Integrated nanozymes: facile preparation and biomedical applications
Jiangjixing Wu,† Sirong Li† and Hui Wei†

Nanomaterials have been viewed as the next generation of artificial enzymes due to their low cost, large specific surface area, and good robustness under extreme conditions. However, the moderate activity and limited selectivity of nanozymes have impeded their usage. To overcome these shortcomings, integrated nanozymes (INAzymes) have been developed by encapsulating two or more different biocatalysts (e.g., natural oxidases and peroxidase mimics) together within confined frameworks. On the one hand, with the assistance of natural enzymes, INAzymes are capable of specifically recognizing targets. On the other hand, nanoscale confinement brought about by integration significantly enhances the cascade reaction efficiency. In this Feature Article, we highlight the newly developed INAzymes, covering from synthetic strategies to versatile applications in biodetection and therapeutics. Moreover, it is predicted that INAzymes with superior activities, specificity, and stability will enrich the research of nanomaterials and pave new ways in designing multifunctional nanozymes.

1. Introduction
Developing robust enzyme mimics has been considered as an ideal solution to overcome natural enzyme limitations, such as laborious production, low stability, and high cost. Among the developed enzyme mimics, nanomaterials with enzyme mimicking activities (termed as nanozymes) have received considerable attention due to the burgeoning development of nanotechnology.1–7 A variety of nanomaterials have been explored to mimic enzymes, including noble metals,8–12 transition metal oxides,14–18 carbon materials19–22 and so on.23–28 Until now, nanozymes have found broad applications, ranging from bioanalysis14,29–35 and diagnosis36,37 to tissue engineering38,39 and environmental protection.40,41

Despite the remarkable achievements accomplished in the last few decades, moderate activity and limited selectivity have impeded the further use of nanozymes. Early studies on glucose detection using nanozymes indicated that high selectivity could be achieved by coupling a nanozyme with a natural enzyme – glucose oxidase (GOx) – for glucose detection.29,42–45 However, such coupled reactions still suffer from a limited overall efficiency because of the spatially separated reactions. Hence, rational design is of great need to advance high performance nanozymes with both improved selectivity and efficiency.

In this regard, nature has evolved a highly elegant strategy to perform cascade reactions by confining sequential enzymes within subcellular compartments. Such a sophisticated confinement enhances the overall efficiency of the cascade reactions by (a) providing a high local concentration of enzymes and substrates, (b) enabling efficient mass transfer, and (c) minimizing intermediate decomposition. These enhancement phenomena are collectively called “confinement effects” or “nanoscale proximity effects”. Various catalytic reactions verified the superiority of the aforementioned effects.46–49 For instance, Travis et al. promoted the efficiency of a 10-step enzymatic tandem reaction to convert glucose into lactate by immobilizing the enzymes on silica nanoparticles (NPs).50 Bao and co-workers have used a bioinspired strategy to develop interface-confined catalysts for a series of reactions (such as carbon monoxide oxidation and the oxygen reduction reaction).51

Such great successes encouraged us and others to apply the “nanoscale proximity effects” to designing integrated nanozymes (INAzymes) (Fig. 1 and Table 1). By encapsulating natural enzymes and nanozymes together within a nanoscaled framework, the designed INAzymes aim to overcome the moderate activity and limited selectivity issues of nanozymes. Numerous INAzymes have been developed and used in versatile applications. To highlight the progress of the INAzymes, this article first discusses the different synthetic strategies for INAzymes. Then, their representative biomedical applications are illustrated. Finally, the perspectives that may advance nanozyme research are discussed.
2. Synthetic strategies

Inspired by biological systems confining multiple enzymes within subcellular compartments, the INAzymes are usually synthesized with the help of a framework to host two or more nanozymes/enzymes. According to the types of frameworks used, the synthetic strategies of INAzymes are categorized as follows.

