Microchimica Acta (2020) 187:423
https://doi.org/10.1007/s00604-020-04390-9

REVIEW ARTICLE

Nanozymes in electrochemical affinity biosensing

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Received: 17 April 2020 / Accepted: 13 June 2020

Abstract

Over the past decade, artificial nanomaterials that exhibit properties similar to those of enzymes are gaining attraction in electrochemical biosensing as highly stable and low-cost alternatives to enzymes. This review article discusses the main features of the various nanomaterials (metal oxide, metal, and carbon-based materials) explored so far to mimic different kinds of enzymes. The unprecedented opportunities imparted by these functional nanomaterials or their nanohybrids, mostly providing peroxidase-like activity, in electrochemical affinity biosensing are critically discussed mainly in connection with their use as catalytic labels or electrode surface modifiers by highlighting representative strategies reported in the past 5 years with application in the food, environmental, and biomedical fields. Apart from outlining the pros and cons of nanomaterial-based enzyme mimetics arising from the impressive development they have experienced over the last few years, current challenges and future directions for achieving their widespread use and exploiting their full potential in the development of electrochemical biosensors are discussed.

Keywords Nanozymes · Mimicked enzyme activity · Electrochemical affinity biosensor · Electrode modifiers · Catalytic labels

Introduction

The high substrate specificity and their catalytic efficiency in biological reactions make enzymes have important applications in medicine, agriculture, chemical industry, and food processing [1]. However, intrinsic limitations (limited versatility and variety of substrates; high cost of preparation, isolation, and purification; low operational stabilities; limited tolerance to harsh environmental conditions (extreme pH, high temperature); protease digestion and solvents other than water; and difficulty in storage and large preparation time) have boosted the development of "artificial enzymes" [1–11]. Among them, nanomaterials with enzyme-like characteristics, called "nanozymes," have emerged as the next generation of enzyme mimics. These nanozymes have attracted continuous attention since the unexpected discovery of magnetic Fe_3O_4 nanoparticles (MNPs) with peroxidase-like activity in 2007. So far, different nanomaterials (metal oxides, metals, and carbon based) have shown to provide catalytic activities similar to those of the enzyme peroxidase, oxidase, catalase, superoxide dismutase, and laccase [7, 12, 13].

Nanozymes, combining the advantages of chemical catalysts and enzymes [8], outperform natural enzymes because they are usually synthetized using low-cost, simple, and mass-production methods and offer high operational stability and self-life, robust catalytic performance (displaying biocatalytic activity even under extreme pH, and/or temperature conditions, and also resisting non-water-based solvents and digestion from proteases) [7, 13–15]. Moreover, the smooth surface modification of nanomaterials provides more room for modifications than the natural enzymes. In addition, their inherent nanomaterial properties impart them both tunable and tenable catalytic activity [9, 16] and allow them to be designed on demand for the intended application [8]. The unique physicochemical properties of nanomaterials along with the significant advances made in the last years by biotechnology, nanotechnology, catalysis science, and computational design to imitate new enzymatic activities, regulate their activities, and elucidate the catalytic mechanisms open new options for the rational layout and applications of nanozymes [4]. Due to their tunable catalytic activities and multifunctionalities, nanozymes and, particularly, peroxidase-like ones have been widely explored as a reliable alternative to natural enzymes in the preparation of electrochemical affinity

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biosensors [3]. The lack of selectivity of nanozymes is compensated using specific bioreceptors (antibodies, peptides, aptamers, oligonucleotides). However, it is important to be aware of the available bio-ligand shortage for emerging analytes and bear in mind that their use compromises both the high stability and the low cost of nanozymes [3].

Affinity ligand-based electrochemical biosensors using nanozymes have been successfully developed and mostly exploited in the clinics seeking to overcome the challenging analytical demands in this field. Nanozyme-based electrochemical affinity biosensors exhibit some excellent merits imparted by the nanozymes such as higher selectivity and sensitivity, lower cost, shorter detection time, and better signal readout [6].

Given this background as well as the recent published overview by Shiddiky's group on nanozyme-based electrochemical biosensors for the determination of disease biomarkers [17], we herein concisely overview recent advances and opportunities offered by nanozymes in electrochemical affinity biosensing. The period since 2015 to present considering applications in food safety, environment, or clinical areas has been reviewed with the aim to provide the reader with a global perspective on the analytical relevance of this research area. Main challenges and future research directions in this field are also outlined.

Nanozymes: fundamentals, types, and mimicked enzyme activity

It is believed that the inherent enzyme-like activity of nanozymes is caused by atoms present both on the surface and in the nanozyme's inside core. Indeed, although factors including size, morphology, surface coating and modification, pH, and temperature can also influence the nanozymes' catalytic activity, the key factor is their atomic composition. Nanozymes can be classified regarding their composition into metal oxide-, metal-, and carbon-based ones [2]. However, to date, a wide variety of nanostructured materials has proven enzyme-like intrinsic activity. Table 1 classifies the nanozymes reported so far regarding the exhibited enzymatic-like activity.

The nanozymes discovered up to now can be broadly classified considering their functions as either scavenging or generating free radicals during the catalytic reaction, respectively, into antioxidants and pro-oxidants. The antioxidant nanozymes include those mimicking superoxide dismutase (SOD) and catalase activity, and the pro-oxidant nanozymes involve peroxidase and oxidase mimetic nanozymes [7]. Moreover, fullerenes, graphene oxides, carbon nanotubes (CNTs), AuNPs modified with catalytic monolayers, and Zr- and Cu-based MOFs mimic hydrolase activity, catalyzing a chemical bond hydrolysis [4]. Cu-based MOFs have demonstrated also laccase-like activity [12].

As it can be seen in Table 1, some nanomaterials (e.g., Pt and CeO_2), called multi-enzyme-mimicking nanozymes, can emulate two or more types of enzymes, which make them more efficient in particular applications. For example, SOD-, catalase-, peroxidase-, and oxidase-like activities of CeO_2 were found depending on the microenvironment and/or pH [4]. PtNP-apoferritin catalase and peroxidase enzyme mimic behavior is dependent on pH and temperature, while AuNPs mimic oxidase and show also peroxidase-like activity with surface charge [23]. Furthermore, it is worth noting that, although out of the scope of this review, the innate magnetic and optical properties of some nanomaterials (e.g., Fe_3O_4 and Au) endow certain nanozymes with multiple functionalities (multifunctional nanozymes) ensuring their separation in an easy way and application in other types of biosensing such as the optical one [4].

Bimetallic nanoparticles have been exploited to achieve single (Bi-AuNPs, Ag heterogeneous nanorods and Ag metallic or bimetallic, Au, Pd and Pt, nanostructures with peroxidase-like activity) or multiple (Au-Pt nanorods) enzyme-mimicking capabilities [23]. In addition, nanocomposites combining different types of nanozymes (Fe_3O_4-graphene oxide (GO), Fe_3O_4-Pt, Au-Pt, and GO-Fe_3O_4-Pt), showing synergistic effects and a significantly enhanced catalytic performance, have been studied intensely [2]. Moreover, it is well documented that the properties of nanoparticles and the intrinsic activity of nanozymes can be refined by modifying their synthetic and surface modification methods thus altering their size, shape, and composition [4, 7]. These strategies have been exploited to create materials with distinct properties or nanozymatic activities. For example, depending on the $Ce^{+3/+4}$ ratio, CeNPs have demonstrated SOD- and catalase-like activities, but once coated with dextran, they displayed oxidase-like activity [7].

