PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2018, 45(2): 129~147
www.pibb.ac.cn

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Nanozymes in Analytical Chemistry: From *in vitro* Detection to Live Bioassays*

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Abstract Nanozymes, the catalytic nanomaterials with enzyme-like properties, have attracted enormous interests in recent years. They have been used for wide range applications from biosensing and bioimaging to therapeutics and environmental protection. In this review, we highlighted the recent progress of nanozymes in analytical applications. We first discussed the *in vitro* applications, which covered the detection of bioactive small molecules, nucleic acids, protein biomarkers, cells, *etc*. We then discussed the *in vivo* applications, which included the monitoring of bioactive small molecules in live brains and tumor tissues, the study of drug efficacy, and the investigation of drug and nanozyme metabolism, *etc*. Finally, we concluded the potential challenges of nanozymes when applying to analytical chemistry and prospected future directions.

Key words nanozymes, *in vitro*, *in vivo*, analytical chemistry, enzyme mimics, biomimetic chemistry
DOI: 10.16476/j.pibb.2017.0469

* This work was supported by grants from The National Natural Science Foundation of China (21722503, 21405081), National Basic Research Program of China (2015CB659400), PAPD Program, Shuangchuang Program of Jiangsu Province, Open Funds of the State Key Laboratory of Analytical Chemistry for Life Science (SKLACLS1704), and Thousand Talents Program for Young Researchers.
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Received: December 18, 2017 Accepted: January 19, 2018

Since the discovery of the unexpected peroxidase mimicking activity of magnetic iron oxide nanoparticles (NPs) by Yan and coworkers, nanozymes researches have been growing at an exponential rate (Figure 1) [1]. Nanozymes, the nanomaterials with enzyme-like activities, possess higher stability and lower cost than natural counterparts and traditional artificial enzymes. In addition, the catalytic activities of nanozymes can be modulated *via* varieties of strategies [2–3]. Several types of natural enzymes have been successfully mimicked by nanozymes, including oxidase, peroxidase, catalase, superoxide dismutase (SOD) and hydrolase, etc.[2–3] Based on these, diverse applications were explored with nanozymes, such as biosensing, cancer diagnosis, tissue engineering, environmental protection and so on [2, 4–64]. Since numerous reviews, chapters, and books on nanozymes have been published, in this current review we summarized the analytical chemistry applications of nanozymes, covering both *in vitro* detection and live bioassays (Figure 2)[2, 65–86].

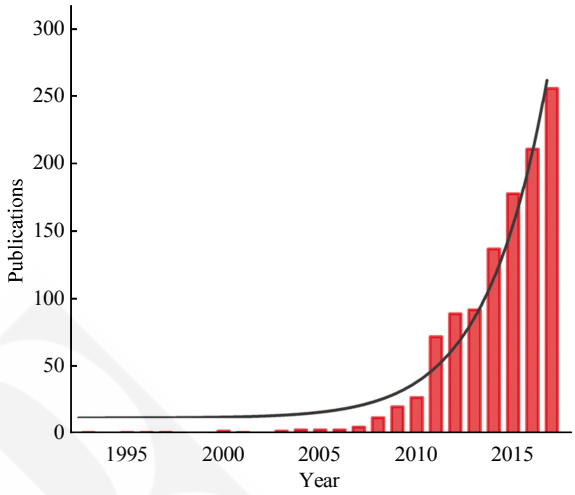

Fig. 1 Number of published papers on nanozymes by the end of 2017

Data was from web of science.

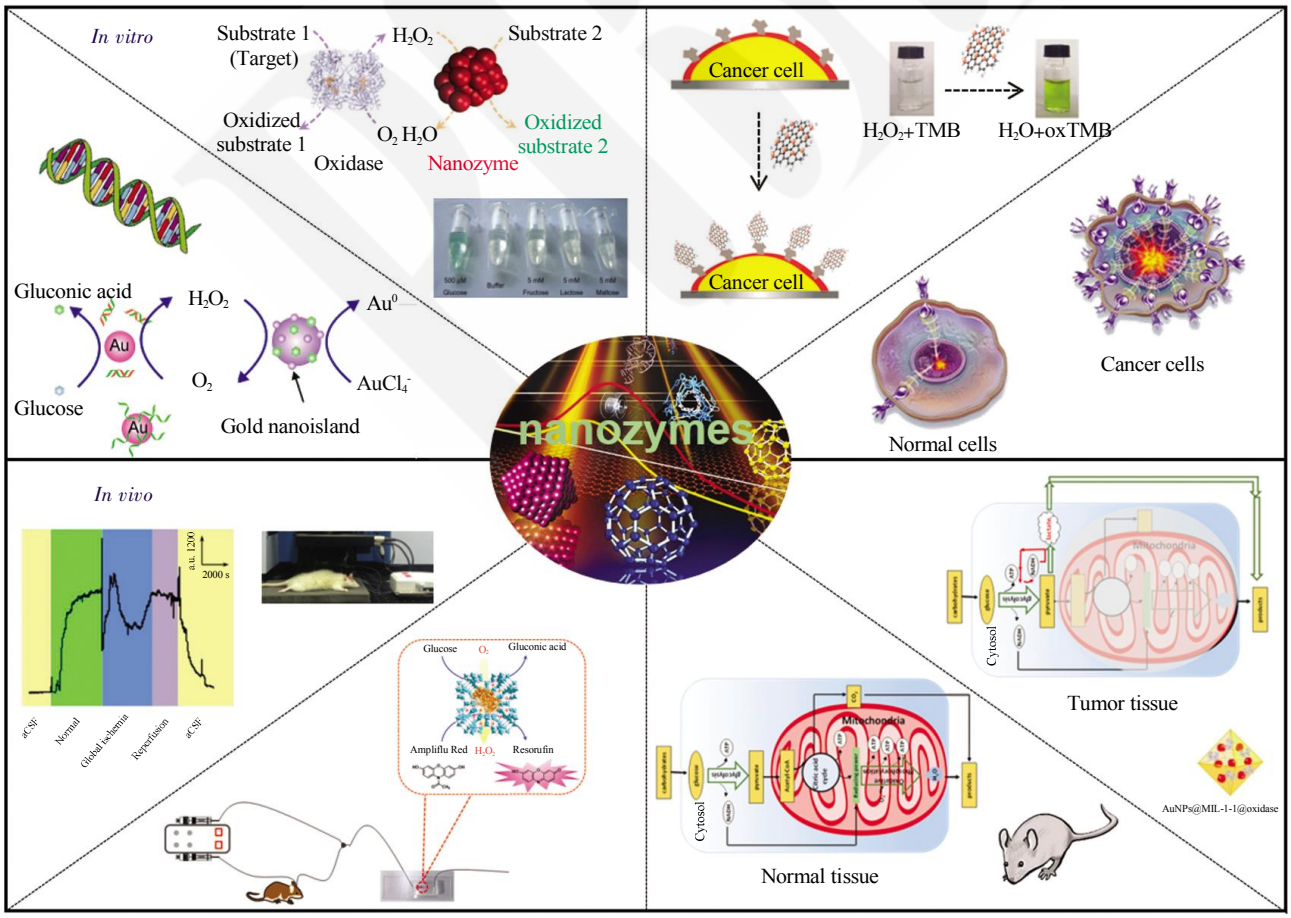

Fig. 2 Illustration of nanozymes applied in analytical chemistry: from in vitro detection to live bioassays

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Note: due to the space limit, only a small number of references are discussed here, readers are therefore referred to the books and reviews mentioned above for further information.