2.1 INAzymes based on metal–organic frameworks

Metal–organic frameworks (MOFs), a class of supporting matrix, were first demonstrated by our group to develop INAzymes through co-assembling enzymes and nanozymes. To integrate enzymes with nanozymes, a compatible porous framework must be chosen to tolerate the different reaction conditions. Due to its biocompatible synthesis, good crystallinity, and high surface-area-to-volume ratio, the zeolitic imidazolate framework (ZIF-8) was chosen as a MOF host for the nanoscaled cascade reaction. Through adding GOx and hemin during the self-assembly of Zn\(^{2+}\) and 2-methylimidazole, GOx and hemin were encapsulated into ZIF-8 simultaneously, as shown in Fig. 2A. The element mapping of Fe and fluorescence labelling of GOx were performed to validate the successful encapsulation of both hemin and GOx within ZIF-8 (Fig. 2B). Later, catalytic activity studies of the INAzyme and a mixture of separated GOx & hemin demonstrated that such confinement of cascade catalysts within a nanoscale space promoted the catalytic efficiency by 600% compared to that of the mixture of hemin@ZIF-8 and GOx@ZIF-8 (Fig. 2D). This enhancement arose from the confinement of multiple catalysts in the MOFs, which provided a high local concentration of enzymes and substrates, reduced the barrier of mass transfer, and minimized the decomposition of H\(_2\)O\(_2\). Additionally, the ZIF-8 framework protected the encapsulated enzymes from thermal denaturation. As shown in Fig. 2E, the INAzyme exhibited 500% higher activity than that of free GOx + HRP (horseradish peroxidase) after heat treatment. Good long-term storage stability of the INAzyme was also demonstrated as 85% activity was retained after 2 months’ storage at 4 °C treatment. (F) Normalized cascade enzymatic activity of freshly prepared and 2 month-old INAzymes. (G) Normalized catalytic activities of the INAzyme after recycling. (Adapted with permission from ref. 52. Copyright (2016) American Chemical Society.)

Table 1 Comparison of properties of INAzymes

<table>
<thead>
<tr>
<th>Enzyme + nanozyme</th>
<th>Cost</th>
<th>Thermal stability</th>
<th>Long-term stability</th>
<th>Recycle</th>
<th>Toxicology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme + nanozyme</td>
<td>Low</td>
<td>High</td>
<td>Months</td>
<td>Several times</td>
<td>Depending on framework</td>
</tr>
<tr>
<td>Enzyme + enzyme</td>
<td>High</td>
<td>Low</td>
<td>Days</td>
<td>Few times</td>
<td>Biocompatible</td>
</tr>
</tbody>
</table>

\(^a\) For comparison, “enzyme + enzyme” encapsulated within a framework is also listed.
LOx was co-assembled with hemin inside ZIF-8 to form a LOx/hemin@ZIF-8 INAzyme. Nearly 400% enhancement in the catalytic activity of LOx/hemin@ZIF-8 was observed compared with the mixture of LOx@ZIF-8 and hemin@ZIF-8. LOx/hemin@ZIF-8 also exhibited remarkable thermal stability relative to free LOx + HRP. Moreover, if another biocatalyst (such as invertase) was added, a triple INAzyme of invertase/GOx/hemin@ZIF-8 could be constructed as well. Such a confinement of cascade catalysts within the nanoscaled space would not only significantly improve the catalysts' stability but also effectively promote the sequential reactions with a 700% enhancement compared to that of the mixture of separated invertase@ZIF-8, GOx@ZIF-8, and hemin@ZIF-8 (Fig. 3B and D). Similarly, other nanozymes could also be encapsulated together with a natural enzyme into ZIF-8 to prepare high-performance INAzymes.\(^{53,54}\) For example, Dong et al. used NiPd instead of hemin as a peroxidase mimic and found that the hollow NiPd nanostructure would help capture more GOx molecules into ZIF-8. Therefore, the catalytic activity enhancement of the GOx/NiPd@ZIF-8 was attributed to the high local concentration of GOx.\(^{53}\)

Besides ZIF-8, other MOFs with porous and thermally stable structures were also used as the frameworks to construct INAzymes. For example, we developed the peroxidase-mimicking nanozyme AuNPs@MIL-101 by \textit{in situ} reducing a gold precursor within MIL-101 and then assembled natural enzymes onto the AuNPs@MIL-101 to obtain INAzymes (Fig. 4A).\(^{55}\) The assembled oxidase oxidized the substrate (\textit{i.e.}, glucose or lactate) to produce H\(_2\)O\(_2\), which would continue to oxidize leucomalachite green (LMG) into Raman-active malachite green (MG) via the AuNPs' catalysis, with surface-enhanced Raman scattering (SERS) signals generated for measurements (Fig. 4B). As expected, the cascade catalytic activities were distinguishable from that of AuNPs@GOx or AuNPs@LOx, owing to the improved stability and promoted cascade efficacy of the INAzymes (Fig. 4C and D).