It is worth noting that despite the important advantages provided by these nanomaterials over natural enzymes, nanozymes do not have the selectivity required in bioaffinity assays. This is due to the lack of a substrate-specific active site as there is in enzymes. The strategies reported to impart nanozymes with target molecules specificity comprise the oxidase-coupled and the surface modification methods. In the first one, peroxidase-like activity nanozymes are coupled with oxidases (producing H_2O_2 only in the presence of specific substrates) to gain selectivity [41]. Regarding the surface modification method, the appropriate bioreceptors (antibodies, aptamers, and oligonucleotides probes) are attached to the nanozyme surface to bestow selectivity toward the target molecule [2]. In order to improve the selectivity, molecularly imprinted polymers (MIPs) have also been grown on the nanozymes. However, as commented above, while the use of bio-

Table 1 Classification of nanozymes regarding their enzymatic like activity

Enzymatic-like activity	Nanozymes
Peroxidase-like nanozymes: catalyze substrate oxidation by a peroxide, such as H_2O_2	- Iron oxide nanomaterials: Fe_3O_4 MNPs [1, 2, 7, 8, 18–21]
	- Iron chalcogenides (e.g., FeS, Fe_3S_4, FeSe, and FeTe), iron phosphates, and Prussian blue (PB) NPs and their structural analogues (e.g., CuFe, FeCoFe, FeCo, $Cu_{1.33}[Fe(CN)_6]_{0.667}$, $Fe[Co_{0.2}Fe_{0.8}(CN)_6]$, $FeCo_{0.67}(CN)_4$, $Fe_{1.33}Fe(CN)_6$) [4, 7, 18, 22]
	- Metal oxides: V_2O_5 nanowires [4, 7, 23], Co_3O_4 nanoparticles [23], CeO_2 NPs [2, 4, 24], MnO_2 nanomaterials [23], and AuNPs [23, 25–28]
	- Metal nanomaterials: Au, Ag, Pt, Pd, Cu, and their multimetallic NPs [2, 4, 18, 23, 29–35]
	- Carbon nanomaterials: single-walled carbon nanotubes, graphene oxide (GO), dipeptide-polyoxometalates (POMs)-graphene oxide (GO) ternary hybrids [36], carbon nanodots (C-dots) or carbon quantum dots (CQDs), graphene quantum dots (GQDs) [37–39], doped C-dots/GQDs, Fe/N-doped carbon, carbon nitride dots, and C-dots/GQDs nanocomposites [2, 4, 23, 40]
	- Metal-organic frameworks (MOFs): Cu-MOF [41, 42], MOF-based multicomponent nanozymes [43–46]
	- Others: $Cu(OH)_2$ supercages [47], graphene-like 2D-layered transition-metal dichalcogenides (MoS_2 and WS_2 nanosheets) [4], nanostructured layered double hydroxide (LDH), CuS superstructures [7], and PtNP-apoferritin [23]
Oxidase-like nanozymes: catalyze substrate oxidation with the assistance of molecular oxygen (O_2) producing H_2O_2 and, in some cases, superoxide radicals [7]	- AuNPs, other supported gold (e.g., Au/Al_2O_3, Au/TiO_2, and Au/ZrO_2), and Au-containing bimetallic/trimetallic NPs [4, 23]
	- TiO_2 NPs [48]
	- MnO_2, CeO_2, and Cu, Mo, Pd- [33] and Pt-based NPs [2, 4]
	- Fe_2O_3 and MnO_2 nanowires [7, 8, 23]
Superoxide dismutase (SOD) activity: eliminate superoxide anion $O_2^{\bullet-}$ through its dismutation reaction to H_2O_2 and O_2	- Fullerene and its derivatives, the hydrophilic carbon cluster (HCC), poly(ethylene glycol) (PEG)-HCC, PEGylated perylene diimides, carbon nitride nanosheets, and nitrogen-doped porous carbon nanospheres, CeO_2 and melanin (Me)NPs [1, 2, 4], AuNPs, and PtNPs [2]
Catalase activity: efficiently decompose H_2O_2 into H_2O and O_2	- Metals (AuNPs, PtNPs [2, 23]), metal oxides (CeO_2, iron oxides, and Co_3O_4 NPs and MnO_2 nanomaterials [7, 23]), PB [1, 4], and PtNP-apoferritin [23]
Laccase activity: catalyze the oxidation of various aromatic compounds (predominantly phenols) and the reduction of O_2 to form H_2O	- MOF based on guanosine monophosphate (GMP)-coordinated copper [12]

ligands compromises the high stability and low cost of nanozymes, MIPs may either not be applicable for certain delicate proteins owing to the polymerization conditions, or not be suitable for multiplexed detection as each nanozyme must be imprinted for the target analyte [3].

Generally, peroxidase nanozymes have been most widely employed in electrochemical affinity biosensing, owing to their convenience for electrochemical detection of H_2O_2 generated during the oxidation of certain substrates [2]. The use of these artificial enzymes lowers the cost of the resulting biosensors and overcomes errors in the sensing processes derived from some critical problems occurring in horseradish peroxidase (HRP)-based devices such as the degradation of the HRP catalytic activity during long-term storage and/or in harsh conditions [2].

Main characteristics and relevant applications of nanozymes have been discussed in some excellent reviews (only the most recent ones are quoted as examples, [1, 4, 7, 11]). Some of them have focused on MOF-based nanozymes [49], nanozyme-based biosensing using aptamers [15] or immunosensors and immunoassays [9] with both optical and electrochemical

detection modes and their application in particular fields such as the detection of disease biomarkers [17] and agrifood industry [50, 51]. So far, nanozymes have been used in food quality and safety mainly integrated in colorimetric- and strip-based biosensing approaches. However, as far as we know, there is not any review article focusing specifically on the coupling of nanozymes with all types of electrochemical affinity biosensing for clinical diagnosis, food analysis, or environment monitoring. Therefore, to highlight this interesting field and help the researchers to better understand the unique opportunities imparted by nanozymes in electrochemical affinity biosensing, this review discusses in depth the progress made in nanozyme-based electrochemical biosensors with a broad application focus. Highlighted approaches are categorized according to the type of affinity bioassay, kind and role played by the nanozyme, and the target analyte. Main challenges to overcome and research trends in exploiting nanozymes to design and develop efficient electrochemical affinity biosensors and in their practical applications' promotion are also commented, with the desire to raise more efforts which advance and catalyze breakthroughs in this highly promising and relatively new field with the potential to impact on many fields of relevance in our society.

Nanozyme-based electrochemical affinity biosensors

Electrochemical affinity biosensors are based on registering changes in electrochemical readouts before and after specific affinity reactions. Since they combine the excellent selectivity and efficiency inherent to affinity reactions with the intrinsic advantages of electrochemical detection to perform multiplexed determinations even in turbid samples and in decentralized environments, they are getting increasing attention to meet the challenging sensing performance that the society demands. Moreover, growing requirements in sensitivity and selectivity have encouraged the development of electrochemical affinity biosensors in combination with ingenious amplification strategies where nanomaterials play a leading role [9].

Nanozymes, being a special type of nanomaterial, can be exploited in electrochemical affinity biosensing as electrode modifiers, nanocarriers, and/or catalytic labels.

By employing a nanozyme replacing a natural enzyme (Fig. 1), the resulting nanozyme-based affinity biosensor is foreseeable to have less cost and exhibit higher stability while keeping or improving the intrinsic features of the enzyme-based biosensor (high sensitivity, selectivity and throughput, etc.) [9]. So, the last few years have witnessed the fast growth of electrochemical affinity biosensors involving the use of nanozymes, most of them with peroxidase-like activity and used mainly as catalytic labels. These strategies have brought significant improvements in terms of analytical performance characteristics and practical usefulness. The main features of representative electrochemical affinity biosensors involving the use of peroxidase-like activity nanozymes reported since 2015 are summarized in Table 2. These are critically highlighted and discussed in the following sections.

Table 2 shows that recently developed electrochemical affinity biosensors use nanozymes primarily as catalytic labels and less as electrode modifiers. Also, their use has extended more to the development of immunosensors than of aptasensors or nucleic acid sensors. It should also be noted that the complexity of nanomaterials is related to the role played by the nanozyme in the biosensor. Thus, while single nanomaterials have been mostly exploited as electrode modifiers, catalytic labels include bimetallic nanoparticles, nanocomposites, decorated MOFs, or multifunctional

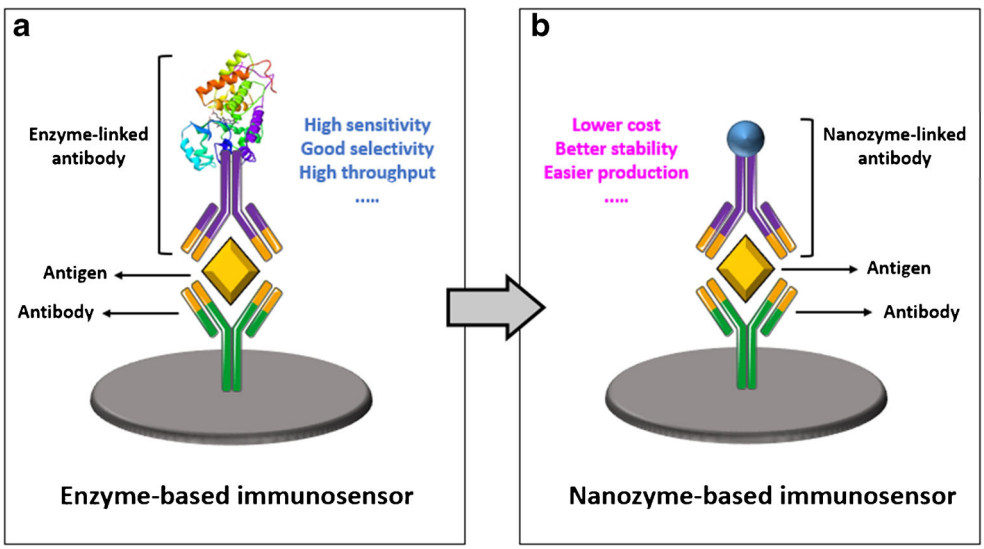


Fig. 1 Comparison of an electrochemical affinity biosensor using a natural enzyme (**a**) or a nanozyme (**b**) as catalytic label. Figure drawn based on [9]