1 Nanozymes for in vitro detection

Inspired by Yan's pioneering work [1], Wei and Wang developed a facile bioassay with iron oxide nanozymes for hydrogen peroxide and glucose detection[87]. Since then, nanozymes have been applied for *in vitro* detection of various important targets, which include bioactive small molecules, nucleic acids, protein biomarkers, cells, *etc*.

1.1 Bioactive small molecules

In their study, Wei and Wang used Fe_3O_4 NPs with peroxidase mimicking activities to develop a facile colorimetric assay for H_2O_2[87]. By catalyzing the oxidation of 2, 2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) with H_2O_2 in the presence of Fe_3O_4 nanozymes, the green colored product (*i.e*., $ABTS^{\cdot+}$) was obtained. The detection of H_2O_2 was therefore achieved using naked eyes or UV-visible absorption spectroscopy. A linear range from 5×10^{-6} to 1×10^{-4} mol/L and a detection limit of 3×10^{-6} mol/L were obtained for H_2O_2 detection with the developed assay. Moreover, they combined glucose oxidase (GOx) with the peroxidase-like Fe_3O_4 nanozymes to enable an enzymatic cascade reaction for glucose detection. As shown in Figure 3, a sensitive and selective glucose assay was achieved with the proposed sensing strategy.

Fig. 3 Fe_3O_4 nanozymes with glucose oxidase for glucose detection

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Since then, various nanomaterials with peroxidase-like activity have been developed, which were usually used for H_2O_2 and glucose detection [31, 91–97]. For example, ultrasmall Pt nanoclusters with peroxidase-like activity were prepared and combined with GOx for glucose detection in human serum [92]. Co_4N nanowires with peroxidase-like activity showed good salt- and temperature-resistance, which were then used to develop facile assay for H_2O_2 and glucose [93]. Recently it reported that H_2O_2 could displace DNA from peroxidase-like ceria nanozymes. Such a displacement would recover the inhibited catalytic activity of nanoceria and enable the catalytic oxidation of a colorimetric (or fluorescent) substrate in the presence of H_2O_2 and nanoceria. On the basis of this interesting phenomenon, a sensing strategy for serum glucose was developed by combing with GOx (Figure 4)[31].

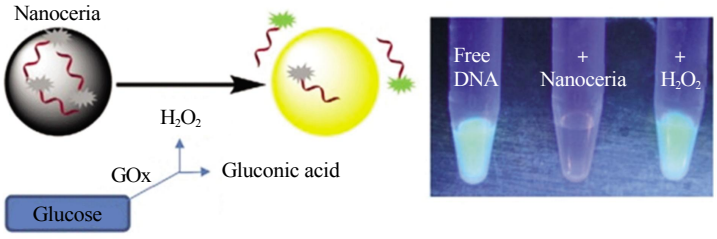

Fig. 4 H_2O_2 displacing DNA from peroxidase-like ceria nanozyme for glucose detection

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Since H_2O_2 is the key product to diverse oxidation processes, in principle, it is universal to use cascade reaction constructed by oxidase and peroxidase to detect a wide range of bioactive small molecules. As hypothesized, choline, cholesterol, galactose and xanthine were quantified by integrating their corresponding oxidase with a peroxidase mimicking nanozyme (Figure 5) [2, 98-106]. Moreover, Yan and coworkers explored a rapid colorimetric assay for organophosphorus pesticide and nerve agent by exploring enzymatic cascade reactions [104]. First, acetylcholinesterase (AChE) catalyzed the hydrolysis of acetylcholine to produce choline. Then, choline was oxidized in the presence of choline oxidase to produce H_2O_2. Finally, detectable color signal would be generated by oxidizing 3, 3', 5, 5'-tetramethylbenzidine (TMB) in the presence of H_2O_2 and peroxidase-like Fe_3O_4 nanozymes. Since organophosphorus reagents were inhibitors to AChE, the presence of them would inhibit the enzyme activity and thus produced less H_2O_2 and weaker color signals. With this method, 1 nmol/L Sarin, 10 nmol/L methyl-paraoxon, and 5 μmol/L acephate were successfully detected.

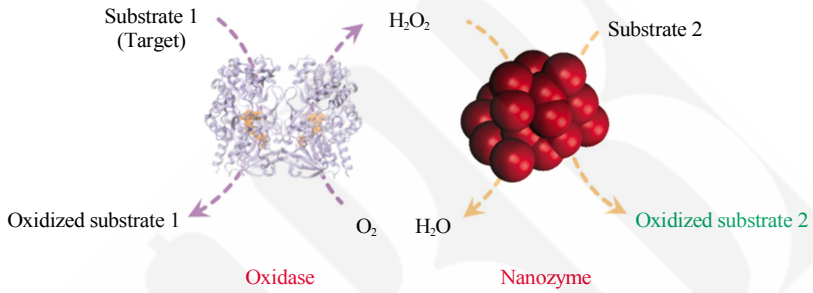

Fig. 5 Target detection by combing its corresponding oxidase with a peroxidase mimicking nanozyme

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Recently, we showed that the oxidase-like activity of nanoceria could be cooperatively modulated by proton and adenosine triphosphate (ATP) [107]. Then using proton-producing/consuming enzymes, we developed self-regulated bioassays to detect the corresponding enzyme activities (Figure 6). More, since the enzyme activities could be tuned by nerve agents (such as methyl-paraoxon), drugs (such as tacrine), and bioactive ions (such as fluoride ion), we further developed assays for determining these bioactive molecules.

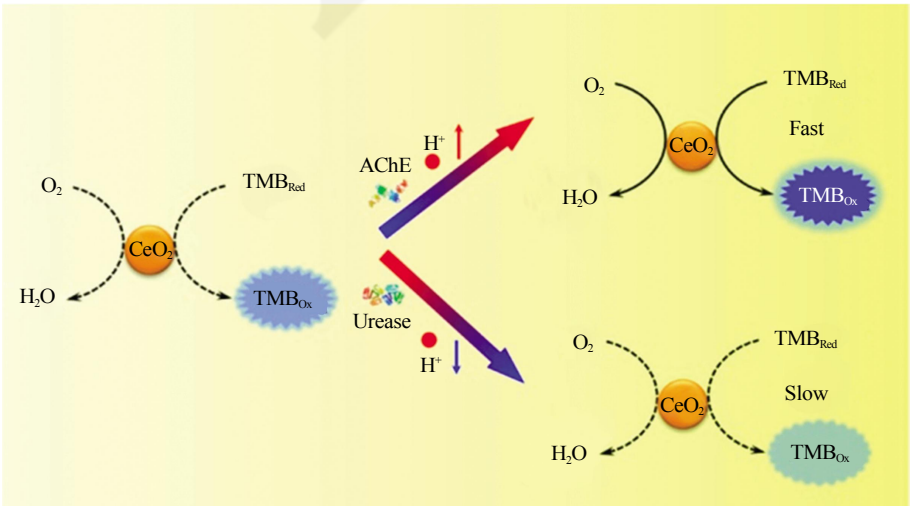

Fig. 6 Modulating oxidase-like ceria nanozymes for self-regulated bioassays

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1.2 Nucleic acids

With huge success in bioactive small molecules, researchers attempted to detect macromolecules under the similar principle, leading to biosensors based on oxidase-like, peroxidase-like, and catalase-like nanozymes for nucleic acid detection [16–17, 108–128]. Dong *et al*. demonstrated that by assembling hemin onto graphene, highly active peroxidase mimic could be prepared [17]. Moreover, they demonstrated the coagulation of hemin/graphene obeyed the 2D Schulze-Hardy rule (*i.e*., the balance between van der Waals attraction and electric double-layer repulsion played a key in the dispersion of hemin/graphene). They further showed that single-stranded DNA (ss-DNA) had stronger affinity towards the hemin/graphene than double-stranded DNS (ds-DNA). Such a difference enabled the distinguishing ss-DNA from ds-DNA using the hemin/graphene. On the basis of these interesting properties of the hemin/graphene, they went on to develop a facile assay for single-nucleotide polymorphisms (SNPs) in disease-associated DNA (such as SNPs in Hepatitis B virus) (Figure 7).

