2-modified electrode can be attributed to the unique nanostructure and uniform pore distribution of the material. The glucose sensor made by the material shows great sensitivity, good specificity, fast response time, and sound reliability. This material has great potential applications in biosensing and green energy systems.

Astuti et al. [72] immobilized peroxidases on mesoporous TiO_2 and SnO_2 electrodes for the development of reagentless electrochemical biosensors for the detection of H_2O_2.

Jia et al. [73] fabricated a novel highly ordered mesoporous titanium oxide (mesoTiO_2) material by the ''acid-base pairs'' route used for the effective immobilization of hemoglobin (Hb) and its bioelectrochemical properties. The meso TiO_2 matrix improved the loading of protein with the retention of bioactivity and greatly promoted direct electron transfer because of its large specific surface area, uniform three-dimensional well-ordered porous structure, suitable pore size and biocompatibility.

Lu et al. [74] prepared a film based on vapor-surface sol-gel deposition of titania and alternate adsorption of oppositely-charged iron-heme proteins. The electrochemical and electrocatalytic activity of the films could be controlled by tailoring the amount of water with which the metal-alkoxide-precursor vapor reacted and the number of bilayers deposited in the assembly.

3.4.2. ZnO. The II–VI semiconductor zinc oxide (ZnO) is a typical semiconductor material with a wide band gap and a large exciton-binding ability (60 meV) [75]. In recent years, ZnO nanostructures attracted much attention in the application of efficient amperometric sensors with many extraordinary properties (e.g., nontoxicity, biological compatibility, chemical and photochemical stability, high electrochemical activities and easy preparation) [76].

In the area of bioscience, the special properties of nano-ZnO have also gradually attracted much attention [77]. Its biocompatibility and fast electron transfer between the active sites of enzyme and the electrode have made the material favored for functioning as the biomimetic membrane to immobilize and to modify proteins. Nano-ZnO also deserves further investigation as an important promising candidate for the supporting material in the fabrication of biosensors [78]. ZnO prepared by Fang et al. is a low-density, loose, porous material with good dispersion and uniformity, and a larger accessible surface area, which is favorable to catalytic application.

Dai et al. [79,80] prepared a tetragonal pyramid-shaped porous ZnO (TPSP-ZnO) nanostructure, which was used for immobilization, direct electrochemistry and biosensing of proteins. The prepared ZnO had a large surface area, good biocompatibility, and sensitivity. Such a nanostructure provided a good matrix for protein immobilization and biosensor preparation. In particular, comparative studies revealed that the nanosheet-based porous ZnO microspheres offered significant advantages with a smooth surface to facilitate direct electron transfer. It is noted that ZnO is unstable in acidic solution, so its application should be in the medium solution.

3.4.3. Nb_2O_5. Highly ordered mesoporous Nb_2O_5 films readily prepared with tailored pore size have also been employed to immobilize biomolecules. An ordered mesoporous Nb_2O_5 film has been considered for biomolecule immobilization, direct electrochemical studies of redox proteins and biosensor applications.

Nanoporous Nb_2O_5 exhibits good photocatalytic and electronic properties, so we expect it to be applicable in electronic and magnetic devices, and biotechnology [81]. It exhibits highly ordered pore structure and very narrow pore-size distribution [79] that make it possible to design the selectivity of these substrates for biomolecule immobilization, such as structure and charge, by varying the pore diameter and by functionalizing the substrate.

The highly ordered mesoporous Nb_2O_5 fabricated by self-adjusted synthesis has been used as immobilization matrices of heme proteins, including cytochrome *c* and HRP, for their large surface areas, narrow pore-size distributions and good biocompatibility [82]. The proteins can be readily assembled onto the mesoporous films with detectable retention of bioactivity. The Nb_2O_5 matrix with a tailored pore size and counterpoised surface charge to that of hemes allows maximum adsorption

capacity of biomolecules. The adsorbed redox molecules exhibit direct electrochemical behavior and give a pair of well-defined quasi-reversible cyclic voltammetric peaks, indicating that the mesoporous Nb_2O_5 matrix can effectively promote direct electron transfer between the protein redox site adsorbed and the electrode surface.

Highly reliable and sensitive DNA biosensors using a thin gold film sputtered on capacitive anodic nanoporous Nb_2O_5 have been reported by Rho et al. [83]. The nanoporous Nb_2O_5 offered a good adhesion and enhanced redox signals by accumulating charges between the gold film and the Nb_2O_5. This method gave a very reliable electrochemical redox signal with sensitivity three times greater than that of a bulk-gold electrode. The mechanism of enhancing the signal by the thin gold film on nanoporous Nb_2O_5 was in part attributed to capacitive Nb_2O_5 and in part ascribed to the bridged thin gold film.

Choi et al. prepared label-free detection of DNA-hybridization events by immobilizing oligonucleotidess on semi-conductive porous Nb_2O_5 [84]. They fabricated new DNA biosensors based on label-free detection techniques involving single-stranded or double-stranded DNA immobilized on semi-conductive porous Nb_2O_5.

3.4.4. Other mesoporous metal oxides. Apart from the mesoporous metal oxides mentioned above, some other mesoporous metal oxides (e.g., Al_2O_3 and ZrO_2) have been used for bioanalysis.