Table 2 Affinity ligand-based electrochemical biosensors reported since 2015 exploiting mostly peroxidase-like nanozymes

Electrode	Nanozyme/function	Affinity biosensing strategy	Detection technique	Target analyte	LR/LOD	Sample	Ref.
SPCE	$(Fe-P)_n$-MOF/ nanocarriers and catalytic labels	Catalytic cleavage of the DNA (GR-5) functionalized $(Fe-P)_n$-MOF in the presence of Pb^{2+} and direct hybridization with a hairpin capture probe immobilized at a SPCE	Chronoamperometry (H_2O_2/TMB)	Pb^{2+}	0.05–200 nM/0.034 nM	Spiked soil samples	[45]
GCE	Strep-FeTCPP@ MOF/catalytic labels	The hairpin DNA attached to the GCE allosteric switched to form a streptavidin aptamer in the target DNA presence and the FeTCPP@MOF-Strep composites bind to the electrode surface	DPV (o-PD)	Target DNA	10 fM–10 nM/0.48 fM	Spiked 10% human serum samples	[46]
AuNPs@N-G-GCE	PdNPs@Fe-MOFs/ nanocarriers + redox probes + electrocatalysts	CHA-based DNA approach	Chronoamperometry (TMB + H_2O_2)	microRNA-122	0.01 fM–10 pM/0.003 fM	Human serum	[43]
Biochip with gold deposited by e-beam	Strep-Fe_3O_4/catalytic labels	Immobilization of biotinylated amplicons generated by solid-phase RPA at thiolated target-specific forward primer-modified biochip and labeling with Strep-Fe_3O_4	Chronoamperometry (TMB + H_2O_2)	Four PC gene targets (*TMPRSS2-ERG* gene fusion, *PCA3*, *SChLAP1*, and *KLK2*)	—/50 copies	PC patient liquid biopsies (urine and serum)	[20]
GCE	β-CD@AuNPs-MWCNT-s/electrode modifiers	ssDNA L2 products dually labeled with MB and Azo at the ends (MB-L2-Azo) produced through aptamer-based target cycling amplification of 8-OHdG were captured on β-CD@AuNPs-MWCNTs-modified GCE	DPV (glucose)	8-OHdG	0.1 pM–10 nM/30 fM	–	[52]
AuE	AuNPs/electrode modifier	Direct aptameric approach	DPV (Thi + H_2O_2)	Kanamycin	0.1–60 nM/0.06 nM	Honey samples	[28]
GCE	rGO/MoS_2 + aptamer-Fe_3O_4NPs/ electrode modifier	Direct aptameric approach	DPV (H_2O_2)	CTCs (MCF-7)	15–45 cells mL^{-1}/ 6 cells mL^{-1}	Spiked human serum samples	[21]
SPCE	AuNPs/electrode modifier	Direct aptameric approach	Chronoamperometry (TMB + H_2O_2)	*Pseudomonas aeruginosa*	60.0–6.0 × 10^7 cfu mL^{-1}/60 cfu mL^{-1}	Water	[25]
SPCE	PtPdNPs/catalytic labels	CHA-based aptameric approach	CV (TMB + H_2O_2)	MUC-1	100 fg mL^{-1}–1 ng mL^{-1}/16 fg mL^{-1}	Human serum	[29]
AuE	Mn_3O_4 nanoflowers + Pd@Pt	NTH-based sandwich aptameric approach	DPV (HQ + H_2O_2)	HER2		Human serum	[30]

Table 2 (continued)

Electrode	Nanozyme/function	Affinity biosensing strategy	Detection technique	Target analyte	LR/LOD	Sample	Ref.
	nanoflowers/-nanocarriers and catalytic labels				0.1–100.0 ng m-L^{-1}/0.08 ng mL^{-1}		
SPGE	Fe_3O_4/PDDA/Au@Pt/ nanocarriers and catalytic labels	NTH-based sandwich aptameric approach	DPV (HQ + H_2O_2)	cTnI	0.01–100 ng mL^{-1}/ 7.5 pg mL^{-1}	Human serum	[53]
GCE	PtNPs/CoTPP/rGO/ nanocarriers and catalytic labels	Indirect competitive immunosensing approach at a GCE modified with AFB_1-BSA	DPV (TMB + H_2O_2)	AFB_1	0.005–5.0 ng m-L^{-1}/5.0 pg mL^{-1}	Naturally contaminated or spiked blank peanut samples	[54]
GCE	Au@Pd NRs at $CoFe_2O_4$/rGO/ electrode modifier	Direct immunosensing approach	Amperometry (H_2O_2)	Estradiol	0.01–18.0 ng m-L^{-1}/3.3 pg mL^{-1}	Spiked rived water	[34]
GCE	Pd/APTES-M-CeO_2-GS/nanocarriers and catalytic labels	Sandwich immunosensing approach	Amperometry (H_2O_2)	AFP	0.1 pg mL^{-1}–50 ng mL^{-1}/0.033 pg mL^{-1}	Spiked human serum samples (100-times diluted)	[24]
SPCE	rGO-NR-Au@Pt/ nanocarriers and catalytic labels	Sandwich immunosensing approach using Fe_3O_4@SiO_2 MNPs for immunocapturing	CV (Thi/H_2O_2)	*Escherichia coli* O157:H7	4.0×10^2 to 4.0×10^8 cfu m-L^{-1}/91 cfu mL^{-1}	Spiked pork and milk samples	[35]
SPGE	MIO/catalytic labels	Direct immunosensing approach	Chronoamperometry (TMB + H_2O_2)	5-mC (global level)	10% difference in global DNA methylation level	Colorectal cell lines (gDNA)	[19]
SPCE	MWCNTs/GQDs/ nanocarriers and catalytic labels	Sandwich immunosensing approach	Amperometry (HQ + H_2O_2)	IL-13Rα2	2.7–100 ng m-L^{-1}/0.8 ng mL^{-1}	Cell lysates and extracts of paraffin-embedded tissues from CRC patients	[37]
SPdCE	MWCNTs/GQDs/ nanocarriers and catalytic labels	Sandwich immunosensing approach	Amperometry (HQ + H_2O_2)	IL-13Rα2 + CDH-17	4.92–100 ng mL^{-1} (IL-13Rα2) and 0.11–10 ng mL^{-1} (CDH-17)/1.4 (IL-13sRα2) and 0.03 ng mL^{-1} (CDH-17)	Cells (both intact and lysed) and paraffin-embedded tissues from CRC patients (protein extracts)	[38]
GO-SPCE	CoZnFeONPs/ catalytic labels	Sandwich immunosensing approach	CV (H_2O_2)	CYFRA 21-1	3.9–1000 fg m-L^{-1}/0.19 fg mL^{-1}	Human serum	[31]
AuE	GQDs/electrode modifier	Direct immunosensing approach	Chronoamperometry (H_2O_2)	*Yersinia enterocolitica*	6.23×10^2–6.23×10^8 cfu mL^{-1}/ 5 (milk) and 30 (serum) cfu mL^{-1}	Milk and human serum	[39]
GCE	FeS_2-AuNPs/nanocarriers and catalytic labels	Sandwich immunosensing approach	DPV (TMB + H_2O_2)	AFP	0.0001–100 ng m-L^{-1}/0.028 pg mL^{-1}	Human serum	[22]
GCE	AuPd-PDA/catalytic labels			APOE4		Spiked goat serum	[32]

Table 2 (continued)