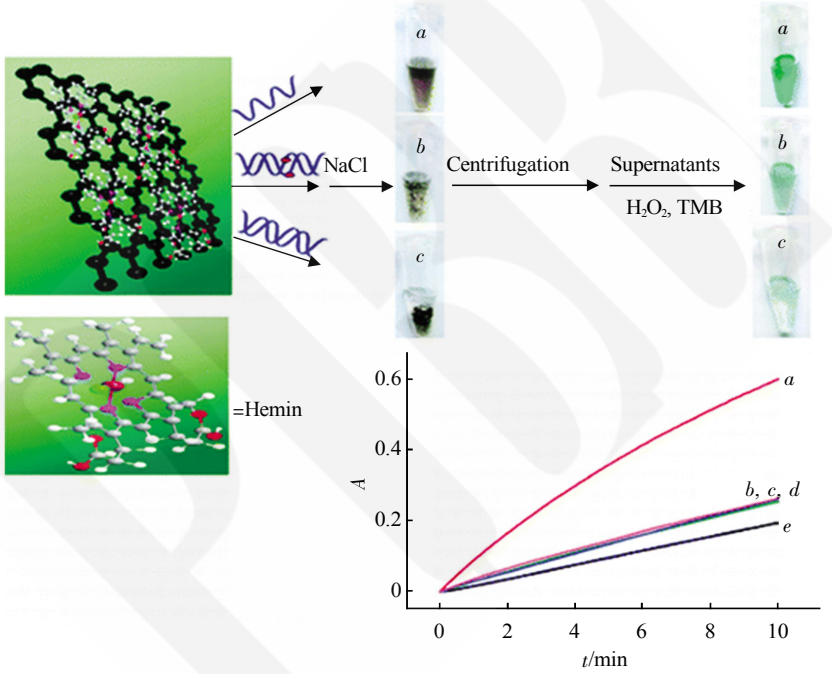

Fig. 7 Hemin/graphene nanozymes for single-nucleotide polymorphism detection

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Citrate gold nanoparticles (AuNPs) with GOx-like activity could be modulated by DNA, as reported by Fan and coworkers (Figure 8) [16]. The AuNPs catalyzed the oxidation of glucose to produce H_2O_2, which would act as a reducing agent to grow gold nanoislands onto the AuNPs. ss-DNA interacted strongly with AuNPs, leading to the inhibition of the catalytic activities and the gold nanoislands growth. On the other hand, the oxidase-like activities of AuNPs were unaffected by ds-DNA due to the negligible interactions between ds-DNA and AuNPs, allowing for the gold nanoislands growth. Moreover, the newly-grown Au nanoislands had a red-shift peak in the surface plasmon resonance (SPR). Therefore, the presence of ss-DNA *vs*. ds-DNA could be differentiated under dark-field illumination. Using an ss-DNA as the probe, the presence of a complementary target nucleic acid (such as an ss-DNA or a microRNA) could be detected with the developed method.

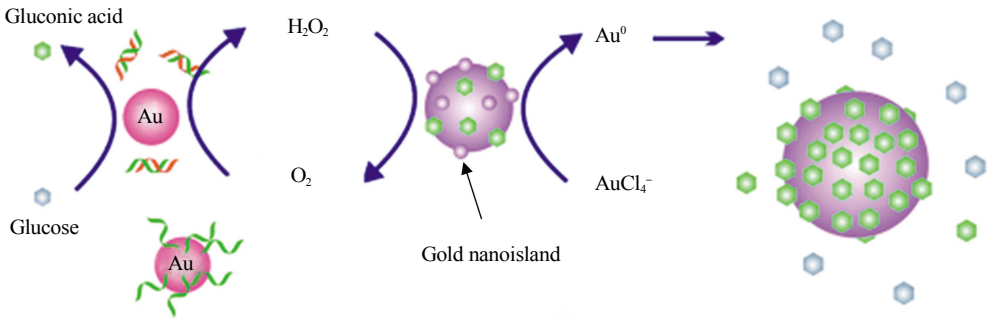

Fig. 8 Regulating the catalytic activity of oxidase-like AuNPs using DNA for DNA detection

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PtNPs with catalase-like activities have been used to fabricate volumetric bar chart chip for DNA analysis (Figure 9)[114]. Catalase-like PtNPs speeded the decomposition of H_2O_2 into O_2. The produced O_2 was then measured by a propelled volumetric bar chart chip, which was directly visualized. By using a sandwich assay format with PtNPs, as low as 20 pmol/L target DNA could be successfully detected.

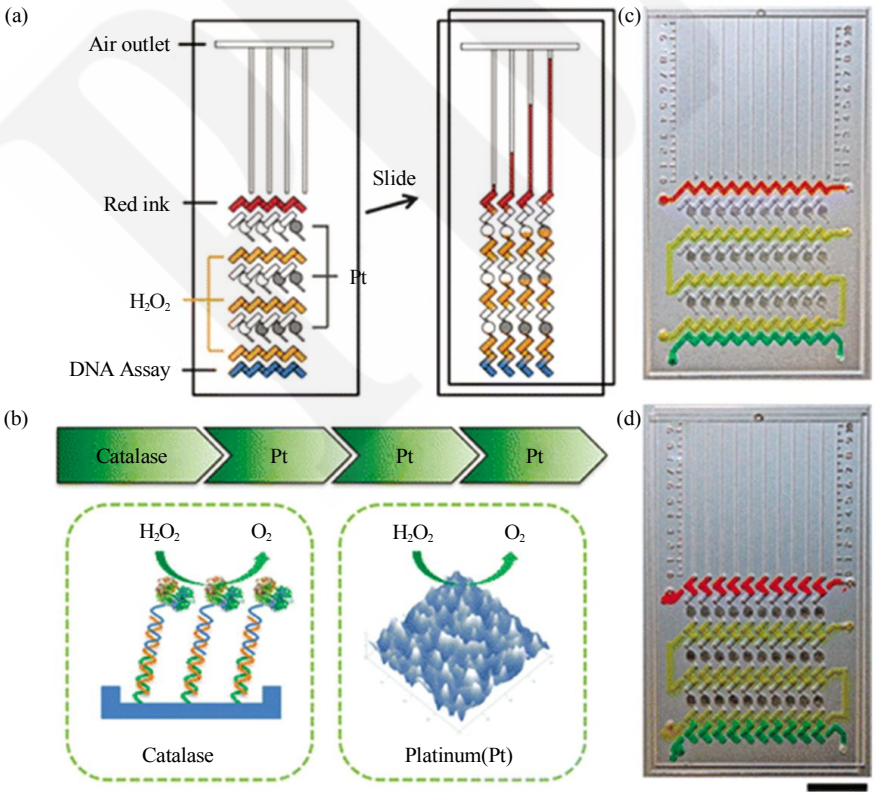

Fig. 9 Catalase-like PtNPs combined with volumetric bar chart ship for DNA detection

