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Review

Recent advances in the fabrication and application of nanomaterial-based enzymatic microsystems in chemical and biological sciences

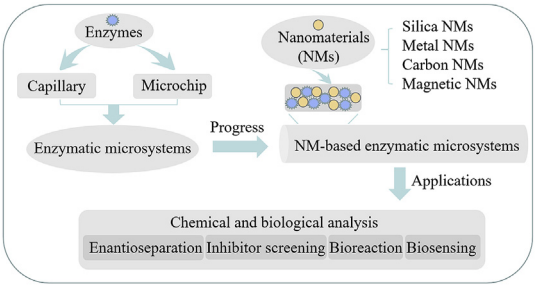
Tingting Hong [a], Wenfang Liu [a], Ming Li [b], Chuanpin Chen [a, *]

[a] Xiangya School of Pharmaceutical Sciences, Central South University, Changsha, Hunan, 410013, China
[b] School of Environmental Science and Engineering, Yangzhou University, Yangzhou, Jiangsu, 225127, China

HIGHLIGHTS

- Discusses nanomaterials used for preparing monolithic capillary-based enzymatic microsystems.
- Reviews nanomaterials used for developing open tubular capillary-based enzymatic microsystems.
- Highlights the applications in enantioseparation, inhibitor screening, and bioreaction.
- Concludes distinct nanomaterials utilized for fabricating microchip-based enzymatic systems.
- Summarizes developments of microchip-based enzymatic systems for bioreaction and biosensing.

GRAPHICAL ABSTRACT



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ABSTRACT

Novel enzymatic microsystems have strong prospects in chemical and biological analysis due to their low consumption of reagents, fast analysis time, easy manipulation and satisfactory portability. Nanomaterials (NMs) provide a favorable platform for integrating enzymes into microsystems with enhanced selectivity and sensitivity. Various NM-enzyme immobilization strategies applied in the fabrication of capillary-based and chip-based enzymatic microsystems are summarized in this manuscript. We focus on highlighting the advantages of employing NM-based enzymatic microsystems for enantioseparation, inhibitor screening, bioreaction and biosensing. Innovative nanocomposites and NM-functionalized monoliths used to construct multienzymatic microsystems are also illustrated. The general

Abbreviations: AChE, Acetylcholine esterase; AGIs, α-glucosidase inhibitors; Ag@SiO_2, Silica-coated silver nanoparticles; antTiO_2-CH, chitosan/anatase titanium dioxide; BlSPase, Sucrose phosphorylase from bifidobacterium longum; BPE, bipolar electrode; BSA, bovine serum albumin; Cat, catalase; CE, capillary electrophoresis; Ch, choline; ChEt, cholesterol esterase; ChOx, cholesterol oxidase; CL, chemiluminescence; CNTs, carbon nanotubes; COD, choline oxidase; c-SWCNTs, carboxylated single-walled carbon nanotubes; ECL, electrochemiluminescence; EDTA, ethylene diamine tetraacetic acid; GLDH, L-glutamic dehydrogenase; GNPs, gold nanoparticles; GO, graphene oxide; GOx, glucose oxidase; HRP, horseradish peroxidase; IMERs, immobilized enzyme reactors; INPs, indium oxide nanoparticles; IrOx NPs, iridium oxide nanoparticles; ISFETs, ion-sensitive field effect transistors; ITO, indium tin oxides; L-Asnase, L-asparaginase; L-Gln, L-glutamine; LODs, limits of detection; MEF, metal-enhanced fluorescence; MNPs, magnetic nanoparticles; MS, mass spectrometer; MT, methimazole; MWCNT, multiwalled carbon nanotubes; NiO, nickel oxide; NMs, nanomaterials; NRs-NiO, nickel oxide nanorods; OECTs, organic electrochemical transistors; OT, open tubular; PAL, phenylalanine ammonia-lyase; μPADs, paper-based analytical devices; PDDA, poly(-diallyldimethylammonium chloride); PDMS, poly(dimethylsiloxane); PEI, polyethyleneimine; PNGase F, peptide-*N*-glycosidase F; POC, point-of-care; poly(GMA-co-EDMA), poly(glycidyl methacrylate-*co*-ethylene dimethacrylate); Pt NPs, platinum nanoparticles; PVA, polyvinyl alcohol; PVDMA, poly(2-vinyl-4,4-dimethylazlactone); SPE, Screen printed electrode; μ-TAS, micro-total analysis system; TG, triglyceride; Tyr, tyrosinase.

* Corresponding author. Xiangya School of Pharmaceutical Sciences, Central South University, 172 TongZiPo Road, Changsha, Hunan, 410013, China.
E-mail address: ccpin2000@hotmail.com (C. Chen).

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Enzymatic microsystem
Microchip
Nanomaterial

development trend identified in this review indicates that the application of NMs has significantly improved enzymatic microsystem performance.

Contents

1. Introduction

Miniaturization has rapidly gained popularity in recent years because of its prominent advantages, including lower reagent consumption, higher automation, faster analysis time, and better portability and disposability. Enzymatic microsystems present enormous potential in the chemical and biological sciences [1]. As a key point in the fabrication of enzymatic microsystems, innovative enzyme immobilization technologies have been investigated to (i) increase the enzyme binding capacity, (ii) improve the enzyme activity and stability, (iii) enhance the analytical efficiency and detection sensitivity, and (iv) develop easy-to-operate procedures. Compared with free enzymes, immobilized enzymes exhibit substantial advantages in terms of reusability and durability [2,3]. To obtain enzymatic microsystems with satisfactory performance, noteworthy efforts have been focused on exploring various immobilization methods, including adsorption, covalent binding, and entrapment [4–6]. Currently, the rapidly expanding development of nanoscience provides novel opportunities for enzyme immobilization [7]. Nanomaterials (NMs) are attracting considerable attention for the development of capillary-based and microchip-based enzymatic systems due to their high surface area and specific electrical, optical, mechanical and magnetic characteristics (Fig. 1).

Capillary electrophoresis (CE) has been regarded as a promising microanalytical technique because of its high efficiency and peak capacity [8]. Capillaries integrated with enzymes offer efficient platforms for online separation applications and enzyme studies. To fabricate novel enzymatic microsystems, NMs have been introduced into monolithic and open tubular (OT) capillaries to construct desirable microenvironments with improved enzyme immobilization capacities. Monolithic capillaries, as an alternative to OT and packed columns, have become useful materials for preparing enzymatic microsystems due to their unique benefits, such as better accessibility of the active site to substrates, lower back pressure, higher mass transfer, and versatile functional groups on the pore surfaces available for enzyme immobilization [9,10]. Therefore, increasing attention has been paid to the immobilization of enzymes onto NM-functionalized polymer monoliths, silica monoliths and organic-silica-based hybrid monoliths. In addition, NMs are favorable for improving the surface ratio of OT columns, and a variety of enzyme-NM conjugates have been synthesized to fabricate enzymatic OT capillaries. Capillary-based enzymatic microsystems offer specific advantages in bioreactions, inhibitor screening, and enantioseparation applications.

Microfluidic systems are prominent candidates for bioanalysis and biosensing, with exceptional merits over traditional devices in terms of portability, the precise control of fluids, low sample consumption, high sample throughput, and the easy integration of various detection platforms [11]. Implementing enzymatic reactions in microchips can combine the high selectivity of enzymes with the effective fluidics of a microreactor, achieving fast and efficient analysis [12]. Since choosing the optimum enzyme immobilization strategy is important for developing enzymatic microreactors with satisfactory activity and stability, innovative NM-based protocols for immobilizing enzymes have been explored due to the large surface area, good hydrophilicity and biocompatibility of NMs. Studies show that the performance of a microreactor can be enhanced by utilizing NMs to immobilize enzymes. Integrating enzymatic biosensors onto microchips can potentially yield more efficient microdevices because of the improved mass transport and lower detection limit [13]. Additionally, enzyme

immobilization methods have an essential influence on the performance of enzymatic biosensors. Since NM-based biosensors possess a high enzyme-coating capacity and desirable electronic transfer properties, ultrasensitive detection can be attained [14]. Recently, microchip-based enzymatic systems fabricated by incorporating NMs into biosensors have attracted tremendous interest.

We have summarized the developments related to preparing NM-based capillaries in separation science [15,16]. In the present review, we further discuss recent advances in the application of NMs in the integration of enzymes into capillaries and microfluidic chips. Shi et al. described the development of novel, easily separated support materials for enzyme immobilization [17]. Herein, innovative NM-functionalized monolithic capillaries and enzyme-NM conjugate-packed capillaries for fabricating enzymatic microsystems will be summarized. The application of NMs in the construction of biosensors and biologically active reactors on microchips will also be discussed in detail. In addition, we will discuss the use of state-of-the-art NM-based enzymatic microsystems for enantioseparation, inhibitor screening, biocatalysis, biotransformation, proteolysis and biosensing. The advantages of miniaturized systems over conventional devices will be highlighted in this article.

2. Capillary-based enzymatic microsystems

The integration of immobilized enzyme reactors (IMERs) with microsystems provides a platform for the online separation and detection of the substrates and products of enzymatic reactions, thereby achieving efficient, high-throughput and fully automated analysis [18–21]. Additionally, NMs have attracted much attention for use in the development of efficient capillary-based enzymatic microreactors to provide a favorable microenvironment for enzyme immobilization. The obtained miniaturized systems facilitate the improvement of the enzyme stability, reduction of reagent cost and availability of enzymes for reuse without the need for complicated isolation procedures. Novel enzyme immobilization strategies for fabricating capillary-based enzymatic microsystems can be classified into two groups according to the selected supports: monolithic capillaries and OT capillaries. Various types of NMs, including gold nanoparticles (GNPs), silica nanoparticles, carbon NMs and magnetic nanoparticles (MNPs), are reviewed in this article (Table 1).

2.1. Enzymes in monolithic capillaries

The monolithic capillary, with a porous 'single-particle' structure, has been considered a promising matrix for enzyme immobilization [22–24]. To further improve the performance of monolithic capillary-based enzymatic microsystems, NMs are introduced to develop suitable enzyme-coated supports. These NM-functionalized monolithic capillaries possess a large surface area, biocompatible microenvironment, high permeability and convective mass transport, enabling enhancement of enzyme activity, stability and immobilization capacity. Herein, we describe the distinct NMs utilized to integrate enzymes into monolithic capillaries.