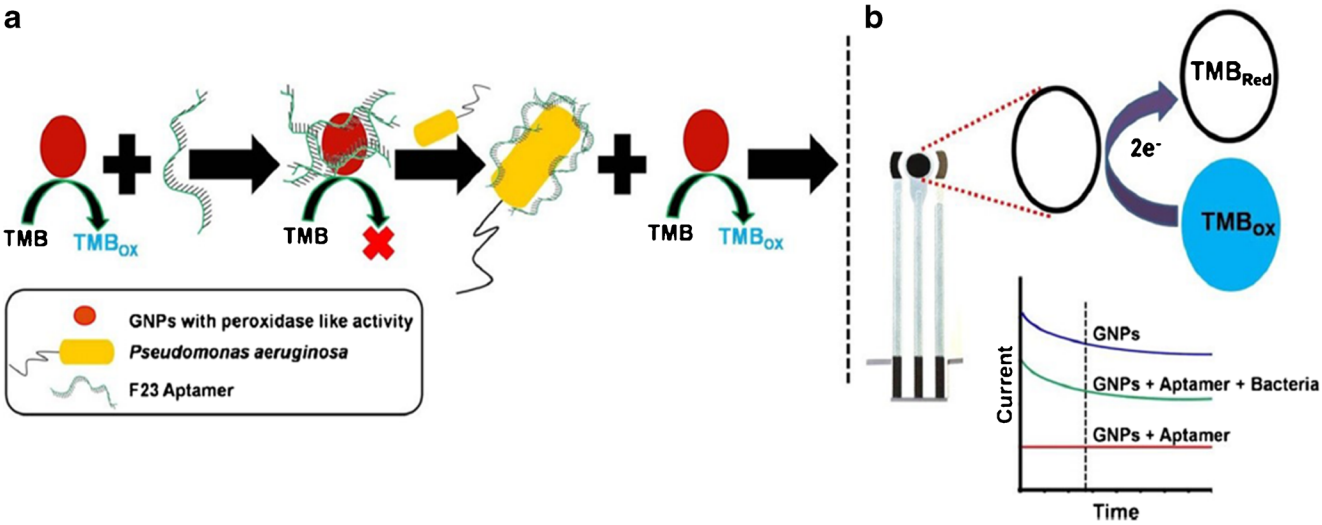
Electrode	Nanozyme/function	Affinity biosensing strategy	Detection technique	Target analyte	LR/LOD	Sample	Ref.
		Sandwich immunosensing approach	Amperometry (H_2O_2)		0.05–2000 ng mL^{-1}/15.4 pg mL^{-1}		
GCE	Au/Fe-MOF/catalytic labels	Sandwich immunosensing approach	SWV (H_2O_2)	PSA	0.001–100 ng mL^{-1}/ 0.13 pg mL^{-1}	Human serum	[44]

AFB1 aflatoxin B1, *AFP* α-fetoprotein, *APOE4* apolipoprotein E4, *AuE* gold electrode, *AuNPs@N-G* AuNPs-functionalized nitrogen-doped graphene sheets, *Au@Pt NPs* gold-platinum core/shell nanoparticles, *Azo* azobenzene, *β-CD@AuNPs-MWCNTs* β-cyclodextrin-functionalized AuNPs/multi-walled carbon nanotubes, *BSA* bovine serum albumin, *CDH-17* cadherin-17, *cfu* colony forming unit, *CHA* catalytic hairpin assembly, *CRC* colorectal cancer, *CTCs* circulating tumor cells, *CV* cyclic voltammetry, *DPV* differential pulse voltammetry, Fe_3O_4*@*SiO_2*NPs* silica-coated magnetite nanoparticles, *FeTCPP* iron(III) meso-5,10,15,20-tetrakis(4-carboxyphenyl) porphyrin chloride, *GCE* glassy carbon electrode, *gDNA* genomic DNA, *GO* graphene oxide, *GQDs* graphene quantum dots, *HER2* human epidermal growth factor receptor 2, *HQ* hydroquinone, *IL-13Rα2* interleukin-13 receptor α2, *LOD* limit of detection, *LR* linear range, *MB* methylene blue, *MIO* mesoporous iron oxides, *MNPs* magnetic nanoparticles, *MOF* metal-organic framework, *MUC-1* mucin 1, *MWCNTs* multi-walled carbon nanotubes, *NRs* nanorods, *NTH* DNA nanotetrahedron, *8-OHdG* 8-hydroxy-2′-deoxyguanosine, *o-PD* o-phenylenediamine, *PC* prostate cancer, *PDA* polydopamine, *Pd/APTES-M-*CeO_2*-GS* Pd octahedral nanoparticles supported on graphene oxide and CeO_2 mesoporous nanocomposite functionalized by 3-aminopropyltriethoxysilane, *PDDA* poly(diallyldimethylammoniumchloride), *PSA* prostate-specific antigen, *PtNPs/CoTPP/rGO* 5,10,15,20-tetraphenyl-21H,23H-porphine cobalt flat stacking on the reduced graphene oxide with platinum nanoparticles, *rGO* reduced graphene oxide, *rGO-NR* rGO functionalized with neutral red, *RPA* recombinase polymerase amplification, *SPCE* screen-printed carbon electrode, *SPdCE* screen-printed dual carbon electrode, *SPGE* screen-printed gold electrode, *Strep* streptavidin, *SWV* square wave voltammetry, *Thi* thionine, *TMB* 3,3′,5,5′-tetramethylbenzidine

nanozymes. To further improve the performance of the resulting biosensors, these nanozymes have been used in aptameric formats in combination with other enzyme-free amplification strategies such as those involving catalytic hairpin assembly (CHA), recombinase polymerase amplification (RPA), and tetrahedral DNA nanostructures (NTHs). Most of the recently described nanozyme-based biosensors have been applied for targeting analytes of relevance in prevalent and high mortality diseases (cancer and cardiovascular and neurodegenerative diseases) at genetic (target genes, methylation events, and oxidative DNA damage), regulatory (miRNAs), functional (protein biomarkers), and cell (circulating tumor cells, CTCs) levels. However, interesting applications have been reported also for targeting analytes of relevance in food quality control and/or environmental monitoring: Pb^{2+}, AFB_1, estradiol, kanamycin, *Pseudomonas aeruginosa*, *Escherichia coli* O157:H7, and *Yersinia enterocolitica.*

The reported biosensors provide good sensitivity and are compatible with the applicability in clinical practice, with limits of detection (LODs) between fg mL^{-1} and ng mL^{-1} for the detection of protein biomarkers, less than 100 cfu mL^{-1} and 10 cfu mL^{-1} for bacteria and cancer cells, respectively, and at the fM-aM level for the determination of target DNAs and miRNAs. They have been applied to the analysis of the target analytes in a wide variety of matrices. Clinical samples include spiked and unspiked liquid biopsies (serum and urine), cancer cells (whole, lysate, and extracted genomic DNA), and paraffin tissues (protein extracts) of patients diagnosed with cancer. Moreover, food such as peanut, pork, milk, and honey and environmental samples such as water and soils have been analyzed.

Focusing on the most relevant characteristics of the representative biosensors summarized in Table 2, the simple label-free strategies proposed for the determination of antibiotics or bacteria using nanozymes as electrode modifiers deserve to be remarked. The strategies developed by Wang et al. [28] and Das et al. [25] for kanamycin and *P. aeruginosa* determination, respectively, merge the artificial peroxidase activity of AuNPs with the selectivity of aptamers. These are off-on strategies in which the peroxidase activity of AuNPs inhibited after adsorption of a specific aptamer on their surface is recovered in the presence of the target bacteria by displacement of the aptamer from their surface (Fig. 2). The peroxidase activity was monitored by DPV or chronoamperometry using thionine (Thi) or 3,3′,5,5′-tetramethylbenzidine (TMB) as redox mediators with H_2O_2. The methods were able to detect selectively 0.73 nM kanamycin in 5-times diluted spiked acacia honey or 60 colony-forming units (cfus) mL^{-1} of *P. aeruginosa* in water in only 10 min. A similar strategy but using graphene quantum dots (GQDs) and an antibody as recognition element was recently proposed for the determination of *Yersinia enterocolitica* in milk and human serum [39]. In this case, a selective antibody



CaptionNumber>Fig. 2 Electrochemical strategy proposed for the determination of *P. aeruginosa* based on the quenching of the intrinsic HRP activity of AuNPs with an adsorbed specific aptamer and its retrieval in the presence of the target bacteria (**a**). Chronoamperometric off-on system monitorization with TMB/H_2O_2 (**b**). Reprinted from [25] with permission

is covalently immobilized through carbodiimide/succinimide chemistry on a gold electrode (AuE) laminated with GQDs. The chronoamperometric response, attributed to the reduction of H_2O_2 by the intrinsic (HRP) activity of the GQDs, decreased after the bacteria immunorecognition. This immunosensor allowed performing the selective and sensitive determination in 10 min, showing a wide linear concentration range and LOD values as low as 5 and 30 cfus mL^{-1} in milk and serum (both 50% diluted), respectively.