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When functional nucleic acids, such as aptamers and DNAzymes, were used to modulate the activities of nanozymes, aptasensors were fabricated [128]. For example, an anti-thrombin aptamer was able to

enhance the peroxidase-like activity of AuNPs. When thrombin was added, the interaction between the aptamer and AuNPs weakened. Therefore, the peroxidase-like activity of the AuNPs decreased. Similarly, an electrochemical biosensor was designed for thrombin detection by labeling an anti-thrombin aptamer with Fe_3O_4 nanozymes. In the absence of thrombin, the flexible aptamer on an electrode produced low electrochemical signals. However, when thrombin was present, it interacted with its aptamer and brought Fe_3O_4 nanozymes closer to the electrode, resulting in increased electrochemical signals. With the developed strategy, thrombin detection was achieved with a linear range of 1.0–75 nmol/L and a detection limit of 0.1 nmol/L [111]. Thrombin has two aptamers, one is a 15-mer and the other is a 29-mer. Yang *et al*. used them to develop a sandwich assay for thrombin (Figure 10). The 29-mer aptamer was used to capture thrombin, and the 15-mer aptamer with Fe_3O_4 nanozyme labels was used for generating detectable signals. With such a sandwich assay, a sensitive and selective thrombin detection was accomplished [109]. Other functional nucleic acids, such as poly(dT), have also been utilized to design nanozyme-based aptasensors[117, 127].

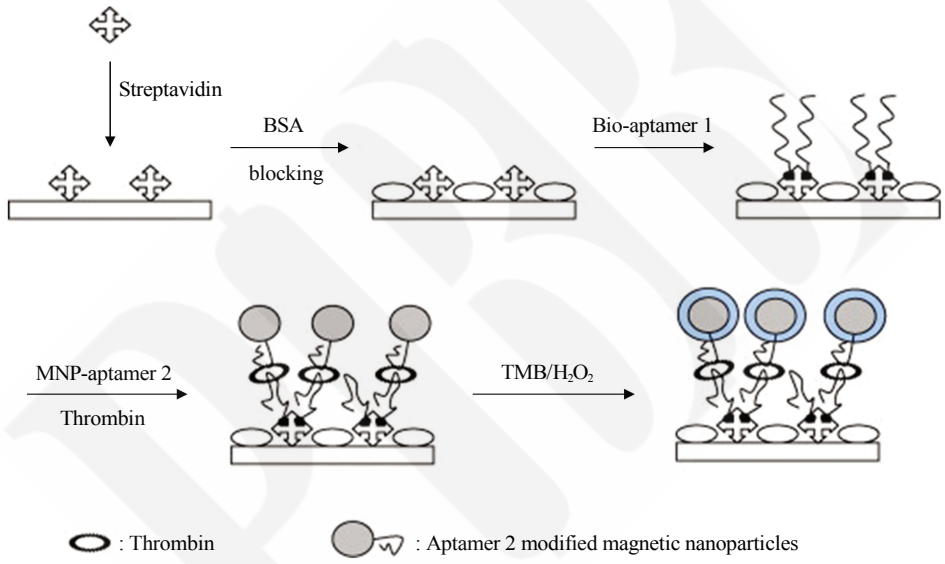

Fig. 10 Fe_3O_4 nanozyme combined with anti-thrombin aptamers for thrombin detection

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1.3 Protein biomarkers

Enzyme linked immunosorbent assay (ELISA) is one of the most useful approaches to analyze protein-based biomarkers. However, the limited stability and high cost of the enzymes (such as horseradish peroxidase) for signaling hinder its applications. To solve this issue, immunoassays have been developed with various nanozymes for detection of protein biomarkers[1, 40, 59, 129–150].

Notably, Yan in 2007 developed an antigen-down immunoassay format and a capture-detection sandwich immunoassay format for the detection of hepatitis B virus surface antigen (preS1) and myocardial infarction biomarker troponin Ⅰ (Tn Ⅰ), respectively[1]. Recently, they established an interesting nanozyme strip for Ebola detection (Figure 11) [147]. With it, the glycoprotein of Ebola virus (EBOV) could be detected as low as 1 μg/L, which was two orders of magnitude better than conventional strip technique. Moreover, the nanozyme strip could be finished within 30 min, providing a rapid and easy-to-use test for urgent Ebola diagnosis.

Numerous sandwich assays have been developed with nanozyme labeled antibodies. For example, highly active gold vesicles encapsulated with Pd-Ir NPs nanozymes with peroxidase mimicking activities were employed to develop an immunoassay for prostate surface antigen (PSA) detection [59]. As shown in Figure 12, due to the amplified signals generated by the nanozymes, the detection limit (*i.e*., pg/L) for PSA

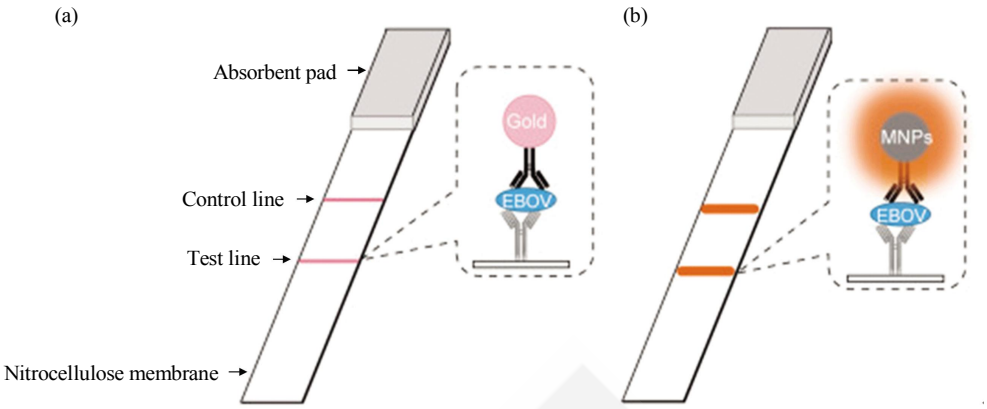

Fig. 11 Nanozyme based strips for Ebola detection

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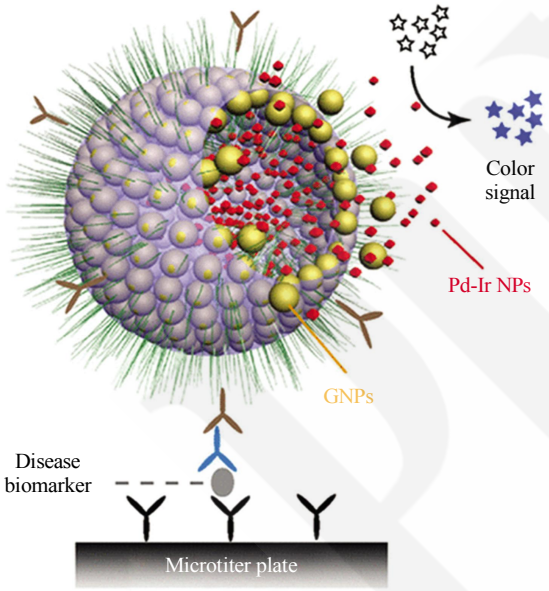

Fig. 12 Pt-Ir NPs nanozymes based sandwich assay for disease biomarker detection

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increased by three orders of magnitude compared with conventional immunoassays.

