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Review

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Filling in the Gaps between Nanozymes and Enzymes: Challenges and Opportunities

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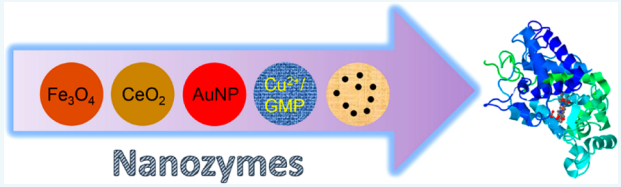
ABSTRACT: Using nanomaterials to mimic the function of protein enzymes is an interesting idea. Many nanomaterials have a similar size as enzymes and they also possess catalytic activity. Over the past decade, a surge of nanozyme work has emerged, likely due to the advancement in the synthesis and characterization of inorganic nanoparticles. Many typical enzymatic reactions mimicking oxidases, peroxidases, laccases, superoxide dismutases, and catalases have been realized by simple metal oxide and metal nanoparticles. In addition, small inorganic catalysts have been loaded in nanoparticles to create another type of nanozyme. The applications of nanozymes in biosensor design, environmental remediation, and therapeutics have been demonstrated. In this Topical Review, we briefly summarize the current status of the field and then focus our attention on some important problems faced by the field. These topics include developing better nanozymes with higher activity, better substrate selectivity, and engineering enzyme-like active sites. For practical applications, reliable methods for bioconjugation of nanozymes with affinity ligands need to be achieved, but not at the cost of losing the activity of nanozymes. Finally, fundamental mechanistic studies are needed to rationally design nanozymes and to obtain key insights into a few model systems.



INTRODUCTION

Enzymes are powerful biocatalysts and it has long been believed that all enzymes are proteins. In the early 1980s, the concept of enzyme has been extended to RNA by the discovery of ribozymes.[1] Since even earlier, developing enzyme mimics has been a very inspiring topic in chemistry. Various molecules have been tested from antibodies,[2] DNA,[3,4] to small molecules for this purpose.[5]

Also in the last few decades, the synthesis and characterization of nanomaterials have developed to an impressive level with exquisite control in size, shape, and composition.[6,7] Some nanoparticles have a size similar to that of proteins and are also catalytically active.[8] A few nanoparticles produce the same catalytic products as natural enzymes using the same substrates.[9,10] The name "nanozyme" has been coined to describe this type of enzyme-mimicking nanomaterial. Compared to typical inorganic catalysts, which often work at high temperature, high pressure, and extreme pH conditions, nanozymes work at ambient and close to physiological conditions with biologically relevant substrates/products.

Nanozyme is an interesting concept since it links nanomaterials with biological systems and functions. The methods and tools of enzyme characterization can be applied to nanozymes. Since nanozymes catalyze the same substrates, assays developed for protein enzymes can be directly grafted to nanozymes. At the same time, nanozymes are much more cost-effective for production and more robust for application.

To date, various types of nanozymes have been reported, and their applications in biosensor development, environmental remediation, and therapeutics have been demonstrated. However, there are still quite large gaps between nanozymes and protein-based enzymes, such as lower activity and turnover, and lack of specificity. In addition, most of nanozyme reactions are redox related. In this Topical Review, we briefly summarize the current status of the field and outline a few scientific and technological challenges that need to be addressed. We hope this discussion can spark future research ideas to address critical problems for advancing this field.

REPRESENTATIVE NANOZYMES

Excellent reviews are available describing different types of nanozymes,[9,10] and here we only introduce a few representative examples to facilitate our discussion. As an early example, iron oxide (e.g., Fe_3O_4) nanoparticles (NPs) have been found to have peroxidase-like activity,[11] meaning that they catalyze substrate oxidation in the presence of H_2O_2. Many standard chromogenic peroxidase substrates can be converted (Figure 1A). Iron oxide is highly attractive since it is biocompatible and magnetic, allowing potential in vivo theranostic applications.[12,13] Later, it was discovered that many nanomaterials have similar peroxidase-like activities.[14]

In comparison, the number of materials with oxidase-like activity (without H_2O_2 needed and directly uses O_2) is much lower, and a representative example is CeO_2 NP (nanoceria, Figure 1B). Nanoceria has a number of enzyme-like activities mimicking oxidase,[15] catalase, and superoxide dismutase,