Other attractive strategies have been implemented by exploiting the use of single (Fe_3O_4NPs [21]) and multicomponent nanozymes (Au@Pd NRs [34] and β-CD@AuNPs-MWCNTs [52]) as electrode modifiers. The methods were developed in connection with specific antibodies or aptamers and were used in the environmental and clinical field for determining estradiol in spiked river water [34] or circulating tumor cells (CTCs) in spiked human serum (Fig. 3a) [21]. An ingenious strategy is that reported recently by Kou et al. [52] for the determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG). Rapid and non-invasive regeneration of high-efficiency cascade amplification of bifunctional nanozymes (β-CD@AuNPs, which exhibit GOx- and HRP-like activity simultaneously) used as GCE modifiers was performed by means of photoswitchable host-guest recognition in

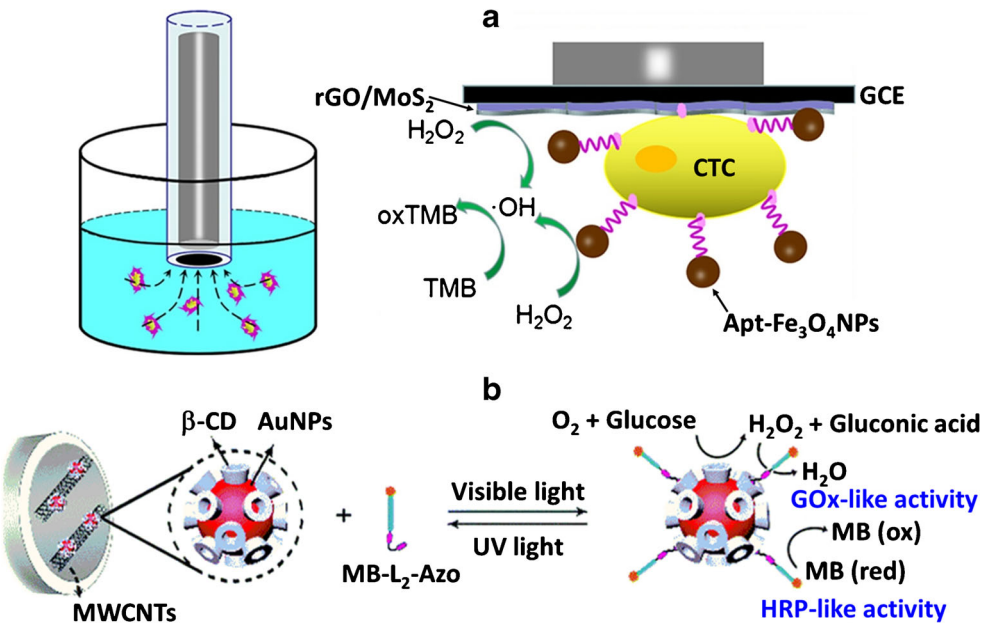


Fig. 3 **a** Direct aptasensing method for the determination of CTCs at a rGO/MoS_2-modified GCE using aptamer-modified Fe_3O_4NPs; **b** modification of a GCE with β-CD@AuNPs for electrochemical analysis of 8-OHdG involving reusable nanozyme cascade amplification. Reprinted and adapted from **a** [21] and **b** [52] with permission

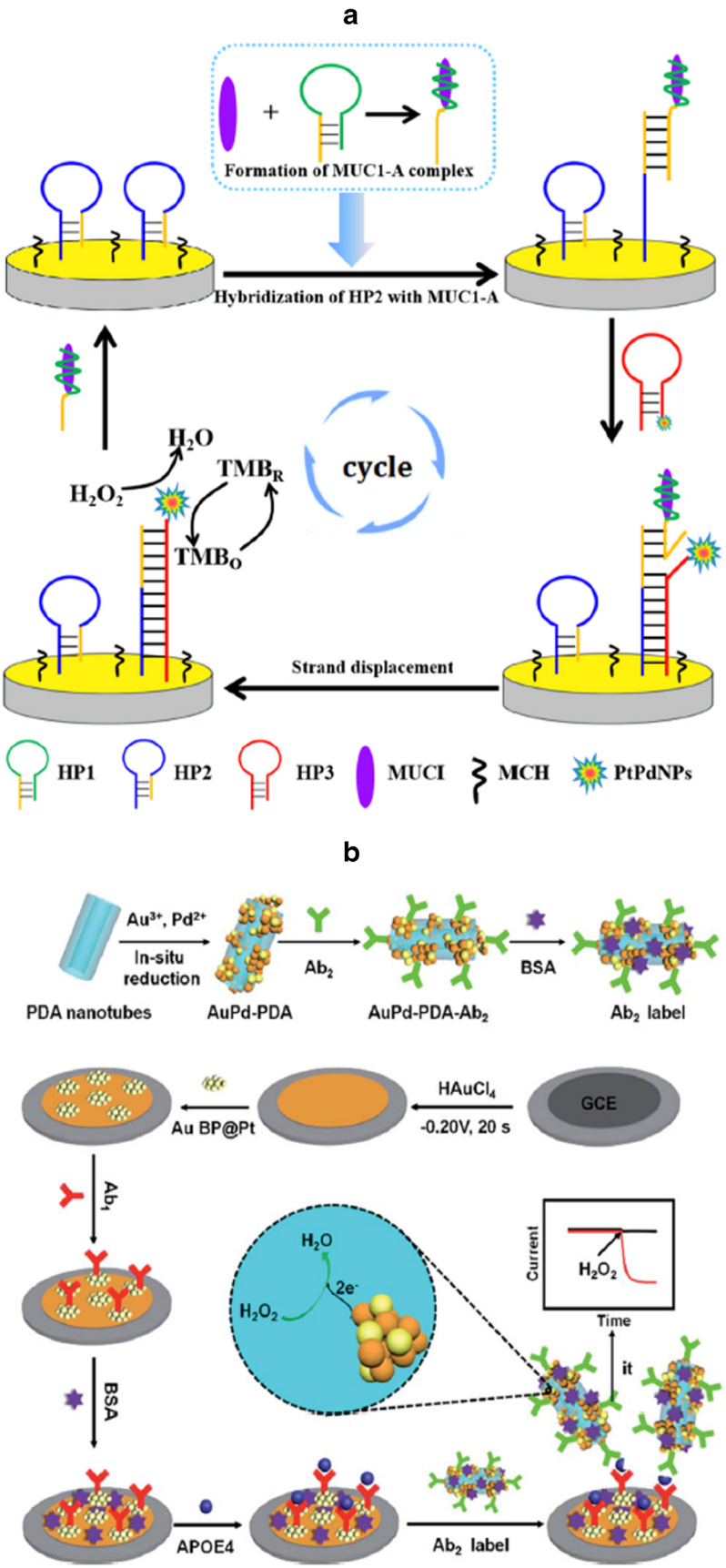


Fig. 4 Schematic display of an aptasensor (**a**) and an immunosensor (**b**) involving the use of nanozymes as catalytic labels. **a** Electrochemical aptasensor for MUC-1 detection based on CHA, strand displacement, and PtPdNP peroxidase-like activity. **b** Sandwich electrochemical immunosensor using gold nanobipyramid-coated Pt (AuBP@Pt) nanostructures as electrode modifiers and AuPd-PDA nanozyme catalytic labels for APOE4 determination. Reprinted and adapted from **a**, **b** [29, 32] with permission