1.4 Cells

Nanozymes have also been used for cell detection [25, 43, 151-155]. For example, better peroxidase mimicking activities were obtained for the *in situ* growth PtNPs on graphene oxide. Then folic acid, an effective recognition moiety, was labelled for specific cancer cell detection (Figure 13). With the nanozyme-based assay, as few as 125 MCF-7 cancer cells were distinguished by naked-eye observation, demonstrating its promising applications in biomedical detection[151].

Recently a sensitive and selective method for qualifying the expression of integrin GP Ⅱ b/ Ⅲ a, an important cell membrane receptor related to platelet aggregation and cancer pathogenesis, was reported [39].

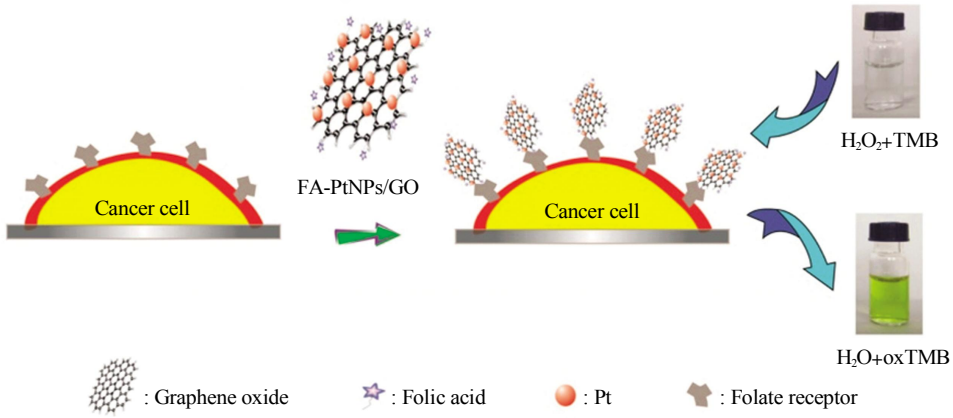

Fig. 13 Schematic of colorimetric cancer cell detection with PtNPs/graphene oxide nanozymes

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As illustrated in Figure 14, AuNPs with peroxidase-like activities were labeled with an integrin GP Ⅱb/Ⅲa targeting peptide. After recognizing the GP Ⅱb/Ⅲa with the AuNPs/peptide, the nanozymes could generate colorimetric signal by catalyzing the oxidation of TMB with H_2O_2. The integrin GP Ⅱb/Ⅲa could also be detected by the NIR two-photon signals of the AuNPs/peptide. With the nanozyme-based method, it determined that around 6.4×10^6 integrin receptors were expressed on a single human erythroleukemiac (HEL) cell.

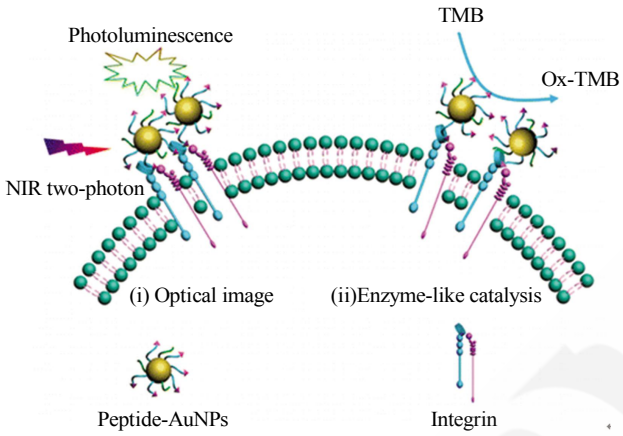

Fig. 14 AuNPs with peroxidase-like activity for selective detection of integrin GP Ⅱb/Ⅲa expression

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1.5 Others

Nanozymes have also been explored for other bioassays, such as cellular imaging, and ions and bacteriumdetection[31, 63, 88, 152−158]. Recently, Rotello and coworkers have designed bioorthogonal nanozymes and used them for intracellular catalysis[32]. As shown in Figure 15, they first assembled molecular catalysts onto a AuNP, and then used CB[7] to complex the ligands on the AuNPs[32]. Such a complexation would inhibit the nanozymes' activities. When nanozymes entered a cell, CB[7] could be released by interacting with 1-adamantylamine (ADA). Therefore, the activity of nanozymes was recovered, which then converted a non-fluorescent probe into a fluorescent one for cell imaging. Alternatively, the nanozymes could also convert a pro-drug into a potent drug for therapy.