Another interesting triple INAzyme of Pt@CuMOFs–hemin/G-quadruplex–GOx was reported by Xu and co-workers.\(^{56}\) The synthetic method was similar to that of Au@MIL-101@GOx. First, CuMOFs with a large surface area and high stability were selected to \textit{in situ} grow a gold precursor within MIL-101 and then assembled natural enzymes onto the AuNPs@MIL-101 to obtain INAzymes (Fig. 4A).\(^{55}\) The assembled oxidase oxidized the substrate (\textit{i.e.}, glucose or lactate) to produce H\(_2\)O\(_2\), which would continue to oxidize leucomalachite green (LMG) into Raman-active malachite green (MG) via the AuNPs' catalysis, with surface-enhanced Raman scattering (SERS) signals generated for measurements (Fig. 4B). As expected, the cascade catalytic activities were distinguishable from that of AuNPs@GOx or AuNPs@LOx, owing to the improved stability and promoted cascade efficacy of theINAzymes (Fig. 4C and D).

### 2.2 INAzymes based on mesoporous silica/carbon

In addition to MOFs, mesoporous silica-based nanomaterials, due to their large surface area, high loading capacity, and good biocompatibility, have been widely used as one of the most favorable biocatalyst supports.\(^{57,58}\) For instance, Shi et al. successfully loaded peroxidase-like small Fe\(_3\)O\(_4\) and GOx into the around 40 nm large mesopores of dendritic mesoporous silica NPs (DMSNs) to form the Fe\(_3\)O\(_4–GOx@DMSNs INAzyme, as shown in Fig. 5A.\(^{57}\) Such an integration would inhibit the decomposition of intermediate H\(_2\)O\(_2\) and result in efficient tumor-cell apoptosis. More details about the use of the INAzyme for tumor therapy are discussed in Section 3.3.

Several studies have also used mesoporous carbon as a framework to synthesize INAzymes.\(^{59–61}\) First, a carbon precursor and iron source were loaded into the mesopores of a silica foam template. Then, thermal treatment and silica removal resulted in magnetic mesoporous carbon. Finally, GOx was assembled into the magnetic mesocellular carbon to obtain the final nanocomposite.
silica hybrid (GS) to construct the GS–hemin–AuNPs nanohybrid (GSHA) INAzyme (Fig. 6A). This GS allowed the controlled spatial assembly of AuNPs and hemin. Due to the π–π stacking, the hemin would adsorb on the exposed graphene surface to form GS–hemin (GSH). Later, AuNPs grew on the silica surface via the electronic interaction between gold and the NH₂ group. Such an integration made the mass transfer efficient and inhibited the decomposition of H₂O₂, thus more than 200% 3,3,5,5-tetramethylbenzidine (TMB) was oxidized by GSHA compared with the mixture of GSH + GS–AuNPs (GSA) (Fig. 6B and C). While for the GS–AuNPs–hemin nanohybrids (GSAH), which grew AuNPs before hemin adsorption, lower catalytic activity was observed due to the inhibition of the GOx-like activity of the AuNPs by hemin. Furthermore, they used a permeable membrane to coat the mesoporous silica nanoparticle (MSN)–AuNPs before hemin adsorption. The obtained MSN–AuNPs–hemin INAzyme improved the catalytic activity by 150% compared to that of the mixture of MSN–AuNPs and MSN–hemin. However, it should be noted that the activity of the INAzyme is still lower than that of the natural enzyme coupled system (Fig. 6C). Thus, the development of new INAzymes with high activity and substrate specificity remains a goal, which we will discuss in the Conclusions and perspectives section.

2.3 INAzymes based on hydrogels

An interesting strategy for encapsulating the nanozymes into an in situ formed framework was reported by Wang and co-workers. As shown in Fig. 7, with the addition of glucose (Fig. 5B). This INAzyme exhibited excellent long-term stability as over 90% of the initial catalytic activity was retained after 2 months of storage at room temperature. In another study, Qu et al. integrated AuNPs (GOx mimic) and hemin (peroxidase mimic) into a graphene–mesoporous silica hybrid (GS) to construct the GS–hemin–AuNPs nanohybrid (GSHA) INAzyme (Fig. 6A). This GS allowed the controlled spatial assembly of AuNPs and hemin. Due to the π–π stacking, the hemin would adsorb on the exposed graphene surface to form GS–hemin (GSH). Later, AuNPs grew on the silica surface via the electronic interaction between gold and the NH₂ group. Such an integration made the mass transfer efficient and inhibited the decomposition of H₂O₂, thus more than 200% 3,3,5,5-tetramethylbenzidine (TMB) was oxidized by GSHA compared with the mixture of GSH + GS–AuNPs (GSA) (Fig. 6B and C). While for the GS–AuNPs–hemin nanohybrids (GSAH), which grew AuNPs before hemin adsorption, lower catalytic activity was observed due to the inhibition of the GOx-like activity of the AuNPs by hemin. Furthermore, they used a permeable membrane to coat the mesoporous silica nanoparticle (MSN)–AuNPs before hemin adsorption. The obtained MSN–AuNPs–hemin INAzyme improved the catalytic activity by 150% compared to that of the mixture of MSN–AuNPs and MSN–hemin. However, it should be noted that the activity of the INAzyme is still lower than that of the natural enzyme coupled system (Fig. 6C). Thus, the development of new INAzymes with high activity and substrate specificity remains a goal, which we will discuss in the Conclusions and perspectives section.