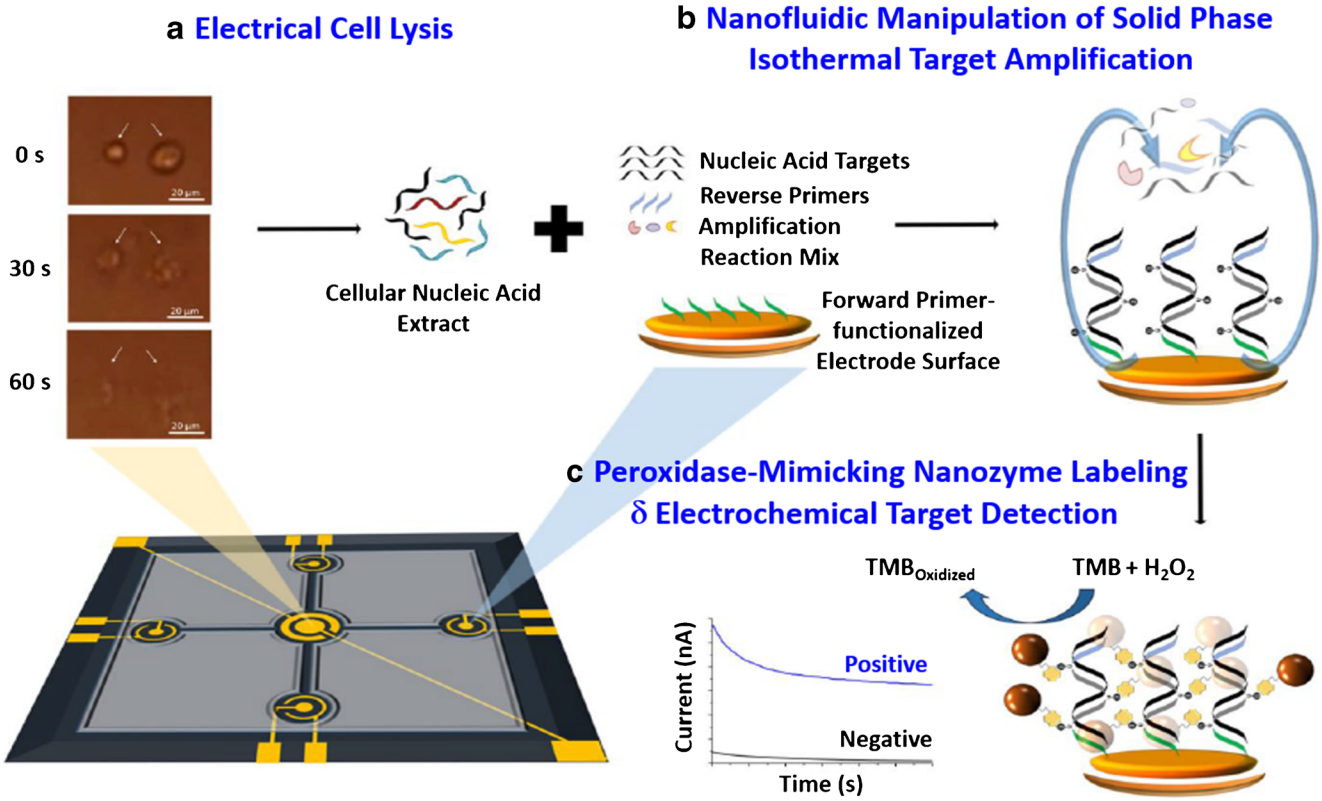


Fig. 5 Integrated biochip involving on-chip electrical cell lysis (**a**), a solid-phase RPA strategy (**b**), and the use of Strep-Fe_3O_4 MNPs as catalytic labels for electrochemical biosensing of four prostate cancer-related genes (**c**). Reprinted from [20] with permission

connection with an aptamer-based target cycling amplification strategy. The method generated ssDNA L2 products dually labeled with MB and Azo at the target 8-OHdG ends (MB-L2-Azo) (Fig. 3b).

As shown in Table 2, PtPdNPs [29], mesoporous iron oxides (MIO) [19], CoZnFeONPs [31], and AuPd alloy-modified polydopamine (AuPd-PDA) [32] nanomaterials have been used as catalytic labels in aptameric (Fig. 4a) and sandwich immunosensing (Fig. 4b) methods. They were applied to the determination of MUC-1, 5-mC, cytokeratin-19 fragment, CYFRA 21-1, and APOE4. Using cyclic voltammetry (CV) and chronoamperometric detection, these biosensors exhibited a high sensitivity for the determination of proteins (LOD values of 0.19 fg mL^{-1} for CYFRA 21-1, 16 fg mL^{-1} for MUC-1, and 15.4 pg mL^{-1} for APOE4) and epigenetic biomarkers and potential to perform the analysis in human/goat serum and genomic DNA (gDNA) extracted from colorectal cells. The use of Strep-Fe_3O_4 MNPs as catalytic labels has been reported for the simultaneous determination of four gene targets (*TMPRSS2-ERG* gene fusion, *PCA3*, *SChLAP1*, and *KLK2*) related with prostate cancer [20]. The method involved the use of an integrated biochip, on-chip electrical cell lysis, and solid-phase RPA and allowed a 30-min sample-to-answer workflow for the analysis in urine and serum of prostate cancer patients. As shown in Fig. 5, the biotinylated amplicons, generated by solid-phase RPA and immobilized at a thiolated target-specific forward primer-modified biochip, were labeled with Strep-Fe_3O_4 MNPs. Chronoamperometric detection was performed in the presence of TMB and H_2O_2.

Nanozymes have also been used as nanocarriers and catalytic labels in aptamers and antibody-based sandwich-type immunoassays. These multifunctional nanozymes, which include PtNPs/CoTPP/rGO [54], Pd/APTES-M-CeO_2-GS [24], rGO-NR-Au@Pt [35], Mn_3O_4 and Pd@Pt nanoflowers [30], Fe_3O_4/PDDA/Au@Pt [23], MWCNTs/GQDs [37, 38], and FeS_2-AuNPs [22], have been decorated with detector antibody (Ab_2) [24, 54], detector antibody (Ab_2) + HRP [35, 37, 38], AuNPs + Ab_2 [22] (Fig. 6), detector aptamer (Apt_2) + HRP [30], or (Apt_2) + HRP + G-quadruplex/hemin DNAzyme [53]. It is important to note that although these nanozymes display intrinsic peroxidase-like catalytic activity, they are often dressed with the natural enzyme to further enhance the sensitivity [35, 37, 38]. The resulting affinity biosensors showed attractive analytical characteristics for the determination of toxins and pathogenic bacteria in food matrices (peanut, pork, and milk) and for the single or dual determination of protein biomarkers of relevance in cancer (diagnosis and

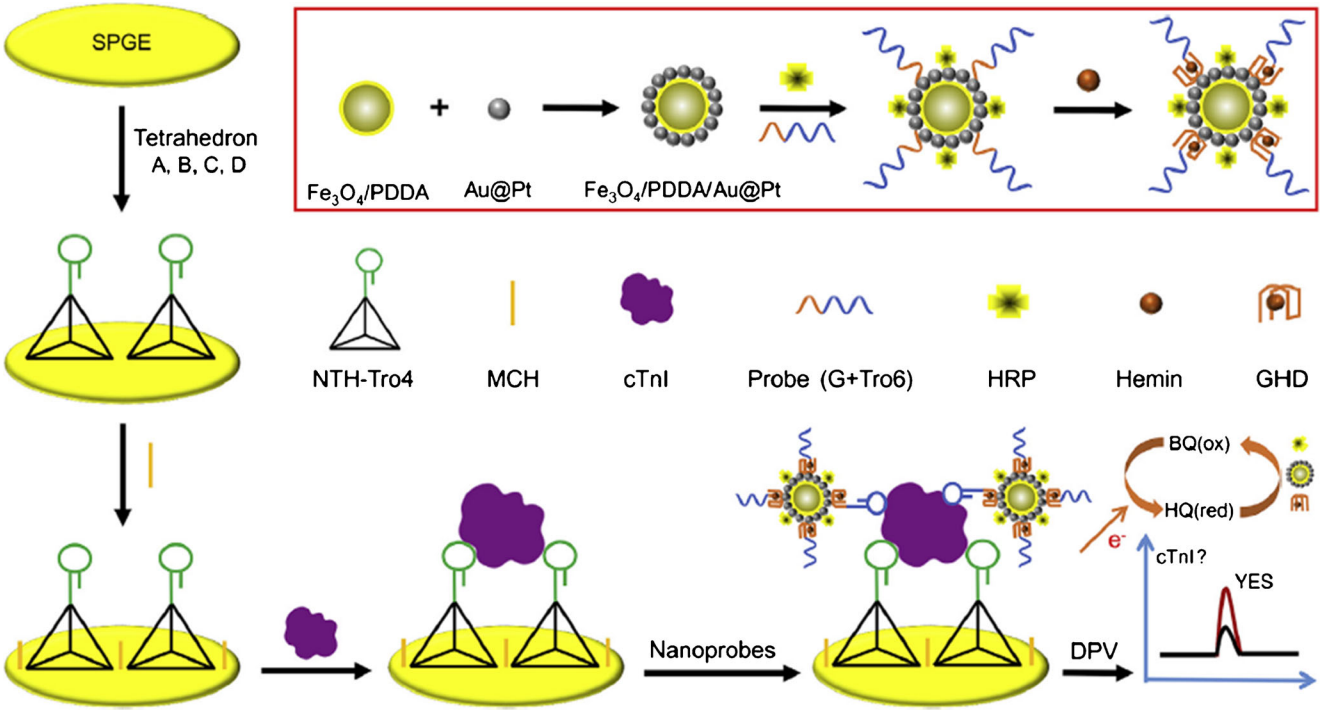


Fig. 6 Dual electrochemical aptasensor for the determination of cardiac troponin I (cTnI) by using NTH capture probes anchored on screen-printed gold electrodes (SPGEs) and multifunctional nanozymes (Fe_3O_4/PDDA/Au@Pt) acting as nanocarriers of HRP, G-quadruplex/hemin DNAzyme, and aptameric detector probe. Reprinted and adapted from [53] with permission

metastasis) and cardiovascular diseases at clinically relevant levels. The methods were applied in samples of varying complexity such as serum, lysate, and whole cells and protein extracts from paraffin-embedded tumors of patients diagnosed with colorectal cancer (CRC).