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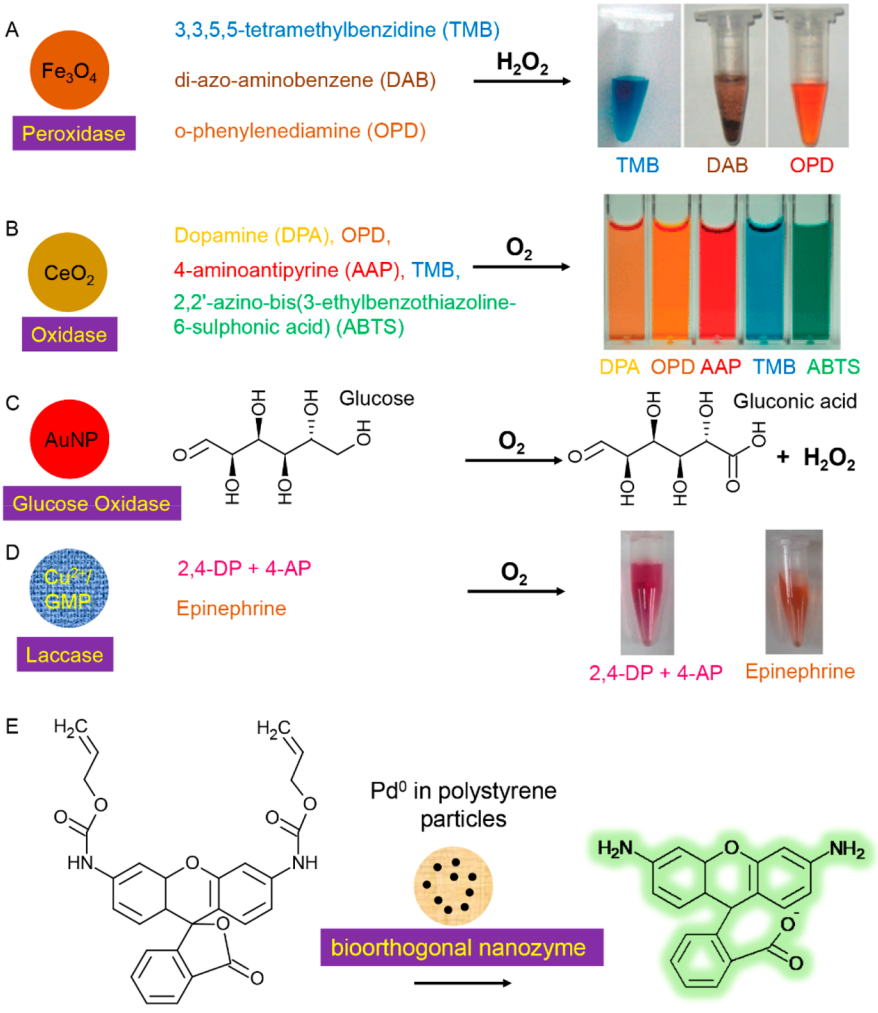

Figure 1. Schemes of representative nanozyme reactions using (A) Fe_3O_4 NPs to mimic peroxidase; (B) CeO_2 NPs to mimic oxidase; (C) AuNPs to mimic glucose oxidase; and (D) Cu^{2+}/GMP coordination NPs to mimic laccase. The color pictures of some typical reaction products are shown and they are adapted with permission from ref 11 for (A), copyright Nature Publishing Group 2007; ref 19 for (B), copyright Royal Society of Chemistry 2011, and ref 18 for (D), copyright American Chemical Society 2017. (E) Nanozymes loaded with a bioorthogonal Pd^0-based catalyst converting a nonfluorescent substrate into a fluorescent compound after the allylcarbamate cleavage reaction.[20]

respectively.[16] Gold nanoparticles (AuNPs) can mimic glucose oxidase (GOx), oxidizing glucose in the presence of oxygen to make H_2O_2 and gluconic acid, the same products as GOx produces (Figure 1C).[17] Cu^{2+} complexed with nucleotides such as guanosine monophosphate (GMP) has laccase-like activity.[18] Laccases are considered to be green enzymes since they do not need or produce H_2O_2 and they can oxide many environmental pollutants (Figure 1D).