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to a mixture of GOx, CuO, and the monomer N,N-dimethylacrylamide, hydroxyl radicals (·OH) would be generated by decomposing H2O2. The ·OH triggered the mild polymerization of the monomer to form a nanozyme–gel INAzyme system under physiological conditions. By changing the GOx/glucose to H2O2, the nanozyme–gel could not be obtained. This is because more oxygen in this system would generate peroxyl radicals rather than hydroxyl radicals, and the former inhibited the monomer polymerization.

### 2.4 INAzymes via a layer-by-layer strategy

Unlike the above-mentioned synthetic strategies, an additional host is not necessary when developing INAzymes through a layer-by-layer strategy. Hence, multi-dimensional nanostructures could be fabricated to realize localized tandem reactions, as shown in Fig. 8. Qu and co-workers used poly-dopamine (PDA) to link V2O5 nanowires (serving as glutathione peroxidase (GPx) mimic) and AuNPs (GOx mimic)65 or MnO2 NPs (acting as superoxide dismutase (SOD) and catalase mimics).66 Therefore, a multifunctional INAzyme was obtained (Fig. 8A). Similarly, Fe3O4 capped by different surfactants could immobilize GOx through the strong hydrophobic interaction67 or electrostatic attraction68,69 between capping agents and GOx to form Fe3O4/GOX INAzymes.

In addition, by directly depositing oxidase-like AuNPs into peroxidase-like 2D MOFs, AuNPs/Cu–TCPP(M) INAzymes (TCPP, tetra(4-carboxyphenyl)porphine; M = Fe, Co) were produced, which were then used for in vitro glucose detection (Fig. 8B).70 It should be noted that the 2D MOFs used here were peroxidase mimics, while the MOFs (i.e., ZIF-8 and MIL-101) chosen in the former INAzymes only served as a support without any catalytic activities.

Likewise, the direct growth of AuNPs onto the surface of Fe3O4 led to Fe3O4–AuNPs INAzymes with both GOx- and peroxidase-like activities. Furthermore, to engineer the dispersibility and stability of the INAzymes, a mesoporous SiO2 (meso-SiO2) layer was introduced onto the surface of the Fe3O4–AuNPs, as shown in Fig. 8C.71 The catalytic activity of the Fe3O4–Au@meso-SiO2 exhibited 300% enhancement compared to that of the mixture of Fe3O4 and AuNPs. With the protection of meso-SiO2, more than 95% activity and the intact structure of Fe3O4–Au@meso-SiO2 microspheres can be maintained after 6 cycles of reaction. While for the mixture of Fe3O4 and AuNPs, serious aggregation of the nanoparticles after only one cycle decreased the catalytic activity by more than 50%.