In addition to combine nanozymes with natural enzymes [35, 37, 38], the sensitivity was improved by using nanozymes together with other nanomaterials [31, 43], NTHs [30, 53], or enzyme-free signal amplification systems such as CHA [29, 43, 53].

MOFs have been widely exploited in electrochemical affinity biosensors as nanocarriers of signaling elements [45, 55–57], tags [45, 46, 58], or electrode modifiers [59, 60]. However, their application as nanozyme labels is still scarce due to their weak conductivity and electrocatalytic properties [43, 44]. Cui et al. [45] and Ling et al. [46] reported the use of $(Fe-P)_n$-MOF and Strep-FeTCPP@MOF as catalytic labels in connection with hairpin probes to determine Pb^{2+} profiting the DNAzyme selectivity (Fig. 7a) [45] or a target DNA through an allosteric switch mechanism (Fig. 7b) [46]. In this latter approach, the FeTCPP@MOF composites can mimetically catalyze the oxidation of o-phenylenediamine (o-PD) to 2,2′-diaminoazobenzene which was detected electrochemically by DPV.

More recently, Li et al. [43] reported the use of multifunctional Fe-based MOFs (PdNPs@Fe-MOFs) conjugated with streptavidin and biotinylated hairpin detector probes working as nanocarriers, redox probes, and electrocatalysts. Based on target-CHA, the target miR-122 is able to trigger the hybridization of thiolated capture probes, attached to a glassy carbon electrode (GCE) modified with AuNPs-functionalized nitrogen-doped graphene sheets (AuNPs@N-G), and the detector probes to be released later, thus initiating the following reaction process. These results in numerous PdNPs@Fe-MOFs anchored onto the sensing interface, which is due to their peroxidase-like activity, reinforce the electrocatalytic oxidation of TMB in the presence of H2O2. The biosensor, which offered a LOD of 3 aM for the synthetic miRNA, demonstrated a potential to determine the endogenous content of miR-122 in serum from healthy individuals. The biosensor recently proposed by Feng et al. [44] for prostate-specific antigen (PSA) implies a sandwich immunosensor in which the capture antibody is covalently immobilized on a GCE coated with methylene blue (MB) thin layer and nanocomposites of reduced graphene oxide and gold (Au-rGO). The detector antibody is conjugated with Au/Fe-MOF nanocomposites (Fig. 8). When H_2O_2 is present, the Au/Fe-MOF nanocomposites induce the Fenton reaction, which degrades the MB on the electrode surface. Therefore, the MB oxidation signal measured by square wave voltammetry (SWV) decreases as the target antigen concentrations (PSA) increases. The immunosensor achieves a LOD of 0.13 pg mL^{-1} and was employed to test human serum samples.

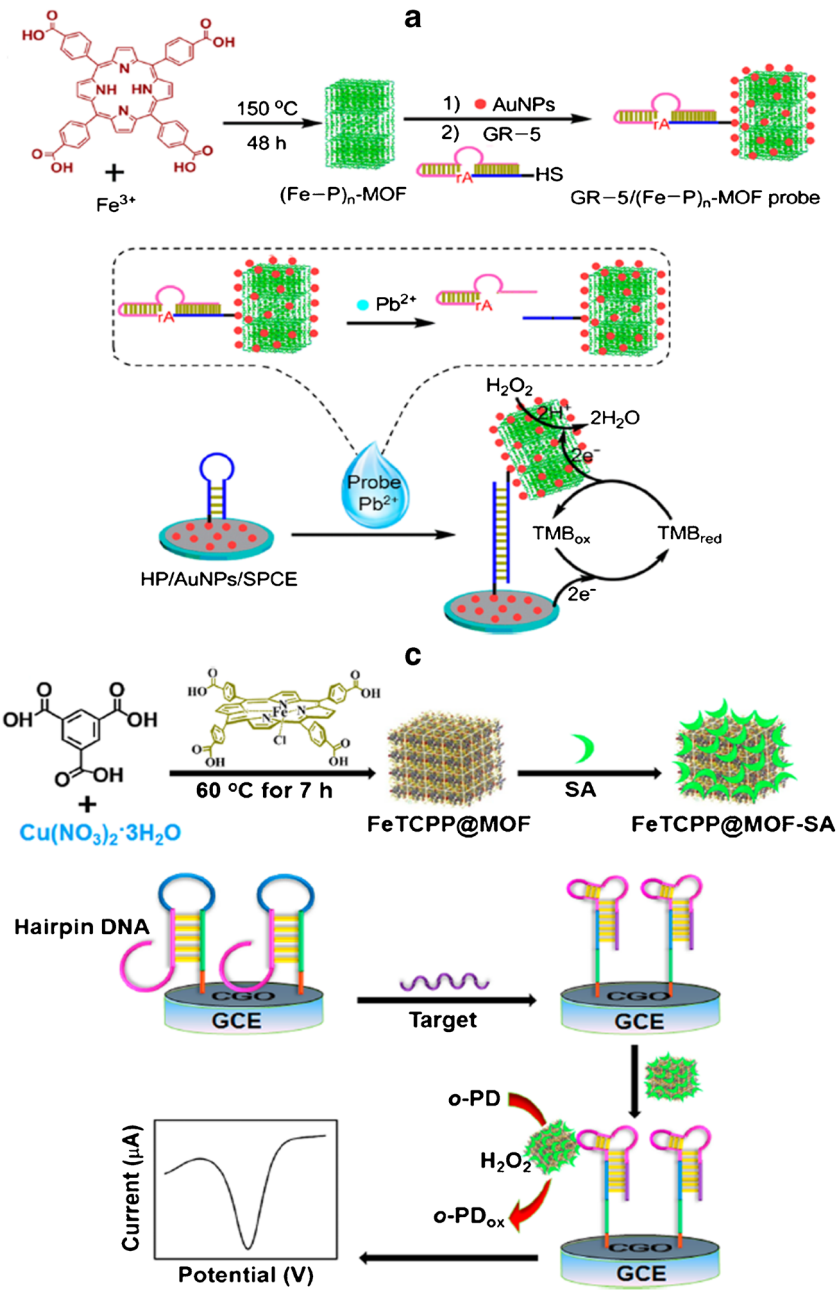


Fig. 7 Electrochemical affinity biosensing strategies involving the use of MOF-based nanozymes as catalytic labels and hairpin probes to determine Pb^{2+} profiting the DNAzyme selectivity (**a**) or a target DNA through an allosteric switch mechanism (**b**). Both panels: nanozyme preparation (top) and bioassay fundamentals (bottom). Reprinted and adapted from **a** [45] and **b** [46] with permission

Opportunities, impact, challenges, and future insights

The use of nanozymes, nanomaterials displaying enzyme-like catalytic features, provides a way to efficiently overcome the shortcomings of high cost and short lifespan that face the use of natural enzymes in electrochemical biosensing. The electrochemical biosensors resulting from replacing natural enzymes with artificial nanozymes have lower cost and easier production and exhibit better stability owing to the striking merits of these artificial nanozymes. This explains why the past few years have seen a thriving progress in electrochemical affinity biosensors using mostly peroxidase-like activity nanozymes, which have grabbed most attention in this field.