2.1.1. Gold nanoparticles

GNPs are a desirable carrier for enzyme immobilization based on the high affinity of gold for thiol and amino moieties. Benefiting from the simple and fast fabrication process of porous polymer monoliths, various GNP-functionalized polymer capillary monoliths have been successfully developed [25,26]. A polymer monolith modified with GNPs provides a satisfactory platform for the subsequent immobilization of enzymes. Therefore, GNP-based polymer monolithic microreactors have specific advantages in biological analysis.

Since enzymes are natural biocatalysts, the construction of enzymatic microreactors for biocatalysis is beneficial for high-throughput analysis under moderate reaction conditions and enables environmentally friendly and economical assays. Despite these merits, a decrease in enzyme activity may occur during the operating process. Therefore, it is necessary to explore enzyme immobilization strategies available for the regeneration of bioreactor activity. To replace an enzyme in situ without losing the support, Svec et al. reversibly immobilized porcine lipase on a porous polymer monolith by using GNPs as intermediate ligands [27]. Compared with traditional methods, this strategy facilitated the regeneration of the deactivated bioreactor by using 2-mercaptoethanol to strip the denatured lipase from the GNPs and subsequently immobilize fresh lipase (Fig. 2). The enzymatic reactor was applied to catalyze the hydrolysis of glyceryl tributyrate. The microreactor lost less than 10% of its activity after the continuous injection of a substrate solution, equaling 1760 reactor volumes. This satisfactory stability could be attributed to the strong affinity of GNPs for thiols and amines, which prevented enzyme leakage during operation. Moreover, the high surface area and biocompatibility of GNPs could improve the enzyme immobilization capacity and maintain the native conformation and catalytic activity of the lipase. The described work provided a general method to develop renewable GNP-based enzymatic microreactors.

In addition to biocatalysis, GNPs can also be used to prepare IMERs for the online enrichment and deglycosylation of glycopeptides. Compared with in-solution deglycosylation, on-column deglycosylation by IMERs exhibits special merits due to the facile integration, high throughput and efficiency of this approach. Since the conventional glycopeptide enrichment method utilizes buffer with a low pH value and a high concentration of acetonitrile, a complex buffer exchange step is essential before the on-column deglycosylation. Herein, GNP-modified polymer monoliths were prepared as matrices for the subsequent immobilization of cysteine and peptide-*N*-glycosidase F (PNGase F), respectively [28]. A satisfactory compatibility of glycopeptide enrichment and deglycosylation was achieved because the PNGase F-functionalized monoliths could be coupled with cysteine-modified columns without pH adjustment and buffer exchange. GNPs could increase the number of reactive sites and the hydrophilicity of the column. These results indicated that a promising strategy for large-scale glycoproteome analysis with high selectivity and sensitivity was developed successfully.

Although colorimetric and fluorometric measurements have commonly been used for enzyme inhibitor screening, these methods are limited to the analysis of complex samples containing various fluorescent or ultraviolet-absorbing compounds. The combination of IMERs and CE techniques has been considered a desirable strategy for screening enzyme inhibitors [29,30]. In drug discovery and development, much more attention has been paid to searching for enzyme inhibitors from medicinal plants. Inline enzymatic bioreactors have been suggested as applicable systems for these complicated sample matrices [31]. For instance, a new method based on CE was established to screen α-glucosidase inhibitors (AGIs) from natural products [32]. AGIs are regarded as essential chemotherapeutic agents for the therapy of non-insulin-dependent diabetes mellitus. In one work, an enzymatic reactor was constructed by attaching α-glucosidase onto a GNP-functionalized poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (poly(GMA-*co*-EDMA)) capillary monolith via the strong affinity of the GNPs for the amino groups of the enzyme. Compared with the free enzyme, the obtained microreactor exhibited high enzymatic activity and satisfactory stability due to the mild immobilization procedure. The results showed that immobilized α-

glucosidase could maintain 80% of its activity after 25 runs. The described study offered an efficient and durable strategy for assaying complex natural products.

2.1.2. Silica nanoparticles

Recently, innovative strategies have been explored to fabricate hybrid capillary monoliths incorporating silica nanoparticles by entrapping nanoparticles in the monolith during copolymerization [33]. The developed organic-silica-based hybrid monoliths combine the advantages of both organic and inorganic monoliths. In this section, the applications of hybrid monolithic enzymatic microsystems for proteolysis and chiral separation are discussed.

In the bottom-up strategy for proteomic profiling, protein digestion is an important step before peptide and protein identification by mass spectrometry (MS) analysis. Traditional proteolysis performed in solution cannot easily achieve a high-throughput analysis and accurate protein identification because of the time-consuming process and incomplete digestion of the low-abundance proteins. To overcome these disadvantages, IMERs with desirable reusability and high enzyme-to-substrate ratios have been widely applied in proteolysis [34]. Among the various matrices, monolithic materials have attracted much attention for enzyme immobilization [35–42]. A trypsin-based enzymatic reactor was developed by incorporating amino-functionalized SBA-15 nanoparticles into a hybrid organic-inorganic monolith [43]. Since SBA-15 could improve the enzyme immobilization capacity, a higher digestion efficiency was achieved than that observed for an enzymatic reactor without the incorporated nanoparticles. In addition to monolithic capillaries, packed capillaries were also employed to prepare trypsin-immobilized microreactors with silica microparticles as enzyme carriers [44]. In this system, single 110-μm silica particles were used as the frits, and trypsin-immobilized spherical microparticles were trapped between pairs of frits. In contrast to particle-packed capillaries, the preparation of NM-based enzymatic capillary monoliths can avoid the frit-making process. In general, trypsin-immobilized microreactors have mainly been utilized to digest proteins containing lysine and arginine residues, and it is difficult to cover the complete sequence of proteins by applying single protease-modified reactors. Therefore, the fabrication of multienzymatic microreactors has attracted tremendous interest [45,46]. Zhang et al. prepared a bienzymatic reactor by immobilizing trypsin and chymotrypsin onto a hybrid organic-inorganic monolith and SBA-15 nanoparticles, respectively [47]. In the referenced work, both immobilization processes provided mild environments to maintain the activity of the enzymes. Moreover, the enzymes coated onto SBA-15 nanoparticles via copper ion chelation could easily be regenerated by removing the Cu^{2+} with ethylene diamine tetraacetic acid (EDTA) solution and introducing fresh solution into the capillary. In comparison with solution-based trypsin/chymotrypsin tandem digestion, the application of bienzymatic reactors shortened the digestion time from 24 h to 50 s. Since trypsin and chymotrypsin possess completely orthogonal specificities, this bienzymatic reactor enhanced the functional analysis of membrane proteins in the rat liver by doubling the number of identified peptides, leading to the identification of more proteins with deep coverage. These results indicated that the obtained SBA-15 nanoparticle-based bienzymatic reactor could achieve the efficient and fast digestion of complex protein samples.

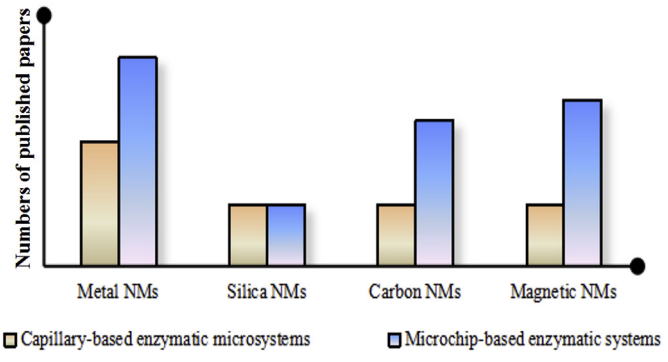

Fig. 1. Summary review of different nanomaterials applied to enzymatic microsystems fabrication.

In addition to the increasing research on bioreactors, enzyme-immobilized capillary monoliths exhibit specific advantages for enantioseparation. Chiral discrimination is an important topic in the analytical chemistry domain since living systems show different physiological responses to distinct enantiomeric forms of a single drug. Enzymes are protein macromolecules containing a variety of chiral centers, and this category of selector is considered a promising stereoselective stationary phase [48,49]. To enhance the separation performance, novel strategies have been investigated to fabricate NM-based monolithic capillaries [50]. Amino-modified mesoporous silica nanoparticles were incorporated into a monolithic capillary to improve the pepsin immobilization capacity, and fifteen basic chiral drugs were successfully enantioseparated. The results suggested that the application of mesoporous silica nanoparticles in the development of enzyme-based chiral stationary phases could provide a favorable enantioseparation strategy.

2.1.3. Carbon nanomaterials

To investigate the use of novel enzymatic microsystems for chiral separation, carbon NMs have also been introduced into polymer and silica monoliths. The methods applied to develop carbon NM-based monoliths can be classified into two categories: incorporating NMs into the monoliths during copolymerization and postmodifying the matrix monoliths with NMs.

Graphene oxide (GO) is a favorable alternative for chemical functionalization because of its unique structures containing various reactive oxygen functional groups [51,52]. Amino-modified silica capillary monoliths were utilized for GO immobilization via SN2 nucleophilic displacement between amino moieties and the epoxy groups of GO [53]. The preparation of affinity capillary monoliths was successfully accomplished by immobilizing a pepsin chiral selector onto GO. GO was a satisfactory material for improving the enantioseparation ability of pepsin-modified capillary monoliths, and the affinity capillary monoliths fabricated by the ethanediamine-based method exhibited better chiral recognition capacity than that exhibited by the other two amino donor-based monoliths. In addition, GO enhanced the proteolysis ability of pepsin-modified capillary monoliths.

Instead of using a postmodification strategy, one-step copolymerization with a reduced number of preparation steps exhibits unique merits in the development of polymer capillary monoliths incorporating carbon nanotubes (CNTs) [54,55]. Pepsin-immobilized polymer capillary monoliths were constructed by applying carboxylated single-walled carbon nanotubes (c-SWCNTs) to improve the enzyme immobilization capacity, and pepsin was immobilized onto monoliths via the glutaraldehyde method [56]. The results indicated that the c-SWCNTs played a dominant role in enhancing the separation performance. Moreover, the obtained affinity capillary monoliths showed satisfactory chiral separation ability for ten pairs of basic enantiomers.

2.2. Enzymes in open tubular capillaries

Generally, electrophoretically mediated microanalysis can be

Table 1
Nanomaterials in preparing capillary-based enzymatic microsystems for chemical and biological analysis.