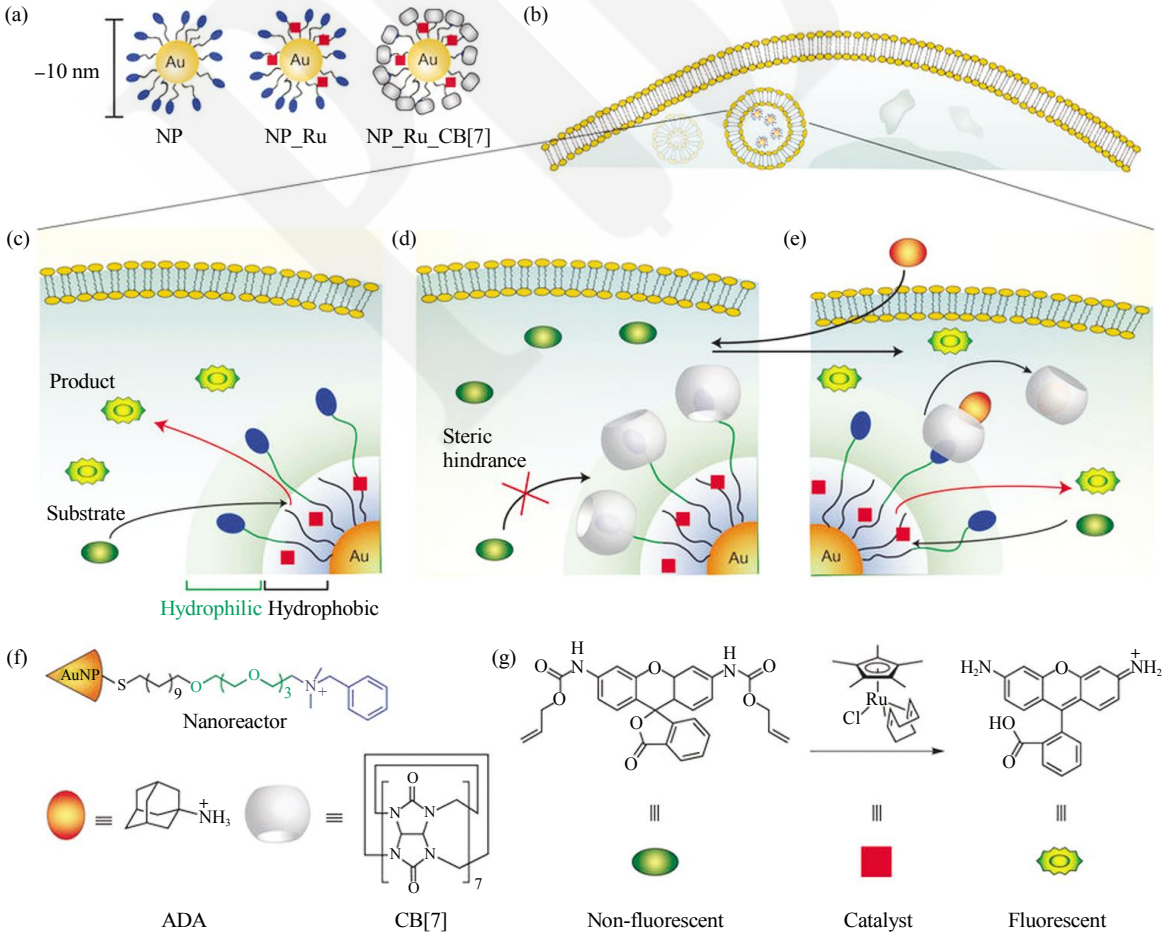

Fig. 15 Bioorthogonal nanozyme design and supramolecular regulation of intracellular catalysis

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Recently, it was found that the oxidase-like activity of nanoceria could be significantly enhanced by fluoride ion capping [156]. By making use of this interesting phenomenon, Liu *et al*. [157] developed an ultrasensitive assay for detection of fluoride ion water and in toothpastes. The oxidase-like activity of Au@Pt nanorods could be selectively inhibited by mercury ions (Hg^{2+}). On the basis of the inhibitory effect, an assay for Hg^{2+} was reported with a detection limit of 55 μmol/L. More, paper chips based gold nanozyme were developed, too[62].

2 Nanozymes for live bioassays

Despite the substantial progress in nanozyme-based bioassays, quite limited studies have been devoted to the live bioassays[89–90, 158–161]. This is partially due to the complicated conditions of live systems as well as moderate activity of the currently developed nanozymes[86, 162–163]. Nevertheless, several recent studies showed that nanozymes could be employed for live assays, such as monitoring bioactive molecules in brains and evaluating the efficacy of therapeutic drugs[89–90, 158–159, 161].

2.1 Live brains

Wei and coworkers recently developed an effective strategy to prepare highly efficient nanozymes by self-assembling a natural enzyme and a molecular catalyst within metal-organic frameworks (MOFs) [89]. The obtained nanozymes, termed as integrated nanozymes, showed better catalytic activities in the cascade reaction as a result of the nanoscale proximity effect. Moreover, they showed the fabricating strategy was universal since it could be used for three catalysts encapsulation. They continued to develop an online sensing platform for monitoring live brain glucose level by immobilizing the GOx/hemin@ZIF-8 nanozyme onto a microfluidic chip (Figure 16). Glucose would be oxidized to produce H_2O_2, which subsequently oxidize a colorimetric substrate (or a fluorescent substrate, such as Amplifu Red) to generate detectable signals (such as Resorufin for fluorescent detection). With the online platform, they were able to monitor the brain glucose levels following ischemia-reperfusion.

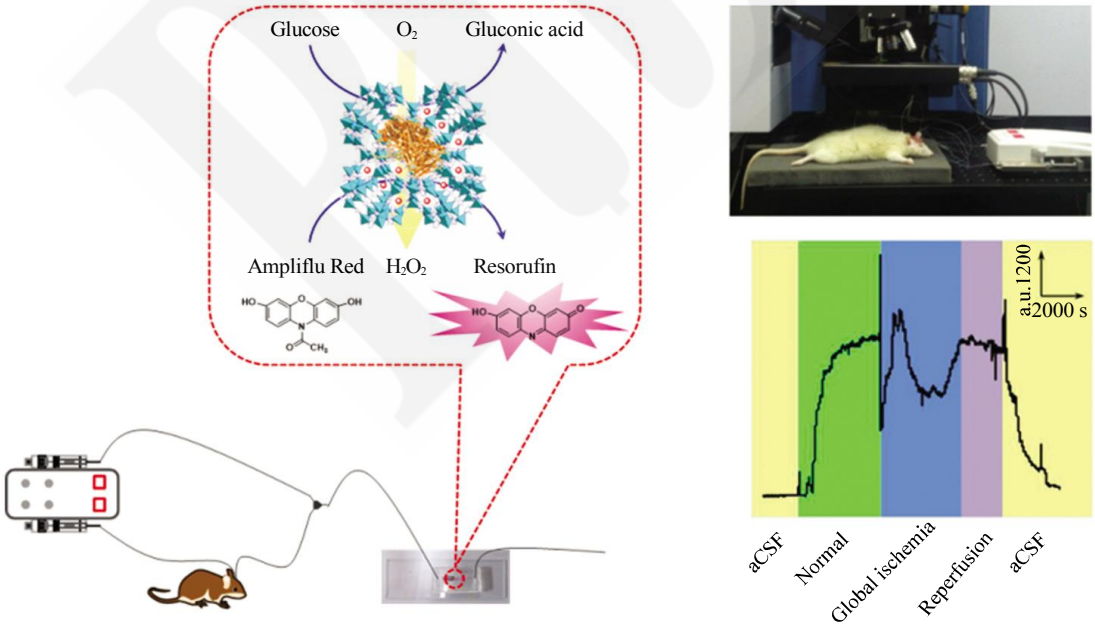

Fig. 16 Real time monitoring glucose level in live brains following ischemia-reperfusion

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AuNPs with both peroxidase mimicking and SERS (surface-enhanced Raman scattering) activities have been developed recently[90]. By integrating natural GOx and LOx with AuNPs@MIL-101, two integrated nanozymes (*i.e.*, AuNPs@MIL-101@GOx and AuNPs@MIL-101@LOx, LOx for lactate oxidase) were fabricated for monitoring glucose and lactate. After evaluating the sensitivity and selectivity for *in*

vitro detection of glucose and lactate with the nanozymes, they were used for monitoring glucose and lactate levels in live brains following ischemia-reperfusion. It showed that the glucose level was lowered while the lactate level was raised after ischemia. Moreover, it demonstrated that the treatment with astaxanthin (ATX) could alleviate the fluctuation of glucose and lactate levels in live brains during ischemia (Figure 17).