### 3. Applications of INAzymes

#### 3.1 INAzymes for in vitro sensing

Benefiting from the nanoscale proximity effect in Fig. 2C, INAzymes perfectly suit the tandem sensing strategy where the products catalyzed by the first reaction would be consumed instantly as reactants of the second reaction to produce the signalling products. As a result, glucose, lactate, and cholesterol could be detected in vitro by integrating GOx/GOx-like AuNPs/LOx/cholesterol oxidase with peroxidase-like nanozymes (Table 2). High specificity and efficiency were obtained with these INAzymes. For example, the V2O5–PDA–AuNP INAzyme displayed good sensitivity and selectivity towards glucose detection, with a detection limit of
<table>
<thead>
<tr>
<th>INAzymes</th>
<th>Target</th>
<th>Method</th>
<th>Linear range</th>
<th>Detection limit</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOx@hemin@ZIF-8</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>0–250 µM</td>
<td>1.7 µM</td>
<td>Substrate: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) Selectivity against: acrobatic acid, lactate, dopamine, 3,4-dihydroxyphenylacetic acid, uric acid, and 5-hydroxtryptamine.</td>
<td>52</td>
</tr>
<tr>
<td>GOx/NiPd@ZIF-8</td>
<td>Glucose</td>
<td>Colorimetric &amp; electrochemical</td>
<td>10–300 µM &amp; 0.1–1.7 mM</td>
<td>9.2 µM</td>
<td>Substrate: o-phenylenediamine Selectivity against: fructose, maltose, lactose, and sucrose</td>
<td>53</td>
</tr>
<tr>
<td>GOx/Fe₃O₄@ZIF-8</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>5–150 µM</td>
<td>1.9 µM</td>
<td>Substrate: o-phenylenediamine Selectivity against: sodium chloride, calcium chloride, potassium nitrate, fructose, mannose, galactose, and bovine serum albumin</td>
<td>54</td>
</tr>
<tr>
<td>AuNPs@MIL-101@GOx</td>
<td>Glucose</td>
<td>Raman</td>
<td>10–200 µM</td>
<td>4.2 µM</td>
<td>Substrate: leucomalachite green Selectivity against: acrobatic acid, dopamine, 3,4-dihydroxyphenylacetic acid, uric acid, and 5-hydroxtryptamine.</td>
<td>55</td>
</tr>
<tr>
<td>Fe₃O₄/GOx/mesoporous silica</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>30–1000 µM</td>
<td>3 µM</td>
<td>Substrate: TMB Selectivity against: lactose, arabinose, galactose, and fructose</td>
<td>58</td>
</tr>
<tr>
<td>MNP/GOx/mesoporous carbon</td>
<td>Glucose</td>
<td>Electrochemical</td>
<td>0.5–10 mM</td>
<td>0.2 mM</td>
<td>The composite was used to construct a carbon paste electrode. Substrate: TMB Selectivity against: fructose, lactose, and maltose</td>
<td>59</td>
</tr>
<tr>
<td>Casein-Fe₃O₄/GOx</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>3–1000 µM</td>
<td>1 µM</td>
<td>Substrate: ABTS Selectivity against: arabinose, cellobiose, galactose, lactose, maltose, raffinose, and xylose.</td>
<td>67</td>
</tr>
<tr>
<td>Poly(diallyldimethylammonium chloride)-GOx-Fe₃O₄ composite</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>30–100 µM &amp; 0.2–1 mM</td>
<td>30 µM</td>
<td>Substrate: ABTS Selectivity against: fructose, lactose, sucrose, sodium chloride, and albumin</td>
<td>68</td>
</tr>
<tr>
<td>Poly(diallyldimethylammonium chloride)-GOx-Fe₃O₄ composite</td>
<td>Glucose</td>
<td>Fluorometric</td>
<td>3–9 µM &amp; 20–100 µM</td>
<td>3 µM</td>
<td>Substrate: amplex ultrared Selectivity against: arabinose, cellobiose, galactose, lactose, maltose, raffinose, and xylose.</td>
<td>69</td>
</tr>
<tr>
<td>GOx/Fe₃O₄@Fe³⁺/adenosine 5′-monophosphate coordination polymers</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>0–100 µM</td>
<td>1.4 µM</td>
<td>Substrate: ABTS Selectivity against: fructose, galactose, sucrose, sodium chloride, and albumin</td>
<td>72</td>
</tr>
<tr>
<td>GOX/Fe₃O₄/graphene oxide nanocomposite</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>0.5–600 µM</td>
<td>0.2 µM</td>
<td>Substrate: ABTS Selectivity against: fructose, lactose, sucrose, sodium chloride, and albumin</td>
<td>73</td>
</tr>
<tr>
<td>AuNPs/Cu-TCPP[M] (M = Fe, Co)</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>10–300 µM</td>
<td>8.5 µM</td>
<td>Substrate: TMB Selectivity against: fructose, lactose, and maltose</td>
<td>70</td>
</tr>
<tr>
<td>Fe₃O₄–Au@meso-SiO₂ microspheres</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>10–130 µM</td>
<td>0.5 µM</td>
<td>Substrate: TMB Selectivity against: fructose, lactose, and maltose</td>
<td>71</td>
</tr>
<tr>
<td>V₂O₅–PDA-AuNP</td>
<td>Glucose</td>
<td>Colorimetric</td>
<td>0–10 µM</td>
<td>0.5 µM</td>
<td>Substrate: ABTS Selectivity against: fructose, lactose, and maltose</td>
<td>65</td>
</tr>
<tr>
<td>AuNPs@MIL-101@LOx</td>
<td>Lactate</td>
<td>Raman</td>
<td>10–200 µM</td>
<td>5.0 µM</td>
<td>Substrate: leucomalachite green Selectivity against: acrobatic acid, dopamine, 3,4-dihydroxyphenylacetic acid, uric acid, and 5-hydroxtryptamine.</td>
<td>55</td>
</tr>
<tr>
<td>Fe₃O₄/cholesterol oxidase/mesoporous silica</td>
<td>Cholesterol</td>
<td>Colorimetric</td>
<td>10–250 µM</td>
<td>5 µM</td>
<td>Substrate: TMB Selectivity against: glycerol, and glucose</td>
<td>58</td>
</tr>
<tr>
<td>Poly(diallyldimethylammonium chloride)-galactose oxidase–Fe₃O₄ composite</td>
<td>Galactose</td>
<td>Fluorometric</td>
<td>2–80 µM</td>
<td>2 µM</td>
<td>Substrate: amplex ultrared Selectivity against: fructose, lactose, and maltose</td>
<td>69</td>
</tr>
<tr>
<td>Poly(diallyldimethylammonium chloride)-choline oxidase–Fe₃O₄ composite</td>
<td>Choline</td>
<td>Fluorometric</td>
<td>20–100 µM</td>
<td>20 µM</td>
<td>Substrate: amplex ultrared Selectivity against: fructose, lactose, and maltose</td>
<td>69</td>
</tr>
<tr>
<td>V₂O₅–PDA-AuNP</td>
<td>Single-stranded DNA Carcinoembryonic antigen (CEA)</td>
<td>Colorimetric</td>
<td>0–0.1 µM</td>
<td>2.3 nM</td>
<td>Substrate: ABTS Selectivity against: 3,3-diaminobenzidine Selectivity against: thombin, human immunoglobulin G, alpha-fetoprotein, and prostate-specific antigen</td>
<td>65</td>
</tr>
<tr>
<td>Pt@CuMOFs–hemin/G-quadruplex-GOx</td>
<td>Glucose</td>
<td>Electrochemical</td>
<td>0.05–20 ng mL⁻¹</td>
<td>0.023 pg mL⁻¹</td>
<td>Substrate: 3,3-diaminobenzidine Selectivity against: thombin, human immunoglobulin G, alpha-fetoprotein, and prostate-specific antigen</td>
<td>56</td>
</tr>
</tbody>
</table>
as low as 0.5 μM. In addition by taking advantage of the different affinities of single-stranded DNA and double-stranded DNA towards AuNPs, the GOx-like activity of AuNPs would be suppressed to a different extent. Therefore, colorimetric sensing of target DNA was achieved with the V$_2$O$_5$–PDA–AuNP INAzymes.