The above examples rely on the intrinsic catalytic properties of inorganic nanomaterials. Another approach loads homogeneous catalysts into nanoscale (or sometimes microscale) particles.[20−22] The particle does not directly participate in catalysis, although it could indirectly influence reaction kinetics.[22] The main role of the particle is for intracellular delivery, increasing catalyst solubility, and lowering toxicity.[23,24] Within this context, the choice of catalyst is extremely versatile. Figure 1E shows such an example where a polystyrene bead was loaded with a Pd^0-based catalyst, catalyzing an allylcarbamate cleavage reaction and producing a fluorescent product.

APPLICATIONS OF NANOZYMES

Being enzyme mimics, typical applications of enzymes can be expected for nanozymes also. Broadly speaking, analytical, biomedical, and environmental applications are the main ones. One of the most explored aspects is biosensor development. For example, nanozymes can replace protein enzymes in bioanalytical assays and the most classic example is enzyme-linked immunosorbance assays (ELISA). Instead of using horseradish peroxidase (HRP) labeled secondary antibodies, iron oxide NPs were used (Figure 2A).[11] Nanozymes can also detect the substrate such as H_2O_2, and the cost-saving aspect is quite attractive.[25] In addition, assays have been developed for detecting both promotors and inhibitors of nanozymes. For example, Hg^{2+} was found to accelerate the peroxidase-like activity of AuNPs,[26] and fluoride strongly promoted the oxidase-like activity of nanoceria (Figure 2B).[27] These discoveries have been used to detect Hg^{2+} and F^-, respectively. Nanozymes were also used for building sensor arrays for pattern recognition based detection.[28]

Another application front has been focused on biology and biomedical science.[13,16] For example, nanoceria has been extensively tested for its ability to remove reactive oxygen

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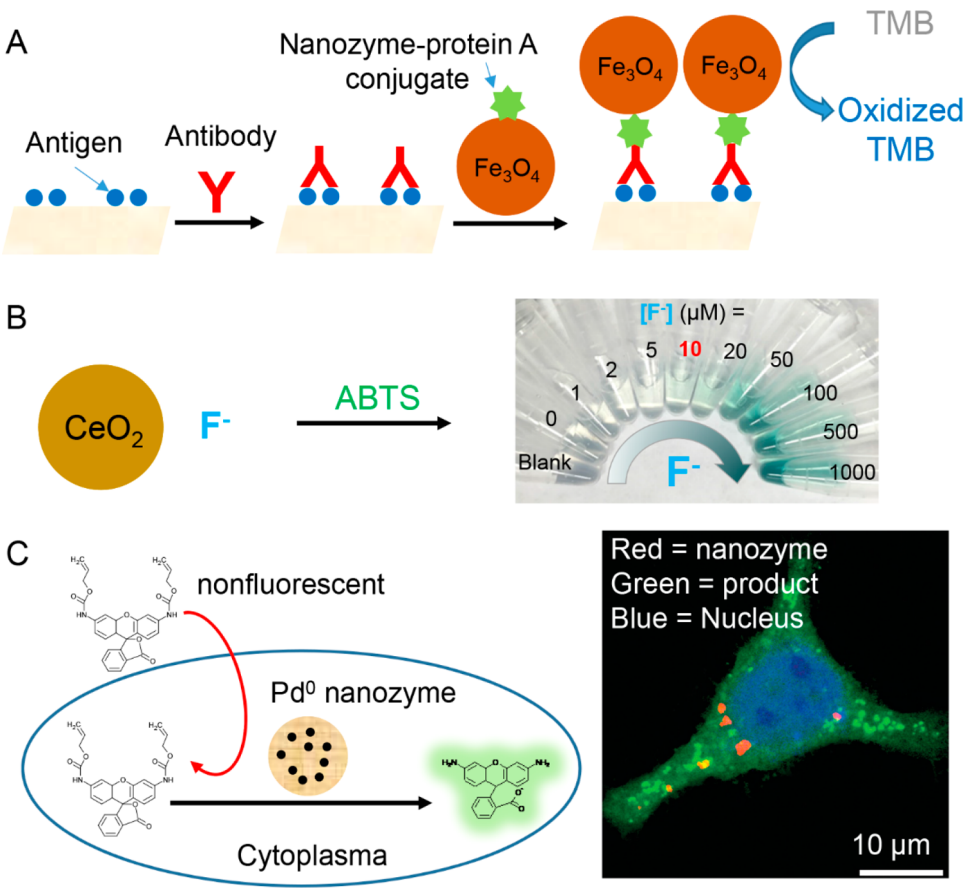