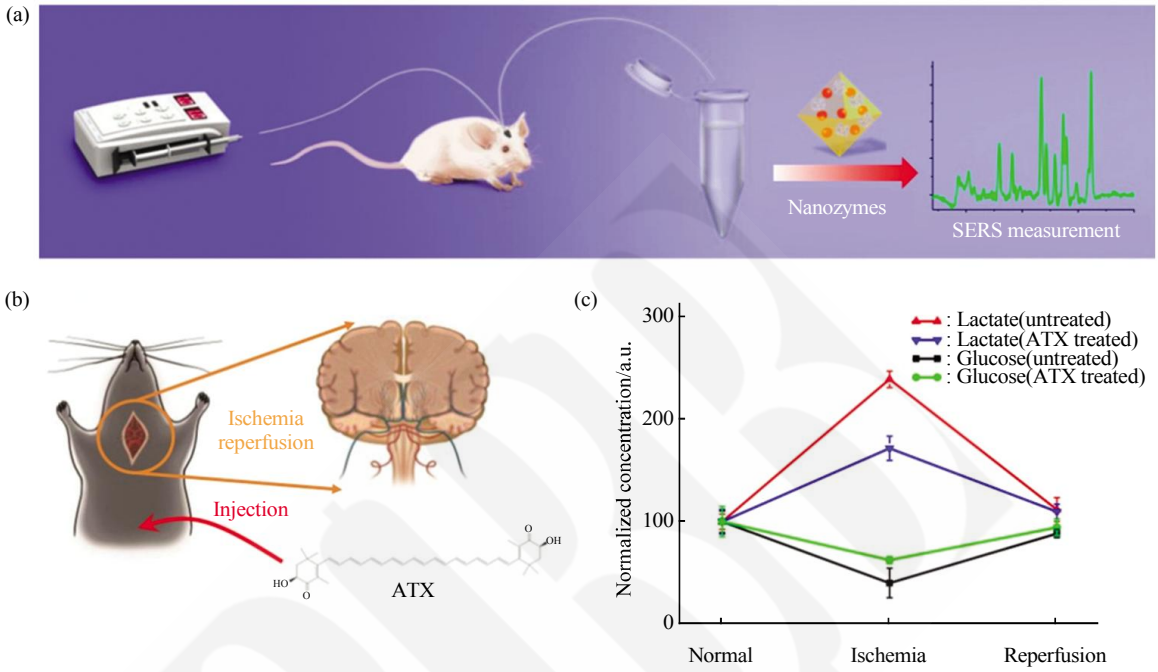

Fig. 17 Monitoring glucose and lactate in live rats' brains with AuNPs@MIL-101@GOx and AuNPs@MIL-101@LOx nanozymes and evaluating the efficacy of ATX for alleviating cerebral ischemic injuries

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An electrochemical biosensor for continuous monitoring lactate in live brains was fabricated[159]. By immobilizing LOx and Pt-ceria on a Pt wire, a microelectrode was prepared for *in vitro* and *in vivo* detection of lactate. Because lactate is an indicator for the tissue oxygen levels, the microelectrode was used to monitor lactate levels during hypoxic conditions. *In vitro* study demonstrated the satisfactory sensitivity and selectivity of the microelectrode. By implanting the microelectrode in the hippocampus of live rats, the continuous monitoring of lactate over 2 h was demonstrated (Figure 18). Compared with LOx alone, the presence of Pt-ceria significantly enhanced the biosensor performance, which could be ascribed to the high catalytic activity of Pt-ceria.

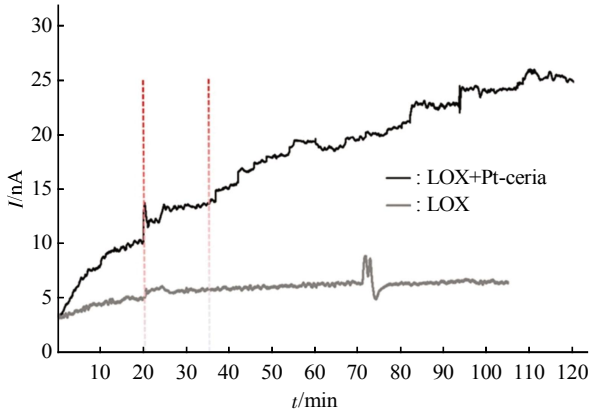

Fig. 18 Continuous monitoring lactate in live brains with Pt-ceria@LOx (LOx alone)

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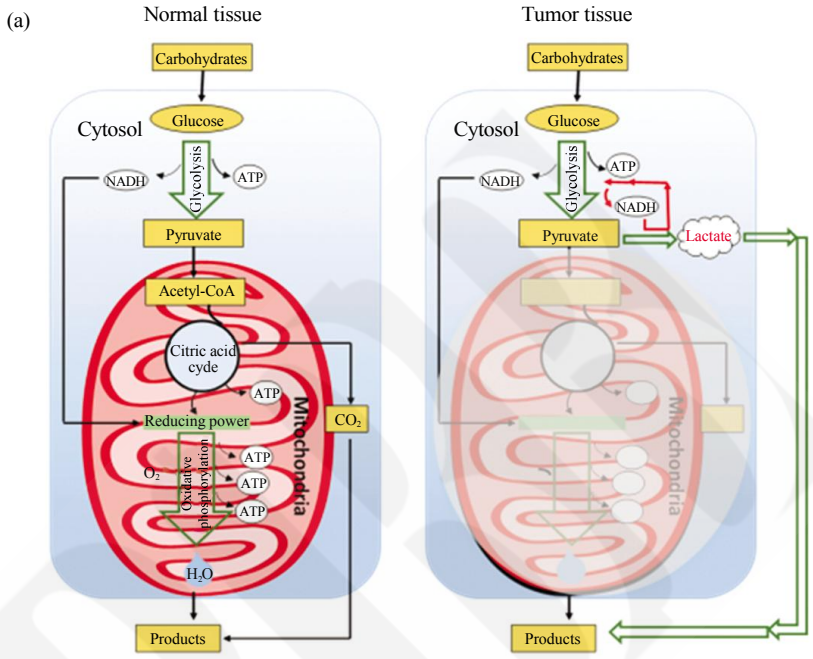
2.2 Live tumor tissues

Since nanozymes were sensitive to bioactive small molecules such as glucose and lactate, the metabolism of glucose or lactate in normal and tumor tissues was studied by using the AuNPs@MIL-101@GOx and AuNPs@MIL-101@LOx nanozymes[90]. As shown in Figure 19, the glucose level in tumor tissue was significantly lower than the normal tissue while the lactate level in tumor tissue increased. Such a difference was probably owing to the Warburg effect, a metabolic hallmark of tumor cells.

(a)



(b)

	c(Glucose)/(μmol·L^{-1})	c(Lactate)/(μmol·L^{-1})
The normal	82	15
	80	12.9
	88	20.5
The tumor	–	192
	–	89
	4.2	282

Fig. 19 Metabolism of glucose or lactate in normal and tumor tissues

(a) Warburg effects in normal and tumor cells. (b) Glucose and lactate levels in normal and tumor tissues. Reprinted with permission from Ref. [90], Copyright (2017) American Chemical Society.

2.3 Metabolism in live animals

It is important to understand nanozymes *in vivo* behaviors (such as biodistribution, pharmacokinetics and organ clearance). Using Fe_3O_4 nanozymes with peroxidase-like activity as a model, Yan *et al*. studied the biodistribution and the organ clearance of Fe_3O_4 nanozymes[158]. They first demonstrated the feasibility of using the intrinsic peroxidase mimicking activity of Fe_3O_4 nanozymes for visualizing the nanozymes in tissues. They then showed that dextran-coated nanozymes were mainly located in liver, spleen, and lung (Figure 20). Moreover, they discovered the rapid clearance of the nanozymes in mice.