Type of NMs	Type of enzymes	Applications	Year	Ref.
Gold nanoparticles	Lipase	Catalyzing the hydrolysis of glyceryl tributyrate	2014	[27]
	Peptide-*N*-glycosidase F	On-line deglycosylation of glycopeptides	2015	[28]
	α-Glucosidase	Screening enzyme inhibitors	2013	[32]
	Trypsin	Protein digestion	2013	[66]
	L-Asparaginase	Hydrolyzing L-glutamine	2013	[65]
	L-glutamic dehydrogenase	Screening enzyme inhibitors	2011	[64]
Silica nanoparticles	Trypsin	Protein digestion	2014	[43]
	Trypsin/chymotrypsin	Protein digestion	2015	[47]
	Pepsin	Chiral separation	2017	[50]
Carbon nanomaterials	Pepsin	Chiral separation	2016/2017	[53,56]
	Trypsin	Protein digestion	2014	[67]
Magnetic nanoparticles	L-Asparaginase	Clinical leukemia treatment	2014	[59]
	Horseradish peroxidase	In-line biotransformation of paracetamol	2010	[60]
	Choline oxidase/catalase	Measuring acetylcholine	2015	[61]

performed by using a capillary both as a reaction chamber and as a separation channel. Enzymes can be easily coated onto the inner wall of the capillary for the fabrication of OT capillary-based IMERs. However, enzyme immobilization onto an OT capillary is limited due to the low surface-to-volume ratio of the capillary. This shortcoming can be overcome by applying NMs for enzyme modification to achieve enhanced efficiency and stability. A variety of innovative enzyme-NM conjugates have been explored for fabricating OT capillary-based enzymatic microsystems. The method of preparing NM-functionalized OT capillaries for further enzyme immobilization has been discussed as well.

2.2.1. Magnetic nanoparticles

MNPs represent a desirable support for enzyme immobilization [57,58]. Since MNPs can be manipulated by applying external permanent magnets, replaceable enzyme microreactors can be developed in a simple way. In contrast to the immobilization strategy for preparing renewable GNP-based and silica nanoparticle-based enzymatic reactors mentioned in Section 2.1, MNP-enzyme conjugates are usually prepared first and then packed into the capillaries.

MNPs functionalized by polymer brushes are favorable carriers for enzyme immobilization due to their high loading efficiency, convenient manipulation, desirable biocompatibility and satisfactory reusability [59]. Considering the multiple binding sites of polymer-grafted MNPs, poly(2-vinyl-4,4-dimethylazlactone) (PVDMA) was chosen for the modification of Fe_3O_4 nanoparticles, followed by conjugation with L-asparaginase (L-Asnase) (Fig. 3). In comparison with conventional polymer-modified MNPs, this novel NM provided a variety of active sites for enzyme immobilization, and the mild reaction was accomplished in a short time. Based on the specific ability of L-Asnase to hydrolyze L-asparagine, the

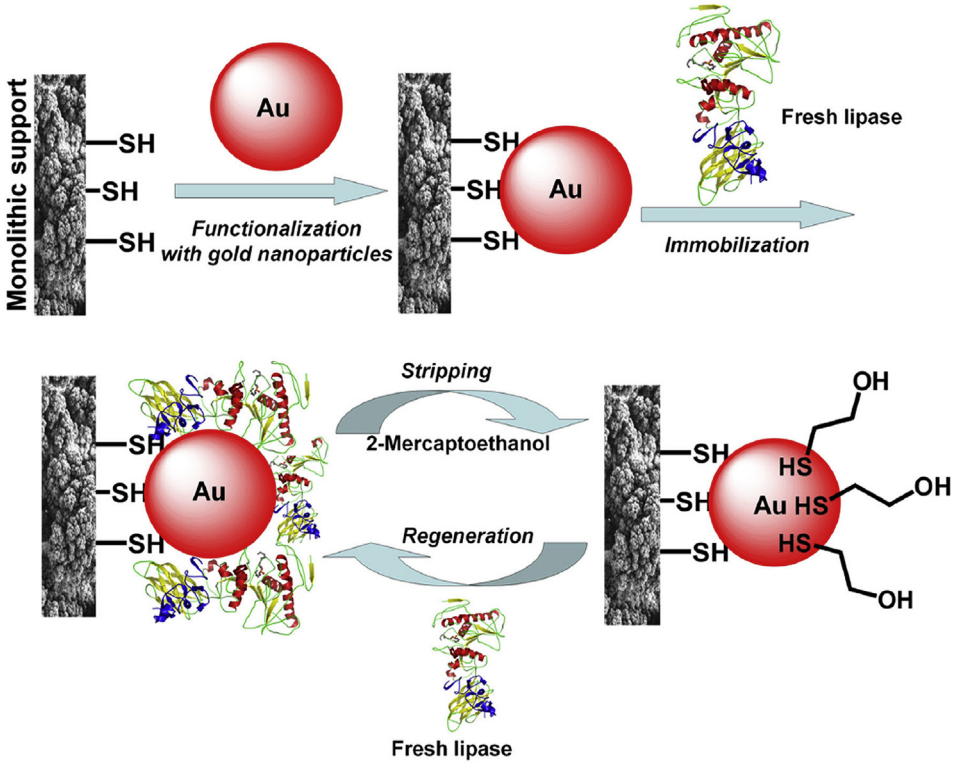

Fig. 2. Process of preparing gold nanoparticle-functionalized monoliths for enzymes immobilization and regeneration (Adapted from Ref. [27]). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

performance of L-Asnase reactors was monitored by the chiral ligand-exchange CE technique. The results indicated that the enzyme immobilization capacity and enzymolysis efficiency improved with a longer polymer chain. The L-Asnase-based reactor exhibited greater affinity than that of the free enzyme and retained more than 72.6% of its activity after 10 weeks of storage. Moreover, since L-Asnase is regarded as an effective antitumor agent and since biocompatible PVDMA-modified MNPs possess a particle size of less than 15 nm, the fabricated enzyme reactors were investigated for clinical applications. As shown in Fig. 3, acute lymphoblastic leukemia cells were incubated with L-Asnase reactors, and the apoptosis of cells was detected via a fluorescence-activated cell sorter. In addition, the enzyme horseradish peroxidase (HRP) was chemically attached to MNPs via the glutaraldehyde method [60]. These enzyme-immobilized MNPs could be injected and magnetically retained in a short part of the capillary without interfering with the inline detection of the paracetamol biotransformation process. The proposed reactor is a satisfactory tool for drug enzymatic transformation studies with low biocomponent consumption and high conversion efficiency.

A renewable and efficient multienzyme microreactor was fabricated by coimmobilizing choline oxidase (COD) and catalase (Cat) onto magnetite Fe_3O_4 nanoparticles and then introducing these modified NPs into a capillary [61]. This on-line reactor was able to remove choline (Ch) and measure the level of acetylcholine (ACh) without Ch interference. Herein, MNPs with a high surface area improved the enzyme loading capacity and enhanced the Ch conversion efficiency. Moreover, the three-dimensional structure of the prepared Fe_3O_4 nanoparticles facilitated the formation of large pores and channel structures among the NMs, increasing the permeability and reducing the flow resistance for the Ch reaction. Furthermore, the obtained reactor was integrated with an in vivo microdialysis sampling system and an acetylcholine esterase (AChE)-COD/Prussian blue electrochemical detector for the selective and continuous monitoring of ACh. In the described study, a low detection limit of 1 μM was observed. Since ACh is an important neurotransmitter involved in neurotransmission processes, ACh measurement plays an essential role in clinical treatment. However, the coexistence of Ch at a high concentration affected the ACh detection. To eliminate the interference of Ch with ACh, the bienzyme reactor fabricated in the study was successfully used for sequential enzymatic reactions, providing a stable and reliable strategy for measuring cerebral ACh release. We compared nanomaterial-based enzymatic microreactors and silica microparticle-based IMERs in Section 2.1.2. In addition to developing OT capillary-based enzymatic microsystems using MNPs, magnetic microparticles were also used to prepare packed capillary-based enzymatic microsystems [62,63]. Since enzyme-immobilized magnetic microparticles can be fixed by a magnetic field, the complex frit preparation step can be circumvented. OT capillary-based enzymatic microsystems exhibited better permeability than packed capillary-based enzymatic microsystems. Moreover, the introduction of NMs improved the enzyme immobilization capacity, overcoming the shortcoming of OT capillaries with a low phase ratio. Therefore, many more studies have focused on fabricating IMERs based on OT and monolithic capillaries instead of packed capillaries.

2.2.2. Gold nanoparticles

As summarized in Section 2.1.1, GNPs are a favorable carrier for enzyme immobilization. In addition to capillary monoliths, GNPs were also used to develop OT capillary-based enzymatic reactors for inhibitor screening [32,64]. In contrast to the method used to develop GNP-functionalized monoliths for subsequent enzyme immobilization, enzyme-GNP conjugates were prepared first and subsequently packed into columns. L-Glutamic dehydrogenase (GLDH) was immobilized onto 38 nm-diameter GNPs to achieve GLDH-NP conjugates. Then, these functionalized GNPs were assembled on a polyethyleneimine (PEI)-modified carrier surface, obtaining an inline GLDH reactor. The GNP-mediated bioreactor exhibited better stability than that of columns without NPs due to the strong bonding between the GNPs and thiol groups in GLDH. In addition, an improved enzyme immobilization capacity was observed because the high surface-to-mass ratio of GNPs facilitated the enrichment of GLDH. The results indicated that the proposed GLDH bioreactor was useful for assaying GLDH inhibitors in medicinal plant extracts.

The strategy for preparing L-Asnase-MNP conjugates is discussed in Section 2.2.1. Qi et al. also utilized GNPs to develop L-Asnase-based reactors [65]. To investigate the cytotoxic effect of L-Asnase in the treatment of acute lymphoblastic leukemia, an L-Asnase-immobilized reactor was prepared for the hydrolysis of L-glutamine (L-Gln). In the referenced study, L-Asnase-GNP conjugates were immobilized onto the inner surface of a capillary that was functionalized with (3-mercaptopropyl)-trimethoxysilane. The GNPs provided a large surface area for L-Asnase attachment, and the biocompatible microenvironment facilitated the hydrolysis process. Satisfactory batch-to-batch repeatability of the obtained reactor was observed, with an RSD less than 5.0%. In comparison with L-Asnase in free solution and L-Asnase-GNP conjugates, the fabricated CE-based IMERs showed the best hydrolysis performance.

To prepare OT capillary-based enzymatic reactors, a majority of strategies have focused on developing enzyme-NM conjugates prior to immobilization onto capillaries. Nevertheless, Jänis et al. developed capillary-based microreactors for protein digestion using two methods [66]. In the first method, trypsin was adsorbed on the GNP surface before attachment to the capillary, and in the second method, unconjugated trypsin was immobilized onto a GNP-based capillary. These reactors exhibited satisfactory repeatability, and no peptide carryover was found between the digestion of different proteins. The described work provided various approaches for fabricating protease-modified microreactors, further broadening the applications of GNPs in proteomic studies.