Figure 2. Representative applications of nanozymes. (A) Using Fe_3O_4 peroxidase nanozyme in immunoassays. (B) Detecting F^- based on its promotor effect in the oxidase-like activity of nanoceria. Partially adapted from ref 27 with permission. Copyright 2016 Royal Society of Chemistry. (C) A scheme of a bioorthogonal Pd^0-based nanozyme. After internalized by cells, the nanozyme then converts a nonfluorescent substrate into a fluorescent product in the complex cell matrix. The molecular structures are in Figure 1E. A fluorescence micrograph of a cell is also shown. Adapted from ref 20 with permission. Copyright 2011 Nature Publishing Group.

species.[16,29,30] The concept of biorthogonal reaction has been quite popular since the early 2000s, and various catalysts have been developed so that specific reactions can take place even in a complex intracellular matrix.[31] By loading such catalysts in nanoparticles, the resulting nanozymes have also been explored for biorthogonal catalysis inside cells. For example, using Pd^0 catalyst loaded polystyrene particles, Bradley and co-workers carried out allylcarbamate cleavage and Suzuki−Miyaura cross-coupling inside cells.[20] Figure 2C depicts such an application, where the nanozyme in Figure 1E was delivered into cells and a nonfluorescent substrate was added.[20] Those diffused into the cells were converted to a fluorescent product by this biorthogonal nanozyme. A fluorescence cell micrograph is also shown in the figure.

CHALLENGES AND OPPORTUNITIES

The last ten years (from 2007 to 2017) has experienced drastic growth in the nanozyme field. The type of reaction and number of nanozymes have both expanded significantly. Various applications and preliminary mechanistic studies have been pursued. With such exciting achievements, the field has also realized some limitations. For example, introducing the concept of nanozyme to biochemists might be problematic. Enzymes have a few basic properties, such as very high activity and excellent substrate specificity, while most nanozymes have yet to reach this level of sophistication. These limitations can in turn be opportunities for future research and development.

Improving Activity. For certain reactions, nanozyme activity can rival that of protein enzymes, such as using small gold nanoparticles to mimic glucose oxidase.[17,32] Some peroxidase-mimicking enzymes can also rival HRP. However, there is still a lot of room for improvement. Since nanozymes' reactions take place on the surface of nanomaterials, surface modification represents a useful way.[33] For example, we discovered that adding fluoride can significantly boost the oxidase-like activity of nanoceria, which was attributed to the adsorption of highly electronegative fluoride.[27] Current discoveries on this front are mainly accidental, and we expect more rational designs by learning from natural enzymes and computational chemistry. For example, Yan and workers studied the activity site of peroxidase enzymes and grafted a histidine on iron oxide surface to mimic the enzyme active site and this indeed boosted its activity (Figure 3).[34] Through rational design, Ni^{3+} was introduced into a nanozyme whose activity was 58-fold than that of NiO.[35] Combinatorial synthesis and screening of materials could be another way moving forward. By systematically doping and surface modification together with measuring the effect of buffer conditions can produce not only a large amount of data but also fundamental insights. In addition, through materials synthesis, it is possible

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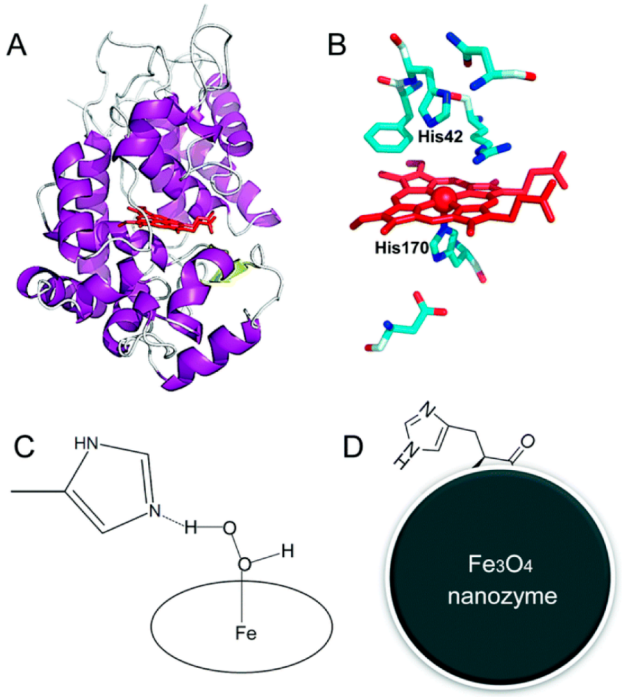