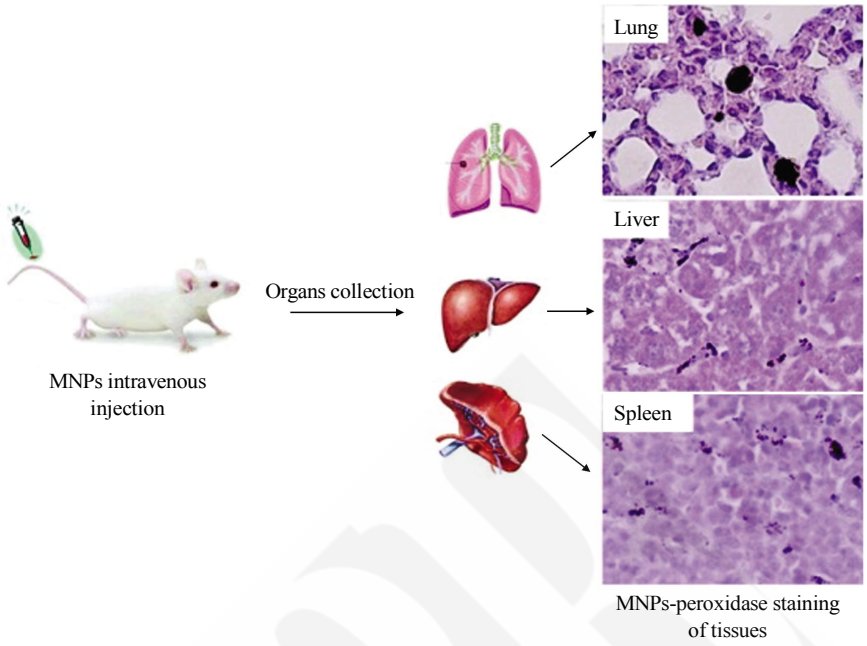


Fig. 20 Ex vivo detection of iron oxide NPs in mice using their peroxidase-like activity

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Very recently, we fabricated 2D MOF nanozymes with peroxidase-like activities using various tetrakis (4-carboxyphenyl)porphyrin (TCPP) ligands and metal ions[161]. A systematic study showed that chelated metal ion in TCCP played a key role in the enzymatic activity. Among the TCCP (M) studied, TCCP (Fe) contained 2D MOF showed the highest peroxidase-like activity. The 2D Zn-TCCP (Fe) nanozyme was then used to develop an assay for monitoring heparin metabolism in live rats (Figure 21). A heparin specific peptide, AG73, was employed to selectively recognize heparin [164–165]. AG73 tended to interact with the 2D

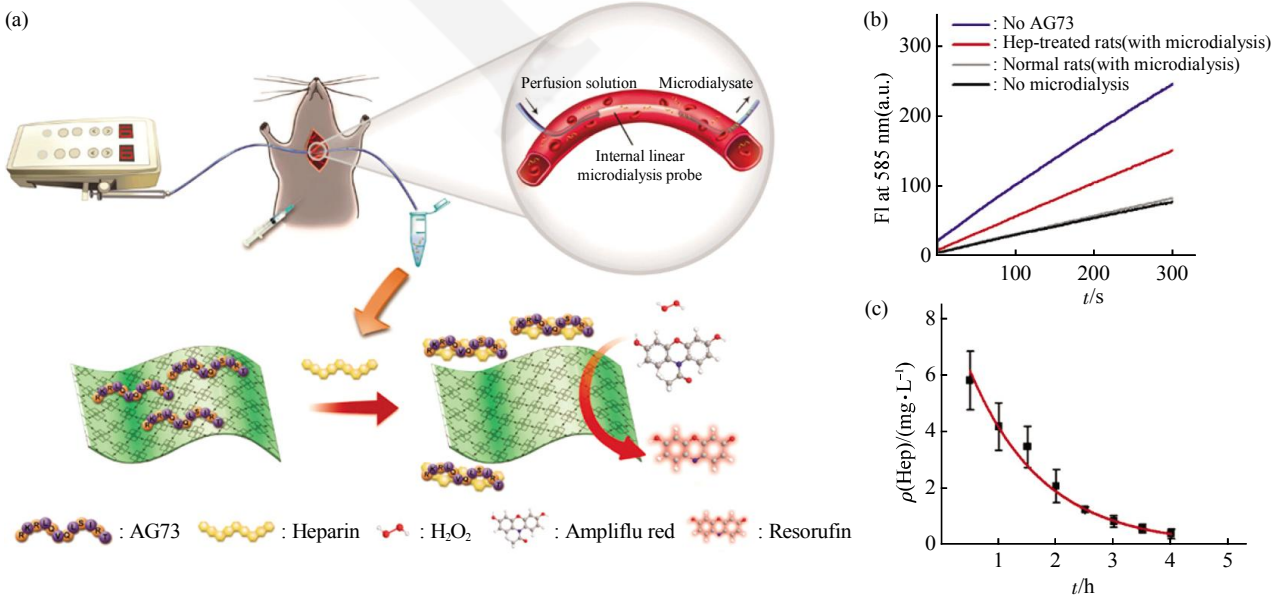

Fig. 21 Monitoring heparin metabolism in live rats with peroxidase-like 2D MOF

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Zn-TCCP (Fe) nanozyme and thus inhibit its activity. The presence of heparin would liberate the nanozyme by interacting with AG73 and recover its catalytic activity. Using such a competing assay, heparin elimination from live rats was successfully monitored.

3 Conclusions and prospects

In this review, we highlighted the recent progress of nanozymes in analytical applications. The promise of nanozymes for both *in vitro* and *in vivo* analytical applications was demonstrated using representative examples. For *in vitro* applications, bioactive small molecules (such as H_2O_2, glucose, lactate, choline, and cholesterol), nucleic acids, protein biomarkers (such as PSA), cancer cells, and ions have been detected with the use of nanozymes. For *in vivo* applications, they have been employed for monitoring bioactive molecules (such as glucose and lactate) in live brains and tumor tissues. They were also used to evaluate the therapeutic efficacy of some drugs and to study the metabolism of both bioactive molecules and nanozyme themselves.

As demonstrated by the exciting progress, the field of nanozyme research has attracted growing interests. Here we suggest several challenges that still remain to be addressed[2, 78]. First, quite limited studies have been devoted to the *in vivo* applications of nanozymes in analytical chemistry. Therefore, more studies are expected in the near future. However, to apply nanozymes for practical live assays, nanozymes with better activity, higher selectivity, and good biocompatibility are needed. Therefore, new design and synthetic strategies should be developed for better nanozymes. Second, the therapeutic applications of nanozymes have not been combined with their analytical ones. By designing and fabricating theranostic nanozymes, such a combination would provide a better way for future nanomedicine.

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纳米酶在分析化学中的应用研究：从体外检测到活体分析 *

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摘要 纳米酶是指具有类酶催化活性的纳米材料．近年来，纳米酶研究引起了人们的极大兴趣．纳米酶已被广泛应用于诸如生物传感、生物成像、疾病治疗和环境保护等众多领域．在本综述中，我们将着重讨论纳米酶在分析化学领域的研究进展．首先将讨论纳米酶在体外检测的应用，将包括生物活性小分子、核酸、蛋白质类生物标志物、细胞等的检测．其后将讨论纳米酶在活体分析的应用，将包括监测活脑、肿瘤组织等的生物活性小分子、药物的药效、药物与纳米酶的代谢等．最后，我们将讨论纳米酶应用于分析化学时面临的挑战和未来研究前景．

关键词 纳米酶，体外，活体，分析化学，模拟酶，仿生化学
学科分类号 O65，Q811 **DOI**: 10.16476/j.pibb.2017.0469

* 国家自然科学基金(21722503, 21405081), 国家重点基础研究发展计划(2015CB659400), 江苏高校优势学科建设工程、江苏省双创计划、生命分析化学国家重点实验室开放基金(SKLACLS1704)和国家千人计划青年项目资助.
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收稿日期：2017-12-18，接受日期：2018-01-19