2.2.3. Graphene oxide

In addition to GNPs, GO containing various functional groups was also used as a coupling reagent to introduce trypsin into a capillary [67]. In the study, enzymatic reactors were developed using a layer-by-layer electrostatic assembly technique, which is a reliable and simple immobilization method. First, a poly(-diallyldimethylammonium chloride) (PDDA) solution was introduced to create a positively charged coating on the inner wall of the capillary. Then, a layer of negatively charged GO was adsorbed onto the PDDA to absorb trypsin. Multilayer IMERs were obtained by repeated coating with GO and trypsin. The prepared IMER was used for the online digestion of bovine serum albumin (BSA), with an incubation time of 30 min, and the obtained products were comparable to those obtained using free trypsin digestion with 12 h of incubation. Moreover, the utilized approach did not affect the enzyme activity. The obtained CE-based IMERs exhibited desirable repeatability and retained 79.5% of the initial activity after more than 100 runs.

A variety of NMs can be applied in the development of enzymatic microsystems by choosing polymer monoliths, silica monoliths, organic-silica-based hybrid monoliths, or OT capillaries as a support. GNPs present substantial benefits in the fabrication of regenerated enzymatic microreactors based on monolithic and OT capillaries. In addition, silica NPs are employed to construct renewable enzymatic microreactors. Carbon NMs with various

functional groups can be introduced into capillaries to immobilize high-capacity enzymes. In the exploration of replaceable enzymatic microreactors by using OT capillaries, magnetic NPs have attracted much research interest. The advantages of these miniaturized systems over conventional devices are summarized in Table 2.

3. Microchip-based enzymatic systems

3.1. Enzymes for fabricating bioreactors

Microchip-based enzymatic bioreactors combine the efficient fluidics of microstructured flow reactors and the high selectivity of enzymes. These bioreactors exhibit substantially better performance than conventional enzyme reactors due to effects resulting from the microscale domain [68–70]. As a benefit of system automation, the reaction output can be controlled by optimizing the flow rate, and better repeatability can be obtained [71–74]. In exothermic reactions, the higher surface-to-volume ratio of microreactors facilitates the formation of a homogenous temperature distribution inside the reactors, enhancing the product yield. In addition, microchannels can construct a desirable microenvironment for maintaining enzyme activity, and microreactors with favorable reusability provide an economical platform for bioreactions. Nevertheless, two major challenges that restrict the utilization of microchip-based enzymatic bioreactors are the fast inactivation and the instability of the enzymes. Based on the prominent advantages of NMs mentioned in Section 2, the efficiency of enzyme microreactors can be further enhanced by immobilizing enzymes onto NMs. Additionally, the combination of microfluidic applications with immobilized enzymes offers potential devices for point-of-care (POC) diagnostics, protein digestion, biocatalysis and synthesis. Herein, we summarize recent progress in the use of MNPs, carbon NMs, silica NMs and GNPs for the development of microchip-based enzymatic bioreactors. Different enzyme immobilization strategies and detection methods are reported in Table 3. Moreover, the advantages of using NM-based enzymatic microreactors instead of conventional devices are discussed in detail.

3.1.1. Magnetic nanoparticles

MNPs are important carriers of biomolecules. Magnetic techniques facilitate the introduction of NMs inside certain sites in microreactors, and enzymatic reactions can be manipulated automatically. MNP-based enzymatic microreactors exhibit satisfactory performance for biocatalysis, biodetection and bioanalysis. Because of the widespread utilization of MNPs, a substantial amount of work has focused on developing innovative nanocomposites for enzyme immobilization.

Poppe et al. developed a novel MagneChip using magnetic techniques to anchor enzyme-immobilized NPs inside multiple chambers of a microfluidic bioreactor [75,76]. Phenylalanine ammonia-lyase (PAL) is an important type of aromatic amino acid lyase that catalyzes the nonoxidative deamination of L-phenylalanine. PAL-coated MNPs were developed and fixed in four individual magnetic compartments of a microfluidic chip. As shown in Fig. 4, two syringe pumps were applied to dispense reagents, and a zoom microscope was used for the optical monitoring of the chip. During the chip filling process, an air pressure of 0.2–0.3 bar was utilized to push the MNPs through the chip, and the MNPs were collected in the first reaction cell, guided by permanent magnets placed in magnet drawers. After the biotransformation reached a constant level of production, the MNPs were released and captured in the next chamber. In the described work, core-shell silica-MNP carriers were prepared by a sol-gel reaction on the MNP surface. Subsequently, epoxy groups were introduced for enzyme immobilization. The obtained particles were accumulated by cells and then released by an external magnetic field. The impact of the nanoparticle size on the enzyme catalytic activity was also investigated. Particles with decreased size possessed higher catalytic activity owing to an increased area-to-volume ratio. A binary mixture of PAL-coated MNPs with diameters of 250 and 600 nm enhanced the enzyme immobilization capacity and achieved better mass transfer, further improving the catalytic activity of the fabricated MagneChip. A fast reaction was obtained due to the short molecular diffusion lengths in this microsystem. The efficient biotransformation of L-phenylalanine and five artificial substrates was achieved using the MagneChip microfluidic device with UV detection, and the productivity

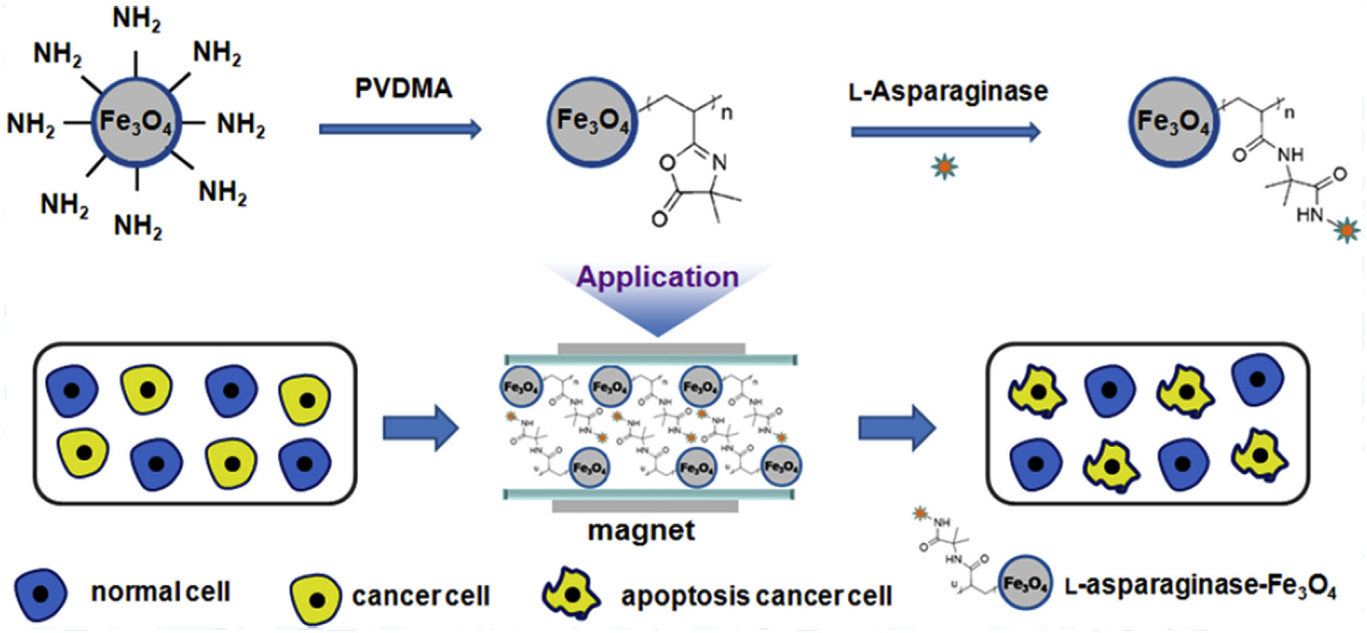

Fig. 3. Synthesis process of L-asparaginase-based microreactors utilized for the simulation of extracorporeal shunt system (Adapted from Ref. [59]).

was more than three orders of magnitude greater than that of shake vials. Moreover, the cyclic operations exhibited satisfactory reproducibility during 14 h of continuous measurements.

Since glucose is an essential marker of diabetes, many efforts have been focused on developing microchip-based enzymatic microreactors for glucose detection. The materials used to prepare enzymatic reaction channels in microfluidics are mainly polymers [77–79]. In comparison with the commonly used polymers, silica capillaries can reduce the adsorption of analytes and improve the separation efficiency. Ju et al. employed a silica capillary as an enzymatic reaction channel [80]. Glucose oxidase (GOx)-MNPs were developed via the glutaraldehyde method and placed in the separation channel of a silica capillary using an external magnetic field. The packed capillary with magnets was then inserted into the sampling reservoir and buffer reservoir retainer and the other side of the capillary was inserted into the detection reservoir retainer. The obtained microfluidic device was applied for the electrochemical determination of glucose in human serum samples without pretreatment. To investigate the glucose detection performance, the microreactor length was easily optimized by changing the magnet length. An optimal detection limit of 11 μM was obtained when a 6 mm reactor was utilized. In the referenced work, high-sensitivity glucose detection was attained because of the sufficient reaction between the glucose and GOx immobilized on the MNPs. Satisfactory inter- and intraday repeatability values of 0.8% and 1.7% for determining glucose were achieved, respectively.

Since the channels of a microchip device are small, a sensitive detection method is necessary for microfluidic assays. In addition to electrochemical detection platforms, chemiluminescence (CL) is a sensitive detection method with prominent advantages, including a wide linear range for quantification, a simple instrumental setup without a light source, and the potential for achieving in-line detection performed on a microfluidic device. GOx-MNPs and HRP-MNPs were used to fabricate inline bioreactors on microfluidic chips [81]. To obtain proper enzyme-MNP conjugates, 3-aminopropyl-triethoxysilane was applied first to modify the MNPs. These amine-functionalized MNPs were then coated with a glutaraldehyde crosslinker. Finally, enzyme immobilization was accomplished via GOx/HRP incubation, and the prepared GOx-MNPs and HRP-MNPs were packed into the chip channel. The enzymatic activity of HRP-MNPs was evaluated by detecting a luminol-H_2O_2 CL signal after two months, and it was observed that more than 90% of the activity was maintained. By comparison, a free HRP solution lost approximately 62% of its activity after one week. An H_2O_2 assay was utilized to determine glucose levels since GOx-catalyzed reactions usually produce H_2O_2. This device exhibited a better detection limit for glucose measurements (limit of detection, LOD = 5.2 μM) than that of the microchip electrophoresis-CL assay (LOD = 0.1 mM). The results demonstrated that the proposed CL bioassay of glucose and H_2O_2 presented satisfactory sensitivity and repeatability.