Figure 3. Improving nanozyme activity by mimicking the active site in enzymes. (A) The structure of HRP from PDB entry: 1HCH. (B) The active site in HRP containing two critical histidine residues. (C) Hydrogen bond between histidine residual and H_2O_2 in the initial state of catalysis of HRP. (D) Adsorption of histidine on Fe_3O_4 nanozyme to better mimic HRP. Figure adapted from ref 34 with permission. Copyright 2017 Royal Society of Chemistry.

to couple a few nanozymes together to form efficient enzyme cascade reactions.[36]

Improving Specificity. Compared to low activity, the lack of specificity is an even more serious concern. Enzyme specificity is one of the fundamental properties of protein enzymes. For example, glucose oxidase only converts glucose, while AuNPs can oxidase many reducing sugars.[37] This is not surprising, since without a substrate binding pocket, potential substrates diffused to nanozymes' surface may all react. This problem can be solved by introducing substrate binding ligands such as antibodies and aptamers. An interesting example was demonstrated by Willner and co-workers who linked aptamers to a DNAzyme (another type of enzyme mimic),[38] and the aptamer target was oxidized faster. While in principle it is also possible to attach aptamers to nanozymes for introducing substrate binding sites, the high cost and low stability of biological ligands defeats the advantages of nanozymes.

We took a different approach by using molecularly imprinted polymers (MIP).[39,40] By adsorbing TMB substrate on iron oxide nanozymes, we grew a layer of imprinted hydrogels. After washing away the TMB (called template), the resulting cavities could selectively rebind TMB over other substrates such as ABTS. By harnessing electrostatic interactions using charged monomers, nearly 100-fold selectivity was achieved for the template substrate (Figure 4). Molecular imprinting is attractive because it uses a cost-effective and robust synthetic polymer, which matches well with the property of nanozymes. While this initial demonstration is encouraging, further development is needed. For example, TMB and ABTS are relatively large molecules, and selective oxidation of smaller molecules without much chemical signature such as glucose might be more challenging. In addition, selective reaction of specific enantiomers for chiral catalysis is desirable. While many attempts have been made, the results are far from satisfactory. For example, Ding and co-workers oxidized glucose using AuNPs adsorbed with various DNA sequences.[41] The assumption was that DNA is a chiral molecule and such chirality might transfer to enantioselectivity in catalysis. While this method worked well with certain small molecule catalysts,[42] the chiral selectivity demonstrated so far with nanozymes was typically less than 1-fold.

One may further improve specificity by learning from the structure of protein substrate binding pockets. Rational construction of protein-like binding pockets in nanozymes has yet to be demonstrated. Given the diverse surface properties of nanozymes, it might be more difficult to realize it in general and each nanozyme needs to be considered separately. We envision a robust model system needs to be developed to gain experience on nanozymes. The use of bioorthogonal nanozyme is another way to achieve specificity, where the catalyst defines the reaction and the substrates are often synthetic compounds.[20]

Bioconjugation. For many applications, an important challenge is bioconjugation. For biosensing, targeted drug delivery, and imaging, it is highly desirable to graft affinity ligands to nanozymes. Protein enzymes can use their lysine and cysteine side chains for this purpose. Since nanozymes rely on their surfaces for activity, densely capped nanozymes might loss catalytic activity. This is of particular concern for those with oxidase likely activity since reactions might need to take place right on the nanozyme surface. While protein peroxidases have well-established mechanisms, inorganic peroxidase nanozymes might involve reactive oxygen species (ROS) from H_2O_2. These ROS may diffuse away from the surface to achieve oxidation and thus make the nanozymes more tolerant for surface capping. Each type of inorganic surface is different, and so far there is no general method to achieve bioconjugation. Using