Although nanozymes have begun to replace progressively the use of natural enzymes in electrochemical affinity biosensing, several challenges and obstacles still remain and should be prioritized to utilize their potential to the fullest:

– Although nanozymes have demonstrated SOD, catalase, oxidase, and peroxidase activity, they are still far from mimicking all the important reactions catalyzed by natural enzymes. Then, additional efforts should be focused

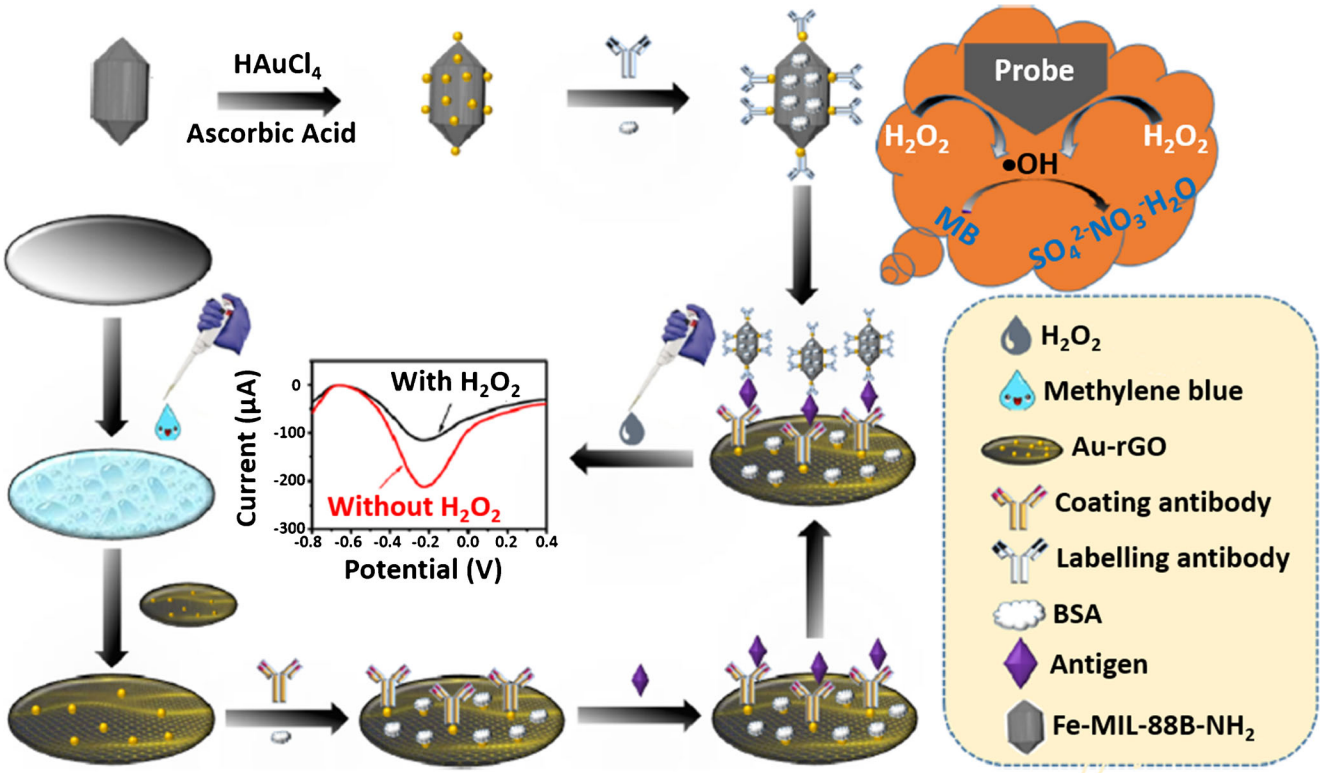


Fig. 8 Electrochemical sandwich immunosensor for PSA using a GCE modified with MB-Au-rGO as scaffold to immobilize the capture antibody and Au/Fe-MOF nanocomposites decorated with the detector antibody. Reprinted and adapted from [44] with permission

on designing nanozymes with new catalytic properties to mimic the activity of hydrolase and synthetase enzymes, among others. Work for creating analogues of active centers in natural enzymes and their incorporation into nanomaterials is a fast and feasible option to achieve this goal. The use of available nanozymes with activity other than peroxidase should also be exploited in electrochemical affinity biosensors. An incursion of electrochemical affinity biosensors in the agro-food and environmental fields as strong as it happened in the clinical field, gaining ground to the colorimetric- and strip-based strategies in these relevant areas, is foreseen.

- Unlike natural enzymes, most nanozymes catalyze more than one specific substrate. Conferring selectivity to nanozymes is one of the main challenges that has been and must continue to be tackled in the future. Although recent developments in nanoparticles' surface modification strategies allow decorating nanozymes' surface with targeting ligands, their catalytic efficiency is often impaired upon modification. Therefore, the development of methods able to produce nanozymes coated with desired biomolecules without compromising their catalytic activity appears to be an attractive field for promoting nanozyme research in electrochemical affinity biosensing.
- At present, the catalytic performance of most nanozymes is still lower than that of natural enzymes. Furthermore, the intrinsic enzymatic-like activity of some nanomaterials is restricted to certain experimental conditions, sometimes not compatible with biomolecules sensing at physiological pH. This makes it necessary to prioritize their activity engineering. Some progress has been made in this direction by using a few identified molecules that can efficiently improve the catalytic activity of nanozymes. However, additional efforts must be made for developing better performance of nanozymes. Some research trends to improve nanozyme activity include mimicking the complex natural enzyme systems, which generally work together as enzyme clusters, by focusing efforts on preparing functional assemblies of several single nanozymes or combining nanozymes and natural enzymes with synergistic catalytic activity, properties, and functionalities of the different components. However, in particular cases, the lifespan and price of the natural enzymes sacrifice those of the whole catalyst system. It is also important to combine smartly nanozymes and other functional nanoparticles possessing special magnetic, optic, electric, and mechanic nanoscale properties to develop multifunctional nanozymes and widen their practical applications for ultra-sensitive and multiplexed biosensing.

- A more rational nanozymes' design is highly required. So far, most nanozymes have been developed by random screening of the enzyme-like activities displayed by the available nanomaterials. However, it can be envisioned that further investigation can play a decisive role in guiding the rational production of the best nanozyme candidates in terms of performance and specific properties. This research may include the following: (i) a deeper understanding of the catalytic mechanism of a nanozyme (structure-activity relationship); (ii) the combination of both experimental and computational studies to decipher the catalytic mechanisms; (iii) the introduction into the field of the emerging single-atom strategy to develop single-atom nanozymes, which offer maximum atomic utilization, thus providing more active sites for reactions; and (iv) the exploration of artificial intelligence techniques. All these lines of research will open new gates helpful to make nanozymes even more advantageous compared with their natural counterparts and should also increase their use.
- The size and composition of most nanozymes (except fullerene-based ones) are not uniform. Currently, nanozymes are synthesized only on a small scale in the research laboratory. This coupled with the dependence of their catalytic activity with different factors makes the reproducibility among different batches of nanozymes not as good as desired. Therefore, further efforts should be devoted to improving the synthesis protocol to synthetize nanozymes with atomically precise structures and, by extension, the assays' reproducibility in which they are used. Moreover, bioconjugated nanozymes are fabricated according to individual considerations and processes, and more efforts are needed to standardize the protocols as with natural enzymes where mature bioconjugation procedures are already well-established. Universal standards should be settled also to fairly assess the catalytic activity of different nanozymes.
- Toxicity of nanozymes is also a key issue to be addressed. In this sense, the adoption of bioinspired and/or "safe-by-design" approaches for their synthesis and/or their coating with biocompatible or transient polymers which have been reported to impart biocompatibility and antibiofouling properties to the nanozymes surface and, therefore, to the biosensors in which they are used should be also explored.

To sum up, despite the remarkable progress made during the latest years, there is still much room for advancement in future research on nanozymes in the development of electrochemical biosensors including their commercial applications for point-of-care testing. Thus, the continuous progress on nanozymes with improved or desired properties will be a hot topic in the coming future and will increasingly attract the attention of the sensor research community. The combination of nanozyme-based electrochemical affinity biosensors with personalized equipment such as smartphones and/or portable low-cost devices will also be an exciting route to move forward in point-of-care testing. This enormous nanozymes' development to achieve catalytic activity and efficiency comparable or even better than natural enzymes will bring a revolution to conventional electrochemical biosensing and more practical applications in other expectation fields. However, identifying unique applications of nanozymes in specific niches is deemed necessary to gain long-term support for their research.

Funding information The financial support of the PID2019-103899RB-I00 (Ministerio de Ciencia e Innovación) and RTI2018-096135-B-I00 (Ministerio de Ciencia, Innovación y Universidades) Research Projects and the TRANSNANOAVANSENS-CM Program from the Comunidad de Madrid (Grant S2018/NMT-4349) are gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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