Magnetic nanocomposites have attracted tremendous attention in the fields of chemistry and biotechnology based on the combination of their nanometer size and unique magnetic responsivity. GO magnetic nanocomposites (GO/Fe_3O_4 MNCs) were prepared to integrate the magnetism of Fe_3O_4 NPs and the advantages of GO, such as a large surface area and satisfactory biocompatibility for AChE adsorption [82]. Since the release of dimethoate pesticides can cause unpredictable environmental contamination, it is meaningful to develop a sensitive method to rapidly detect dimethoate. In one study, the satisfactory detection of dimethoate pesticide (LOD = 0.18 μg L^{-1}) was achieved by the enzymatic inhibition of AChE. The favorable stability of GO/Fe_3O_4/AChE MNCs might be due to the biocompatible microenvironment provided by the GO, which could prevent the leakage of AChE and maintain enzyme activity. Therefore, magnetic graphene nanocomposites offer a desirable platform for developing replaceable and stable on-chip enzymatic microreactors. To combine the merits of polymer monomers with the specific magnetic properties and high dispersibility of magnetic particles, magnetic polymer nanocomposites were prepared via a simple method [83]. In the referenced study, multienzyme-modified magnetic nanocomposites were developed by the coimmobilization of lipase, glycerokinase and glycerol-3-phosphate oxidase onto chitosan/Fe_3O_4 composite nanoparticles. The obtained enzyme/chitosan/Fe_3O_4 nanocomposites were introduced into the microchip channels using a magnetic field. The results suggested that the integration of a multienzymatic reactor into a microchip could sensitively determine triglycerides (TGs). Moreover, the effect of the enzyme carrier length on the TG evaluation was discussed, and a low LOD of 6.0 mM was achieved when a 3.0 cm enzyme carrier was utilized. Satisfactory stability with 70% activity was maintained over 40 days of storage. The described work offered a promising strategy to detect TG expression in serum and diagnose human vascular disorders.

Proteomics research has recently attracted tremendous

Table 2
Advantages of using capillary-based enzymatic microsystems.

Type of capillaries	Type of NMs	Advantages	Ref.
Polymer monoliths	Gold nanoparticles	•Available for developing regenerated enzymatic microreactors Helpful in improving enzymes immobilization stability and capability Enhanced hydrophilicity of the columns for preparing immobilized enzyme reactors	[27,28,32]
	Carbon nanomaterials	•Available for increasing chiral recognition and protein digestion ability of enzymes	[56]
Silica monoliths	Carbon nanomaterials	•Rich functionalities for enzymes immobilization with high capacity	[53]
Hybrid monoliths	Silica nanoparticles	•Mild microenvironments for enzymes immobilization Helpful in fabricating microreactors for efficient proteolysis Promising in the construction of renewable enzymatic microreactors Satisfactory potential in increasing enantioseparation ability of enzymes	[43,47,50]
Open tubular capillaries	Magnetic nanoparticles	•Helpful in preparing replaceable microreactors Improved enzymes loading capacity for increasing biotransformation efficiency Available for constructing enzymatic microreactors for efficient clinical applications	[59–61]
	Gold nanoparticles	•Biocompatiable environment for maintaining enzymes activity Enhanced enzymes immobilization capacity and stability Helpful in screening enzymes inhibitors of complex samples Desirable repeatability	[64–66]
	Graphene oxide	•Various active groups for enzymes immobilization Simple immobilization process Helpful in preparing microreactors for protein digestion with satisfactory repeatability	[67]

Table 3
Nanomaterials in preparing microchip-based enzymatic reactors.

Type of NMs	Type of enzymes	Immobilization strategies	Advantages	Detection methods	Ref.
Magnetic nanoparticles	Phenylalanine ammonia-lyase	Covalent binding	Enhanced Biotransformation productivity and fast reaction	UV–Vis detection	[75,76]
	Glucose oxidase, horseradish peroxidase	Covalent binding	Improved glucose detection sensitivity and satisfactory repeatability	Electrochemical detection, chemiluminescence detection	[80,81]
	Acetylcholine esterase	Adsorption	Sensitive detection of dimethoate pesticide and improved stability	Electrochemical detection	[82]
	Lipase/glycerokinase/glycerol-3-phosphate oxidase	Covalent binding	Determine triglyceride with lower LOD and enhanced stability	Electrochemical detection	[83]
	Trypsin	Covalent binding	Enhanced proteolysis performance in short time	Mass spectrometry detection	[84]
Carbon nanomaterials	Naringinase	Adsorption	Improved isoquercitrin production and operational stability	UV–Vis detection	[87]
	Glucose oxidase/horseradish peroxidase	Adsorption	Increased glucose detection sensitivity	Colorimetric detection	[88]
Silica nanomaterials	Sucrose phosphorylase	Incorporation	Improved glycoside and sugar phosphate productivity	Confocal laser scanning	[89]
	Glucose oxidase/Lactate oxidase/L-glutamate oxidase	Adsorption	Lower LOD for determining lactate, glucose and glutamate	Colorimetric detection	[91]
Gold nanoparticles	Trypsin	Adsorption	Fast and efficient on-line digestion	Mass spectrometry detection	[92]
	Glucose oxidase	Covalent binding	Improved glucose detection sensitivity	Electrochemical detection	[93]

attention. In bottom-up analysis, immobilized enzymatic microreactors provide an efficient tool for fast proteolysis. However, microchannels with low surface areas are not sufficient for directly immobilizing enzymes. Zhang et al. prepared amine-modified MNPs via a one-pot method, and trypsin was then immobilized onto the obtained particles using a glutaraldehyde crosslinker [84]. These MNPs with a high enzyme capacity were packed onto a glass microchip by the application of a magnetic field. The performance of the bioreactor was evaluated using BSA, cytochrome *c* and myoglobin as model proteins, and complete protein digestion was achieved in a short time (10 s). Moreover, magnetic microparticles were utilized to develop microchip-based enzymatic microsystems for proteomics applications [85,86]. In this process, particle retention was the difficult aspect of the microchip packing, and packings with optimum compression needed to be adjusted by accurately positioning magnets.

3.1.2. Carbon nanomaterials

Biological reactions utilizing immobilized enzymes in microchips have enabled the easy separation of enzymes from their products with a low enzyme cost. However, this technique also suffers from certain disadvantages. To avoid the fast inactivation of the enzymes in microchannel catalysis, moving and unsinkable graphene sheets were investigated for enzyme immobilization [87]. The findings suggested that the size and density of the nanoparticles were two important factors that affected enzyme immobilization in microchannels. Nanoparticles with a larger size blocked the channels, and a higher density led to deposition as the nanoparticles flowed through the channels. The results indicated that a better dispersion of the immobilized enzymes was achieved in the microchannels due to the lower density of graphene. Moreover, the enzymes provided more active sites for the combination of substrates, further improving the catalytic efficiency. In the referenced study, naringinase was immobilized onto a graphene sheet surface by physical adsorption. Graphene presented a high enzyme adsorption capacity, and the maximum adsorbed enzyme loading was approximately 4 g/g of support. This graphene-based naringinase biocatalytic microreactor was developed for isoquercitrin production. Ten cycles of enzymatic reaction were successively achieved, with an enzyme activity of $85.51 \pm 2.76\%$. Enzymes immobilized on microchips exhibited higher operational stability and catalysis yields than those of free enzymes. This result indicated that the highly repeatable immobilization of enzymes on fluid and unsinkable graphene sheets was promising for microfluidic biocatalysis.

As presented in Section 3.1.1, glucose measurements are important for diabetes and glycosuria monitoring. Paper microfluidic devices are regarded as desirable microsystems for POC applications. To improve the analytical signal of colorimetric glucose detection, three types of NMs, including Fe_3O_4 nanoparticles (MNPs), multiwalled carbon nanotubes (MWCNTs) and GO, were applied to increase the available superficial area of microfluidic paper-based analytical devices (μPADs) [88]. The immobilization of GOx and HRP was achieved without affecting the enzyme activity. Since NMs were incorporated into cellulose fibers without using other spacers, the influence of additional interactions on the enzyme activity was avoided. The modification of cellulose fiber with NMs enhanced the analytical performance and decreased the washing effects in lateral-flow analysis based on colorimetric measurements. In the referenced work, an optimal color signal and uniformity were obtained for paper soaked with 100, 10, and 100 $\mu g\,mL^{-1}$ MNPs, MWCNTs and GO, respectively. However, higher concentrations corresponded to a strong background color intensity, which could affect detection. As shown in Fig. 5, there is a noticeable enhancement of the color

intensity in NMs-μPADs when compared with native μPADs. The morphology of native and treated μPADs is presented as well. The specific tubular structure of MWCNTs is shown in Fig. 5, and the long edges of GO are observed on the surface of the cellulose fibers. The results indicated that the combination of μPADs with MNPs and carbon-based NMs provided a promising tool for clinical diagnosis.

3.1.3. Silica nanomaterials

We have discussed the application of silica NMs for enhancing the enzyme immobilization capacity in monolithic capillaries [43–49]. The modification of silica NMs on microchannel walls is also a favorable strategy for fabricating enzymatic microchips [89]. Among the various candidates, nanosprings possess a highly solvent-accessible surface with desirable permeability and mechanical stability. In addition, nanospring-based matrices containing a sufficient number of silanol groups facilitate further modification reactions. Therefore, nanosprings can be patterned into microdevices to immobilize biomolecules. In the described work, silica nanosprings were introduced into microchannels to improve the surface area for sucrose phosphorylase attachment. As shown in Fig. 6, sucrose phosphorylase from *Bifidobacterium longum* (BlSPase) was functionalized with two silica-binding modules, Z_{basic2}, and this immobilized enzyme is referred to as Z BlSPase. The obtained Z BlSPase was then modified with nanospring-SO_3 to enhance the enzyme immobilization capacity. The enzymes penetrated the ~70 μm-thick nanospring layer and maintained a uniform distribution due to the variable pore size (from the nm to μm scale) of the layer. Since the enzymes were integrated into the nanolayer, enhanced operational stability was obtained. The enzyme activity was increased up to 10-fold over that of microchips without NMs by functionalizing the surface with sulfonate-modified nanosprings, and the productivity was improved accordingly. This microreactor provided a novel platform for the effective biosynthesis of glycosides and sugar phosphates. As conventional chromatographic packing materials, silica microparticles were easily chosen as solid supports for enzyme immobilization. PNGase F-coated silica beads were packed into a microchip, and the resulting enzymatic reactor was further coupled with a microfluidic glycan cleanup chip and a commercially available HPLC chip [90]. This integrated design facilitated the incorporation of all steps of the glycan profiling analysis, including glycoprotein deglycosylation, cleanup, glycan capture, separation, and detection. The results indicated that silica microparticles were another desirable choice for constructing microchip-based enzymatic microreactors. Moreover, silica NMs could be introduced into μPADs for the immobilization of enzymes due to the specific properties of NMs.