An innovative concept for an electrochemical impedimetric aptasensor for carcinoembryonic antigen (CEA) was reported by using the Pt@CuMOFs–hemin/G-quadruplex–GOx triple INAzyme. The CEA aptamers (Apt1 and Apt2) were immobilized onto an AuNP-modified electrode and the triple INAzyme, respectively. In the presence of the target CEA, a sandwich-type assay was fabricated with INAzymes captured onto the electrode surface, and then nonconductive insoluble precipitates would form to give the impedimetric signal. With the developed electrochemical impedimetric aptasensor, the response signal was efficiently amplified by the enhanced catalytic activities of the INAzyme. As low as 0.023 pg mL$^{-1}$ of target CEA was detected, which made clinical detection promising.

3.2 INAzymes for in vivo sensing

As mentioned above, the promoted activity and enhanced stability of INAzymes offered the possibility of functioning under complex physiological conditions. Recently, we have engineered an INAzyme for in vivo neurochemical monitoring in living brains. First, the dynamic linear range from 0 to 250 μM obtained from in vitro sensing enabled the further measurements of cerebral glucose in living brains, as this range covered the physiological levels of microdialyzed glucose. Then, an off-line detection of glucose in living brains was developed with the help of in vivo microdialysis (Fig. 9A). Through mixing the microdialyzed samples with the INAzyme and substrates, the products catalysed by cascade reactions would be generated for signalling. For the GOx@hemin@ZIF-8 INAzyme, both 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and Ampliflu Red were used for producing green-colored and fluorescent signals, respectively. However by using AuNPs@MIL-101@GOx, Raman spectra of malachite green would be produced for signalling. Both INAzyme systems showed a decreased glucose level after global cerebral ischemia, as the blockage of cerebral blood flow resulted in an enhanced metabolism consumption.