Al_2O_3 sol-gel has two functions. The hydrophilic, porous, and positively charged Al_2O_3 mesoporous matrix not only provides a friendly microenvironment for the enzyme to retain its functional activity to a large extent but also acts as an effective promoter for the electron transfer between *o*-quinone and the electrode [85].

A tyrosinase biosensor has been developed for the sub-nanomolar detection of phenols, based on the immobilization of tyrosinase in a positively-charged Al_2O_3 sol-gel membrane on a GCE [86]. It has been found that Al_2O_3 sol-gel is perfectly beneficial to the immobilization of tyrosinase, because it not only possesses the general advantages of mesoporous materials but also is an effective promoter of the biosensor. The large microscopic surface area, porous morphology, and hydrophilic property of the sol-gel matrix result in high enzyme loading, and the enzyme entrapped in this matrix retains its activity to a large extent.

ZrO_2 has general affinity for the binding of proteins because the amine and carboxyl groups on the surface of an enzyme can act as ligands to ZrO_2 [87]. Therefore, the usage of glutaraldehyde, which can denature the enzyme, can be avoided. In addition, the nanoporous structure of ZrO_2 greatly enhances the active surface available for enzyme binding over the geometrical area [88].

ZrO_2 is an attractive matrix for the immobilization of enzyme to fabricate biosensors. This can be due to the good biocompatibility and the negligible swelling of the ZrO_2 in aqueous. A large number of hydrogen bonds in the composite film is favorable to maintaining the configuration of enzyme molecule [89]. This composite material can also be conveniently extended to the

Table 2. Limits of detection (LODs) of small molecules based on enzymes immobilized on mesoporous materials

Mesoporous material	Immobilized enzyme	Analyte	Detection method	LODs	Ref.
Mesoporous silica	Tyrosinase-HRP	Phenol catechol p-cresol	Amperometry	4.1×10^{-9} M 7.8×10^{-10} M 3.9×10^{-9} M	[30]
Mesoporous silica	Hb	H_2O_2 NO_2^-	Amperometry	1.86×10^{-9} M 6.11×10^{-7} M	[7]
Mesoporous silica	HRP	H_2O_2	Amperometry	1.7×10^{-9} M	[32]
Mesoporous silica	Mb	H_2O_2 NO_2^-	Amperometry	6.2×10^{-8} M 8.0×10^{-7} M	[31]
Mesoporous silica	GOx	glucose	Amperometry	0.18 mM	[29]
Nay zeolite	Cytochrome c	H_2O_2	Amperometry	0.32 μM	[5]
β-Zeolite	Tyrosinase	phenol	Amperometry	0.5 nM	[22]
Graphitized ordered mesoporous carbon	Hb	H_2O_2	Amperometry	0.1 μM	[48]
Mesocellular silica–carbon nanocomposite foam	GOx	glucose	Amperometry	34 μM	[4]
Tio_2 nanotube	HRP	H_2O_2	Amperometry	1.2 μM	[69]
Mesoporous tio_2	HRP	H_2O_2	Amperometry	0.04 μM	[72]
Mesoporous sno_2	Cyt-*c* peroxidase	NO_2^-		1 μM	
Mesoporous tio_2	Hb	H_2O_2	Cyclic voltammetry	1 μM	[73]
TPSP-zno	HRP	H_2O_2	Amperometry	0.12 μM	[79]
TPSP-zno	GOx	Glucose	Cyclic voltammetry	0.01 mM	[80]
Mesoporous Nb2O5	Cyt-*c* HRP	H_2O_2	Amperometry	0.01 mM	[82]

immobilization of other biomolecules. GOx and Hb were chosen as the model enzymes. The mesoporous ZrO_2 is highly homogeneous. GOx and Hb can be effectively entrapped in the film with higher bioactivities.

In summary, the LODs of small molecules based on enzymes immobilized on the mesoporous materials are listed in Table 2.

4. Conclusions and future perspectives

Nanoporous materials have been explored as the immobilization matrix for a variety of enzymes and other biologically-active agents. This review summarizes their functionalization with enzymes, and applications in biosensing technology. Enzymes encapsulated or entrapped in ordered nanoporous materials can retain their biocatalytic activity and are more stable than enzymes in solution.

Further progress in controlling the pore size and morphology of nanoporous materials will facilitate the immobilization of larger proteins and expand their applications in various areas (e.g., bioanalysis and biotechnology, bioprocessing, food and environmental control, and biosensing). We expect future work to study systems and devices based on nanoporous materials and to address their suitability for practical implementation in these areas. Nanoporous materials can be the key to obtaining robust, miniaturized, and portable devices for field applications.

Although some instances have shown highly sensitive detection of biomolecules with femtomolar concentrations, the transducers described in this review are currently less sensitive than conventional ELISA. Furthermore, optical signal transduction is inherently affordable and requires only inexpensive device components (e.g., a white light source).

We expect that further developments in patterning and miniaturization will open a path to the development of small, highly parallel mesoporous materials-based biochips for the simultaneous detection of multiple analytes. Also, the biocompatibility of mesoporous materials might enable the design of implantable biosensors that are able to monitor biological systems in real time and *in vivo*.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under research projects (21175069, 21121091), the program for New Century Excellent Talents in University of the Chinese Ministry of Education (NCET-09-0159), the Foundation of the Jiangsu Education Committee (11KJA150003), the Research Fund for the Doctoral Program of Higher Education of China (20113207110005), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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