As discussed in Section 3.1.2, μPADs have attracted tremendous attention based on their specific advantages in terms of safe disposal, compatibility with various fabrication techniques, and suitability for the semiquantitation of different analytes. Many detection methods for μPADs, such as electrochemical, electrochemiluminescence, CL and colorimetric detection, have been performed on the basis of selectively oxidizing the analytes, followed by the oxidation of a chromogenic agent catalyzed by a peroxidase-based reaction, leading to a color change. Nevertheless, the heterogeneity of the color distribution in the detection zones

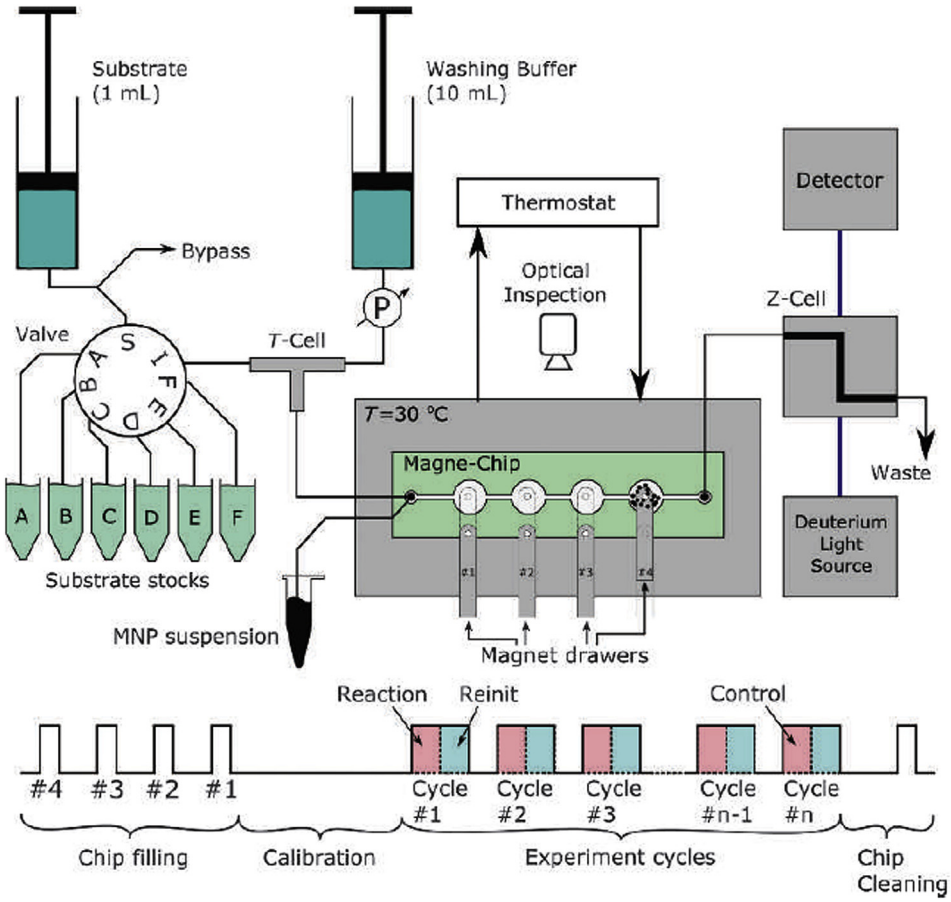

Fig. 4. Schematic diagram of the fluid control system (Adapted from Ref. [76]).

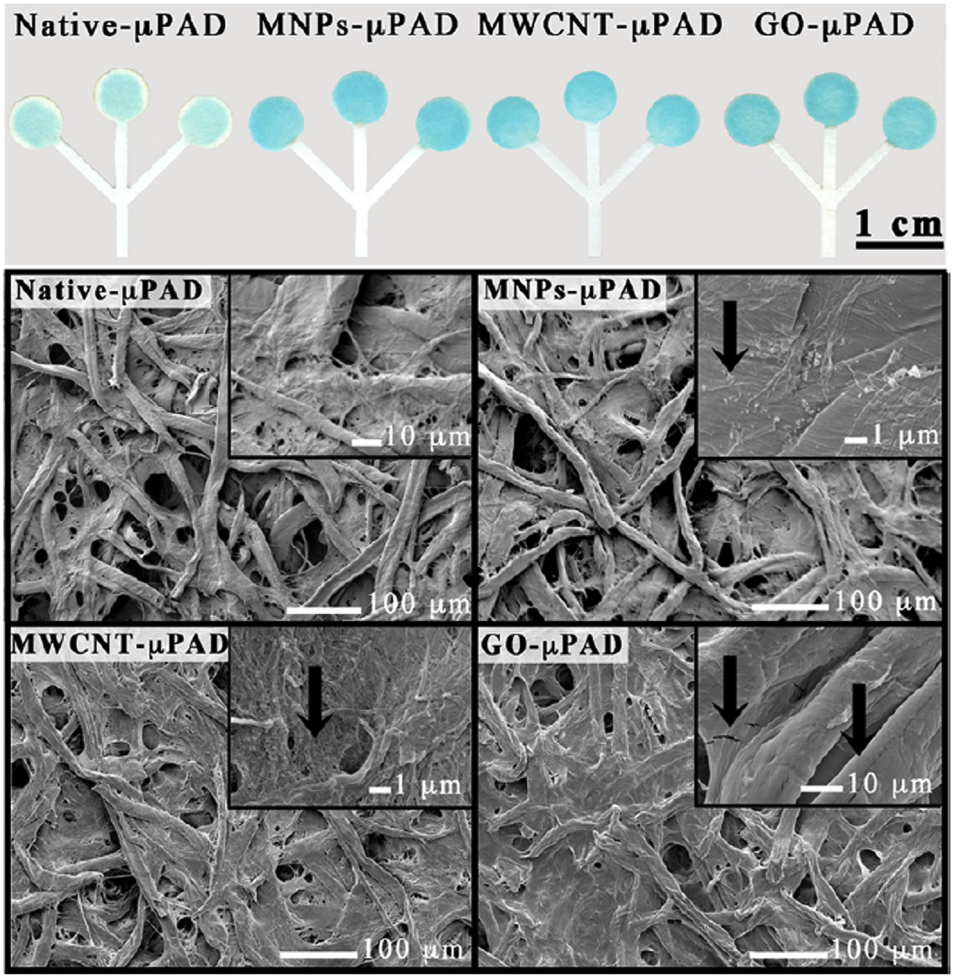

Fig. 5. Optical micrograph of paper-based analytical devices after addition of 1 mM glucose sample and field emission scanning electron microscopy images of native and treated devices with magnetic nanoparticles, multiwalled carbon nanotubes, and graphene oxide (Adapted from Ref. [88]).

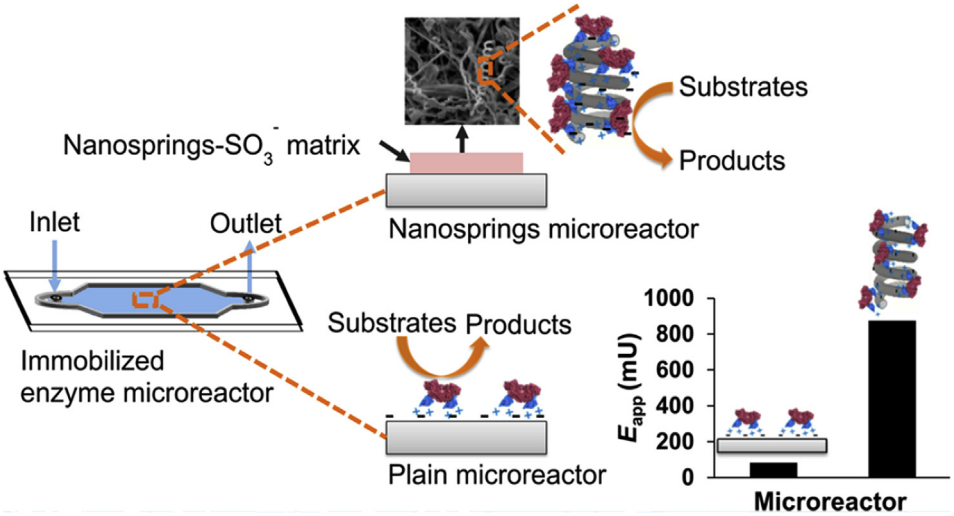

Fig. 6. Process of fabricating silica nanospring-based enzymatic microreactors applied for biocatalysis (Adapted from Ref. [89]).

has limited the application of μPADs. To solve this problem, silica nanoparticles modified with 3-aminopropyltriethoxysilane were introduced into μPADs for the adsorption of enzymes [91]. The results suggested that the integration of silica nanoparticles could prevent the washing away of immobilized enzymes, further improving the color uniformity and intensity of the colorimetric measurements. The satisfactory semiquantitative detection of lactate, glucose and glutamate was achieved, with LODs of

0.63 mM, 0.50 mM, and 0.25 mM, respectively.

3.1.4. Gold nanoparticles

To preserve the enzyme bioactivity, trypsin was entrapped in a GNP network [92]. In the referenced study, the GNP network offered a biocompatible microenvironment similar to that of the enzymes in their native states and gave the enzymes more freedom in their orientation. Furthermore, a nanostructured surface coating was formed in the microchannel by the sequential layer-by-layer adsorption of anion-coated GNPs and the cationic polyelectrolyte PDDA. The obtained microchip was coupled with MALDI-TOF MS analysis to identify complex proteins extracted from mouse macrophages and low-level (16 fmol) protein samples. The above-mentioned study provided a fast and efficient online digestion strategy that was needed for the further development of proteomics. In addition, a GNP-poly(dimethylsiloxane) (PDMS) composite was synthesized in situ, and GOx was integrated into the microchannel to prepare an enzymatic reactor [93]. GNPs, as a type of metal NM, have exhibited excellent merits for fabricating enzymatic microreactors. The majority of metal NMs with specific electrical characteristics have also been utilized to develop microfluidic-based biosensors.