Furthermore, the SERS active INAzyme was applied for evaluating the therapeutic effects of potential drugs like astaxanthin (ATX). If ischemic stroke occurred, the striatum glucose level would decrease while the lactate level would increase. When an anti-oxidation drug like ATX was used for treatment, both the glucose and lactate fluctuation level should be suppressed. Fig. 9B and C demonstrate that ATX could be an effective drug for alleviating cerebral ischemic injuries. In addition to monitoring glucose and lactate in living brains and evaluating the therapeutic effects, this effective bioassay based on a SERS INAzyme could also discriminate abnormal metabolism in tumors from that in normal tissues. Concentrations of 83.3 ± 4.7 and 16.1 ± 4.1 μM were measured for glucose and lactate in normal tissues. Attributed to the Warburg effects of tumors, the concentration of glucose decreased as low as to be barely measurable. However the lactate amount displayed was 187.7 ± 98.7 μM, nearly 10 times higher than that of normal tissues.

Though the INAzyme could be used for effective detection of glucose in living rats, the off-line sensor still suffered from a poor temporal resolution, which hence limits future practical applications. To overcome this limitation, we went on to construct an online measurement platform by immobilizing the INAzyme into the channel of a microfluidics chip. When assisted with in vivo microdialysis, the dynamic changes of cerebral glucose following global ischemia and perfusion could be successfully monitored with the INAzyme-based analytic platform (Fig. 10A). Due to the fast reaction and strong fluorescence of the oxidized products, Ampliflu Red was chosen to meet the requirements of continuous in vivo measurements. As shown in Fig. 10B, the glucose levels decreased to 49.1 ± 12.7% of the basal level after global ischemia, which was consistent with the off-line measured values 50.2 ± 8.3%. Later, the value was restored to 98.4 ± 10.1% of the basal level during the reperfusion process. This prototypical device may aid researchers to explore with profundity the mechanism of an unclear illness by real time monitoring.

Another important aspect of in vivo sensing is the biomedical imaging of a cell or organ, which was demonstrated by Shi et al. The Fe$_3$O$_4$–GOx@DMSNs INAzyme (Fig. 5A) was used to produce harmful hydroxyl radicals in situ in cancer cells through the designed cascade reaction. Then, the produced hydroxyl radicals were imaged by using a reactive oxygen species (ROS) fluorescent probe, 2′,7′-dichlorofluorescein diacetate. And a quantitative evaluation of the generated hydroxyl radicals and mapping of the glucose distribution in cancer cells could be achieved through analysing the corresponding fluorescence intensity.

3.3 INAzymes for therapeutics

Nanozymes have been exploited for potential therapeutics via eliminating ROS, such as H$_2$O$_2$, *OH, and *O$_2$. To provide an
enhanced antioxidative effect, Qu and co-workers developed a V2O5–PDA–MnO2 INAzyme to mimic natural ROS defenders with improved catalytic activity and efficiency.66 For this INAzyme, MnO2 NPs served as SOD and catalase mimics while V2O5 acted as a GPx mimic, which mimicked the intracellular antioxidant defense process where SOD, CAT, and GPx co-participated. Even PDA was also found to be an efficient antioxidant. Owing to the excellent ROS scavenging abilities of assembled INAzymes, an anti-inflammation model was successfully built (Fig. 11). After the treatment of the V2O5–PDA–MnO2 nanocomposites, the fluorescence intensity of the phorbol 12-myristate 13-acetate (PMA)-treated ear decreased.

In contrast to eliminating ROS for anti-oxidation/anti-inflammatory effects, several studies reported the applications of nanozyme-produced $^{15}$OH for anti-bacteria or anti-tumor use. For instance, Wang and co-workers fabricated an INAzyme based on a hydrogel system with $^{15}$OH for antibacterial effects. The cell wall structure of the bacterial membrane could be destroyed through oxidizing the unsaturated bonds of the phospholipids by $^{15}$OH.64 Recently, Shi et al. demonstrated that $^{15}$OH could do harm to cancer cells.57 As shown in Fig. 12A, ultrasmall Fe3O4 together with natural GOx were encapsulated in dendritic mesoporous silica nanoparticles forming GOx–Fe3O4@DMSNs nanocatalysts (GFD NCs). After being taken up by cancer cells, H2O2 produced from oxidation of glucose by GOx decomposed into harmful hydroxyl radicals during the catalysis process of Fe3O4. Meanwhile, the consumption of glucose would also starve the cancer cells. Hence, therapeutic INAzymes with the ability to kill cancer cells were established.