3.2. Enzymes for fabricating biosensors

The applications of microchip-based enzymatic systems in biosensing have recently attracted tremendous attention due to their exceptional benefits over traditional devices in terms of portability, automation, response time, reagent cost and power consumption [94,95]. Distinct NMs, as immobilization carriers and sensing elements, are introduced into enzymatic biosensors to enhance the electrochemical detection sensitivity [96–98]. Since biological processes usually involve charge transfers and electrostatic interactions accompanying enzymatic reactions, charges can be readily transferred from/to NMs to transform biological reactions into electronic signals. Moreover, the fluorescence detection protocol is advantageous in enzymatic biosensing because of the specific optical characteristics of NMs. We now review the state-of-the-art microfluidic techniques for enzymatic biosensing via the incorporation of metal NMs and carbon NMs.

3.2.1. Metal nanomaterials

Since nanorods can offer direct channels for transporting electrons from the active sites of enzymes to collectors, this type of NM has been regarded as a satisfactory candidate for preparing biosensors with high enzyme loading. Nanostructured nickel oxide (NiO) is an antiferromagnetic semiconductor, and NiO nanorods (NRs-NiO) possess high stability in the environment and do not degrade during enzyme immobilization. Moreover, NiO nanorods can improve the intensity of electrochemical detection signals. Malhotra et al. prepared NRs-NiO using a standard coprecipitation technique [99]. Furthermore, cholesterol esterase (ChEt) and cholesterol oxidase (ChOx) were coimmobilized onto NRs-NiO electrodes for the fabrication of microfluidic biosensor chips. In the described work, the total cholesterol in human blood was determined with improved sensitivity via electrochemical detection. Since NiO nanoparticles possess poor electrical conductivity and a relatively low enzyme immobilization capacity, MWCNTs were integrated with NiO nanoparticles to improve the biosensing performance [100]. The results indicated that MWCNTs with high electrochemical reactivity exhibited fast electron transfer for use as electrodes in electrochemical biochips. Herein, nanocomposites containing NiO nanoparticles and MWCNTs were developed to fabricate microchips for cholesterol determination. ChOx and ChEt were coimmobilized onto electrodes via a reaction between the carboxyl groups on the MWCNTs and the amino groups of the bienzyme (Fig. 7). This NiO-MWCNT composite integrated microchip showed a better detection limit for cholesterol measurements than that of NiO-based biochips and achieved faster response times.

In addition to NiO-based nanocomposites, other innovative nanocomposites were also investigated for biosensor fabrication by combining the benefits of nanocomposites and microfluidics. Malhotra et al. introduced chitosan/anatase titanium dioxide nanoparticle (antTiO_2-CH) electrodes into PDMS microchannels for cholesterol sensing [101]. The active functional groups of nanoporous antTiO_2-CH provided appropriate sites for bienzyme (ChOx and ChEt) immobilization. In addition, nanostructured TiO_2 is a desirable transducer material for biosensing based on its short diffusion length, long-term chemical stability and large surface-to-volume ratio. Point defects (Ti^{3+}, Ti^{2+}, Ti^{0}, etc.) in antTiO_2 improved the heterogeneous electron transfer efficiency between the enzyme and the electrode, enhancing the electrochemical sensitivity. Moreover, to improve the enzyme immobilization capacity, a biopolymer CH was used to enhance the film formation ability and the dispersion of antTiO_2 nanoparticles at the electrode surface. A low detection limit of 0.011 mmol L^{-1} was achieved by applying this biochip for cholesterol evaluation. An HRP-modified electrode was inserted into a microfluidic channel to fabricate the biosensor [102]. In the described study, the sensing electrode was a gold wire coated with carboxylated GNPs, and HRP was immobilized onto glutathione-GNPs via a reaction between the carboxyl groups and HRP lysines. The highly sensitive amperometric detection of H_2O_2 was achieved in unmediated biocatalysis. This GNP-modified biosensing electrode could provide a common platform for fabricating various biosensors based on amidization reactions between enzymes and carboxylated GNPs.

We summarized the applications of NM-based enzymatic reactors for glucose determination in Section 3.1. In this section, NM-immobilized GOx for biosensor fabrication is discussed. An ion-sensitive conductometric glucose biosensor array was developed by self-assembling indium oxide nanoparticles (INPs) and silica nanoparticles on the multisite channel areas of resistors [103]. This design enabled statistical analysis with a single sample delivery shot. The obtained sensor array possessed a high sensitivity of 4–12 nA/mM for glucose determination. Since organic electrochemical transistors (OECTs) have attracted much attention based on their high sensitivity and noninvasive detection, OECTs containing GOx/lactate oxidase and poly(n-vinyl-2-pyrrolidone)-capped platinum nanoparticle (Pt NP)-modified gate electrodes were integrated into microfluidic channels [104]. The conventional OECT preparation technique involves complex steps requiring a voltage bias from a three-terminal potentiostat. Instead of the traditional electrodeposition method, a simple two-step dip-coating strategy was used to deposit Pt NPs onto the gate electrodes to enhance the device performance. Moreover, to achieve fast detection, the channel was directly designed on top of the OECT sensor. To accomplish the sensing of multiple analytes, two single sensors were integrated into two separate channels of one chip. The sensitive determination of glucose and lactate was attained with detection limits of 10^{-7} and 10^{-6} M, respectively. Furthermore, glucose levels in human saliva samples were tested using this microsensor, and the real-time detection of glucose was achieved by connecting this device to a smartphone via Bluetooth.

Metal-enhanced fluorescence (MEF) has been widely utilized in fluorescence detection applications based on the interactions of fluorophores with metallic nanoparticles. Silver nanostructures possess specific merits for MEF due to their intense surface plasmon resonance and simple preparation method. Silica-coated silver nanoparticles (Ag@SiO_2) were introduced into a GOx- and

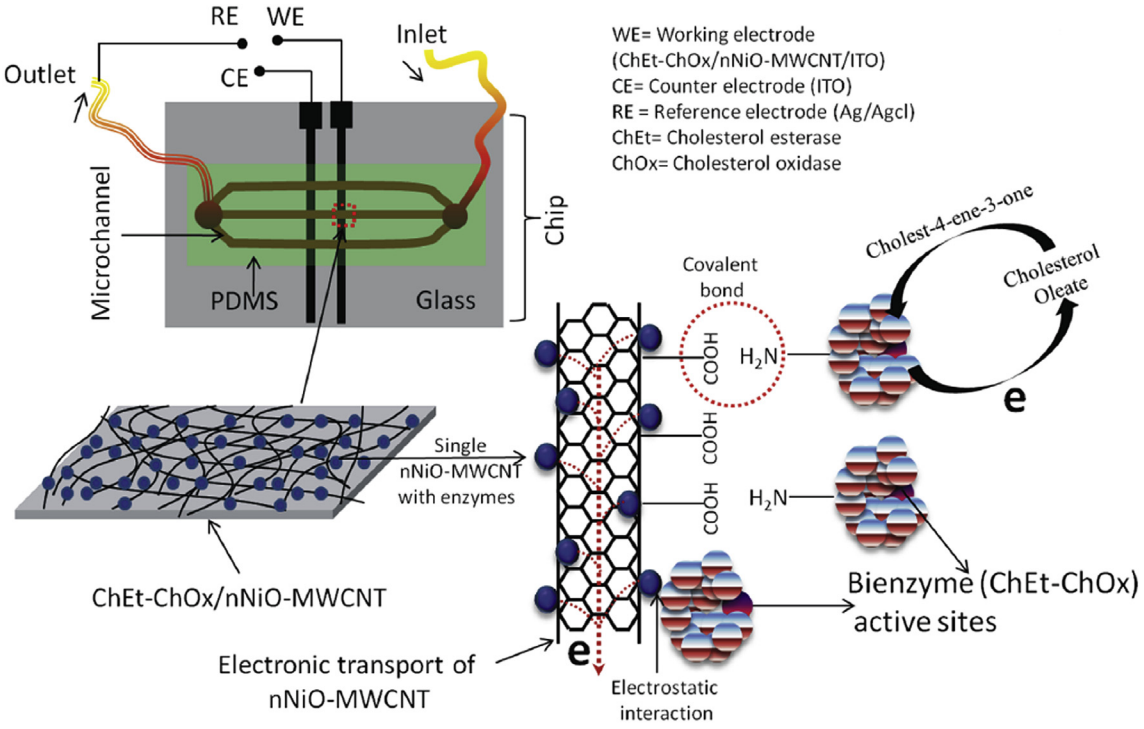

Fig. 7. Schematic of nickel oxide-multiwalled carbon nanotube-based enzymatic microsensors used for total cholesterol detection (Adapted from Ref. [100]).

peroxidase-entrapped hydrogel microarray to enhance the fluorescence signal [105]. The advantages of using silver cores for MEF effects were investigated by optimizing the Ag@SiO_2 amount and the thickness of the silica shells. In comparison with the properties of glucose sensors without Ag@SiO_2, the sensitivity was significantly improved by using this microarray system. Furthermore, the Ag@SiO_2-containing microarray was incorporated into a microfluidic device to develop an MEF-based micrototal analysis system (μ-TAS). The results indicated that the Ag@SiO_2 MEF-inducing hydrogel microarray provided a satisfactory platform for microsensing with high sensitivity.

A biocomposite of MNPs, iridium oxide nanoparticles (IrOx NPs) and tyrosinase (Tyr) was immobilized onto a screen-printed electrode (SPE) by applying a permanent magnet [106]. Since enzyme inhibition systems can be used for the biosensing of different analytes, a methimazole (MT) biosensor based on the inhibition of Tyr via copper chelation at the active site was fabricated with an integrated SPE. Due to the high conductivity of IrOx NPs, the biosensor presented improved analytical performance with a low LOD of 0.003 μM for MT detection. Compared with the batch mode, this lab-on-a-chip system exhibited prominent advantages, including a low sample consumption, fast response, and desirable reusability and automation.

3.2.2. Carbon nanomaterials

Carbon NMs are a promising candidate for electrochemical sensing owing to their desirable electron transfer rate, high surface area and electrical conductivity. CNTs have been widely utilized as sensors in a variety of biosensing applications [107–109].

To achieve multiple-analyte biosensing, bipolar electrode (BPE) arrays are considered a desirable choice, enabling concomitant determinations at different transducer sites. Since MWCNTs exhibit high carrier mobility, tensile strength and quantum electron transport ability for fabricating biosensors, a biocompatible MWCNTs/chitosan composite was used to immobilize different types of enzymes, including GOx, lactate oxidase and COD, via a simple physical mixing method [110]. The obtained enzyme/MWCNT/chitosan mixture was then coated onto one end of an indium tin oxide (ITO) electrode surface. Finally, a BPE array containing three ITO microband electrodes was integrated into a microfluidic chip. During the operation process, liquid droplets of glucose and luminol were added into the chip channels, and the electrochemiluminescence (ECL) reaction between the in situ-generated H_2O_2 and luminol occurred when an external voltage was applied to the two driving electrodes. This sensing array design avoided the cross-contamination of BPEs modified with different oxidases and integrated into the same microchannel, facilitating the multiplexed quantification of glucose, lactate and Ch by a combination of bipolar electrochemistry and ECL imaging. Combining the satisfactory electrotransfer merits of MWCNTs and the film-forming characteristics of chitosan, the fabricated microfluidic biochip provided a simple and efficient platform for the simultaneous detection of biomolecules. Satisfactory LODs of 7.57 μM, 43.19 μM and 8.25 μM were achieved for measuring glucose, Ch and lactate, respectively.

Cholesterol determination has become much more important as the rate of clinical disorders involving abnormal blood cholesterol levels has increased [111]. The integration of cholesterol sensors into microchips can achieve instant blood cholesterol determinations due to the continuous extraction and detection process occurring on the microchips. Developing electrochemical cholesterol sensors has attracted tremendous attention due to the specific advantages of such sensors. In one work, a CNT working electrode was integrated into a polydimethylsiloxane/glass microfluidic chip, and ChOx prepared in polyvinyl alcohol (PVA) solution was attached to hydrolyzed PVA-functionalized CNTs via an in-channel flow technique [112]. The high-throughput detection of cholesterol was achieved with more than 60 samples per hour. The results indicated that the functionalized CNT electrode presented satisfactory repeatability for

Table 4
Advantages of using microchip-based enzymatic biosensors.

Type of enzymes	Type of NMs	Immobilization strategies	Advantages	Ref.
Cholesterol esterase/cholesterol oxidase	Nickel oxide nanorods	Adsorption	Enhanced detection limits and faster response time for determining cholesterol	[99–101]
	Nickel oxide-multiwalled carbon nanotubes	Covalent binding		
	Titanium dioxide nanoparticles	Adsorption		
Cholesterol oxidase	Carbon nanotube	Entrapment	Improved sensitivity for detecting cholesterol	[112]
Horseradish peroxidase	Gold nanoparticles	Covalent binding	Improved sensitivity for the amperometric detection of H_2O_2	[102]
Glucose oxidase	Indium oxide nanoparticles	Adsorption	High sensitivity for glucose determination	[103,115]
	Graphene	Covalent binding		
Glucose oxidase/lactate oxidase	Platinum nanoparticles	Entrapment	Sensitive determination of glucose and lactate simultaneously	[104]
Glucose oxidase/peroxidase	Silica-coated silver nanoparticles	Entrapment	Improved sensitivity and limit of detection for glucose determination	[105]
Glucose oxidase, lactate oxidase, choline oxidase	Multiwalled carbon nanotubes	Adsorption	Sensitive measurement of glucose, choline and lactate	[110]
Tyrosinase	Iridium oxide nanoparticles	Adsorption	Lower LOD for methimazole detection	[106]

cholesterol measurements in a microflow injection microfluidic system.

Diabetes is a prevalent chronic disease, and glucometers provide powerful POC devices for home monitoring and clinical diagnosis [113,114]. A POC biosensor platform was fabricated for glucose measurement [115]. Compared with CNT-based sensors, graphene can avoid signal interference from metals and sensitively detect the surrounding microenvironment. Moreover, improved enzyme loading can be obtained because of the high biocompatibility of the graphitic metal-free edges. To combine the electrical properties of graphene and the selectivity of enzymes, GOx was modified onto a graphene surface. During the preparation process, a linker molecule (1-pyrenebutanoic acid succinimidyl ester) was used to functionalize graphene via π-π interactions. Then, the amino groups on GOx reacted with this linker to achieve enzyme immobilization. Furthermore, graphene-based field-effect transistors were integrated into a microfluidic device. For measurements, enzymatic reactions were transduced by direct electron transport.

NMs provide a promising alternative for fabricating different enzymatic biosensors. Different NM-enzyme immobilization strategies have been discussed in this section, and the results demonstrate that microchip-based biosensors present specific merits over traditional sensors (Table 4).

4. Conclusions and perspectives

We reviewed the application of various NMs in the fabrication of novel capillary-based and chip-based enzymatic microsystems. For the development of enzyme-immobilized monolithic capillaries, the studies have mainly focused on developing GNP- and carbon NM-functionalized polymeric monoliths. Moreover, silica nanoparticles have been investigated to prepare hybrid monoliths for enzyme immobilization. Since NM-functionalized monolithic capillaries display a high surface area and biocompatibility, the enzyme activity and immobilization capacity can be improved. The influences of NMs on the performance of monolithic capillary-based enzymatic microsystems have been discussed in detail. To prepare enzyme-immobilized OT capillaries, NMs have been introduced to overcome the shortcoming of OT capillaries with a low phase ratio. Because MNPs can be easily manipulated by using external permanent magnets, replaceable enzyme microreactors have been fabricated by preparing novel MNP-enzyme conjugates and then packing the conjugates into OT capillaries. We believe that more effort should be focused on investigating novel renewable enzymatic microreactors with enhanced stability and activity. GNP-functionalized bioreactors possess desirable stability owing to the strong affinity of GNPs for the thiols and amines present in enzymes. In addition, GO containing various functional groups is a satisfactory candidate to fabricate enzyme-immobilized OT capillaries with enhanced enzyme immobilization capacity. Enzyme-immobilized monolithic and OT capillaries exhibit specific advantages over IMERs based on microparticle-packed capillaries.

The variety of microchip-based enzymatic systems summarized in this review suggest that the application of NMs is suitable for improving the performance of bioreactors and biosensors. New enzyme-MNP conjugate-packed microchips have been described in detail, and the substantial advantages of magnetic nanocomposites in enzymatic microreactor fabrication are included in the discussion. Moreover, carbon NMs, silica nanoparticles and GNPs are desirable carriers for constructing microchip-based enzymatic reactors. Studies indicate that enzymes immobilized on microchips show higher operational stability and catalysis yields when compared with free enzymes. Furthermore, NMs, including MNPs, carbon NMs, and silica nanoparticles, have been incorporated into paper microfluidic devices to optimize the selectivity and sensitivity of μPADs. We believe that novel NMs need to be efficiently exploited to broaden the applications of enzyme-based μPADs for portable measurements. To develop biosensors, distinct enzyme-modified NMs have been integrated into microchips. Carbon and metal NMs with intrinsic electronic and optical characteristics facilitate the achievement of sensitive detection.

Different NMs utilized in fabricating enzymatic microsystems for bioreaction, biosensing, enantioseparation and inhibitor screening are reviewed (Fig. 8). The results suggest that NM-functionalized enzymatic reactors have received considerable

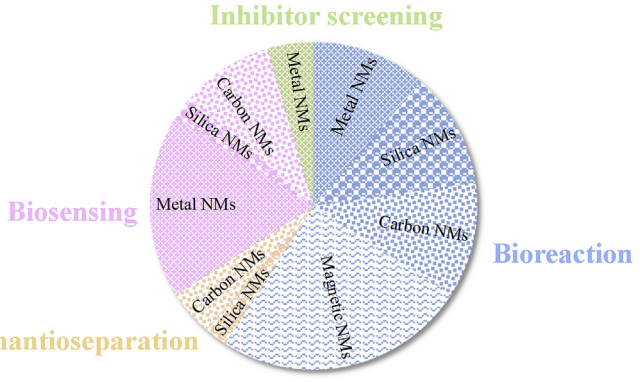

Fig. 8. Summary review of various nanomaterial-functionalized enzymatic microsystems applied for chemical and biological analysis.

research interest. We foresee the application of NM-functionalized monolithic capillaries in the development of multienzymatic microreactors. Although much attention has been paid to the development of enzymatic bioreactors, NM-functionalized enzymatic microsystems possess promising potential for use in chiral separation. Further studies can explore innovative NMs for the synthesis of enzyme-based chiral stationary phases and discuss the effects of NMs on chiral recognition. Moreover, microchip-based enzymatic systems exhibit promising potential in POC diagnostics. We believe that considerable efforts will continue to be made to develop innovative NM-functionalized enzymatic microsystems for use in the chemical and biological sciences.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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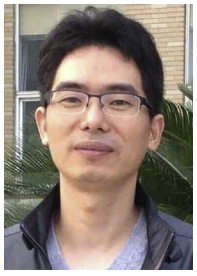
Tingting Hong obtained her Ph.D. degree from China Pharmaceutical University in 2017. In 2016–2017, she worked as a CSC joint-PhD student in Vrije Universiteit Brussel. She is currently a postdoc with a research mainly focus on preparation and evaluation of capillary monoliths for separation science in the group of Prof. Dr. Chuanpin Chen at Central South University. She has published several papers about capillary electrochromatography in Journal of Chromatography A, Analytica Chimica Acta, Analyst, Journal of Separation Science, Analytical Biochemistry, Electrophoresis and Trends in Analytical Chemistry.

Wenfang Liu obtained her master degree from Central South University in 2009. She is currently a teacher at Xiangya School of Pharmaceutical Sciences in Central South University. Her research interest mainly focuses on the preparation of microchips for pharmaceutical analysis and the applications of SERS in pharmaceutical analysis. Wenfang Liu has published several papers in Analytical Chemistry, Analytical Methods, Microsystem Technologies and so on.

Ming Li obtained his Ph.D. degree from Kyoungpook University of Analytical Chemistry in 2004. He is currently a professor at Yangzhou University. His research interest focuses on the development of microfluidic sensor devices for environmental analysis, with several publications in Analytical Chemistry, Lab on a Chip, Microchemical Journal, Biomicrofluidics and so on.



Chuanpin Chen obtained his Ph.D. degree from Pohang University of Science and Technology in 2007. In 2008–2011, he worked as a postdoc at CSIRO (Commonwealth Scientific and Industrial Research Organization) in Australia. Chuanpin Chen is currently a professor at Xiangya School of Pharmaceutical Sciences in Central South University. Professor Chen's research interests include the fabrication of microchips for pharmaceutical analysis, preparation of drug-loaded micro/nanoparticles, applications of SERS in pharmaceutical analysis, with several publications in Analytical Chemistry, Electrophoresis, Analytical Methods and so on.