Before therapy, the in vivo biosafety issue and pharmacokinetics of intravenously injected GFD NCs were investigated. Compared with the control group, the administration of GFD NCs did not show any significant effect on the mouse growth and organ functions, indicating the high biocompatibility of such INAzymes. Besides, the effective accumulation in tumors, the increasing distribution kinetics in the whole blood, as well as the 2.65 h circulating half-life all demonstrated the improved pharmacokinetics.

Then, to verify the therapeutics effects, 4T1 mammary tumor xenografts on specific pathogen-free BALB/c nude mice were treated with GFD NCs both intravenously and intratumorally (Fig. 12B). Fig. 12C–H demonstrates that 4T1 mammary tumor
Effective tumor therapy. Besides, the biodegradation studies of INAzymes were further demonstrated on another tumor xenograft (U87 glioblastoma model), which provided a promising strategy for the tumor-suppression rates were estimated to be 64.67% and 68.89%, respectively. And the general applications of therapeutics have been summarized and discussed in detail. Many exciting advancements have been achieved with high-performance INAzymes showed a similar high biocompatibility and better pharmacokinetics profiles, and immunogenicity should be fully investigated to further advance the field. Some preliminary results about the toxicity and pharmacokinetics have been reported, which indicated the potential biocompatibility and good pharmacokinetics profiles of INAzymes. Compared with human recombinant enzymes and enzymes encapsulated/modified with biocompatible and FDA-approved polymers, the INAzymes showed a similar high biocompatibility and better pharmacokinetics. Further immunogenicity should also be exploited.

4. Conclusions and perspectives

Nanozymes, as one of the most emerging branches in the field of nanotechnology, though gaining achievable outcomes, still face numerous challenges. Integrating different nanozymes/enzymes as a whole has partially solved the limitations of insufficient activity and poor selectivity. In this Feature Article, synthetic strategies and biomedical applications of INAzymes have been summarized and discussed in detail. Many exciting advancements have been achieved with high-performance INAzymes, including engineering INAzymes for in vivo cerebral sensing. However, some critical issues still need to be addressed for the further development of INAzymes.

(a) We speculate that by taking advantage of the multiple enzyme mimicking activities of nanozymes, single component INAzymes could be constructed to realize a cascade reaction. Such all-in-one nanozymes may reveal insights into the ultimate form of INAzymes, but the mechanism of multi-enzyme mimicking nanozymes remains to be investigated.

(b) While the robustness of single component INAzymes may save cost, the specificity is sacrificed without the involvement of natural enzymes. Therefore, the rational construction of protein-like binding pockets or the utilization of molecularly imprinted polymers may be considered in further INAzyme design.

c) The efficiency and activity of INAzymes was enhanced, but is still lower than those of natural enzymes. As the activity of nanozymes mainly comes from the nanomaterials themselves, high-performance INAzymes could be developed by engineering the physical and chemical properties of nanozymes.

d) Although some achievements have been made for the in vivo biomedical applications of INAzymes, various problems such as clinical toxicity, poor pharmacokinetics profiles, and immunogenicity should be fully investigated to further advance the field.

(e) Currently, the therapeutic studies are mainly focused on cancer. Therapeutics for other diseases could be investigated by modulating the ROS levels with INAzymes. Besides sensing and therapeutics, other interesting applications could be explored. For example, SERS active INAzymes have been applied for evaluating the therapeutic efficacy of the ATX drug for ischemic stroke. Therefore, further drug screening would be possible with the help of INAzymes. Developing INAzyme based biomedical devices could be another interesting direction. Besides the biomedical field, the application of INAzymes should also be explored in the environment, agriculture, and national security fields in future studies.

(f) Still, the types of nanozymes and INAzymes constructed by nanomaterials are mainly focused on redox catalysis.
In a broader sense, it is important to design and prepare new nanozymes and iNAzymes for other important biocatalytic reactions, supplementing natural and re-combinant enzymes for practical applications.28

Conflicts of interest
There are no conflicts to declare.

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Notes and references