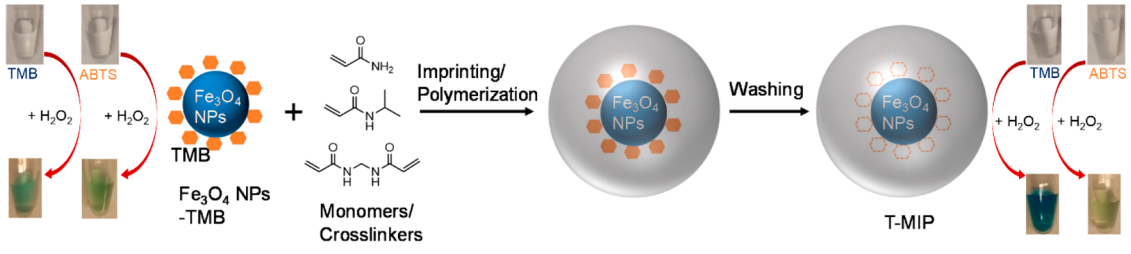

Figure 4. Fe_3O_4 peroxidase-mimicking nanozyme has a similar activity for TMB and ABTS. After imprinting with TMB, its selectivity for TMB is drastically improved. Figure adapted from ref 39 with modification. Copyright 2017 American Chemical Society.

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polysaccharides such as dextran is a quite popular method for nanoceria,[15] but AuNPs lose their glucose oxidation activity with polymer adsorption.[37] We envision that a different strategy needs to be developed for each type of nanozyme. Nanozymes formed by loading homogeneous catalysts into organic particles might be easier to conjugate. For example, Bradley and co-workers grafted a tumor targeting peptide on a Pd nanoparticle loaded polystyrene by forming an amide bond.[43] Rich knowledge already exists in functionalizing inorganic materials,[44] and such wisdom from previous work can be useful for nanozymes. Research on this front is likely to grow, pushed by practical applications of nanozymes.

Exploring New Activities beyond Redox. So far, most reported nanozymes catalyze redox reactions. A logic desire is to expand the range of reaction to others such as hydrolysis, ligation, and transesterification. This would require a more careful design of materials composition and surface properties. For example, metal ions are required for DNA-catalyzed RNA cleavage.[4,45] With metal species on surface (e.g., in metal oxides), it would be interesting to see the same reaction catalyzed by nanozymes. With a Zn^{2+} complex grafted on the surface, AuNPs were able to catalyze RNA cleavage.[46] In this case, the main catalytic role is likely from the metal complex while the gold surface only plays a support role. Loading such metal complexes represents another way to expand the reaction range. Most current work has focused on bioorthogonal reactions. Given the versatility of small molecular catalysts, we expect to see more growth on this front.

For the current redox nanozymes, most experiments were performed on a few model chromogenic or fluorogenic substrates such as TMB, ABTS, and dopamine. While these are convenient for activity assays and useful for biosensor development, it is also important to look at other substrates such as pollutants for environmental remediation and metabolites for in vivo applications. To monitoring such reactions, it would require different assay methods including high performance liquid chromatography (HPLC) and mass spectrometry.

Enzyme-like Reaction Mechanisms. So far, our discussion has mainly focused on observation of enzyme-like activities and applications. Another important aspect to push this field forward is fundamental mechanistic studies. Essentially, nanozymes' reactions are surface reactions, typically involving a few steps of substrate adsorption, diffusion, chemical transformation, and product desorption. These steps have been carefully followed in many gas phase reactions on crystalline surfaces. The nanozyme systems are typically more complex in aqueous solutions with buffers and electrolytes. So far, most of reported nanozymes catalyze redox reactions, and the mechanisms of such electron transfer reactions are often quite difficult to study. Nevertheless, some mechanistic studies have also been performed, and the best studied nanozyme is probably nanoceria. Gao believed that nanoceria worked by dissolution.[19] Catalytic cycles with the interconversion of Ce^{3+} and Ce(IV) were proposed in a review paper,[16] while an electron sponge model was proposed in another study.[47] From this example, it is clear that we are far from reaching a conclusive understanding of its mechanism. We expect more studies to be carried out, and a technical challenge on the gap between the wet nanozyme reaction conditions and surface characterization techniques (e.g., dried in ultrahigh vacuum) needs to be overcome. The results obtained from vacuum measurements need to be interpreted carefully to ensure that the information can describe solution phase reactions.

Using nanomaterials as a carrier for homogeneous catalysts has also enabled detailed mechanistic studies by independently tuning the property of the carrier. For example, Rotello and co-workers used ~2 nm AuNPs with a protection ligand layer to load a bioorthogonal ruthenium catalyst.[48] By tuning the structure of the ligand, they observed different kinetic behaviors of the nanozyme. In this case, the probed mechanism was related to the ligand shell, while the catalytic mechanism of the chemical reaction is often clear for such reactions. This work will likely inspire future engineering of nanozyme materials and also provides a method for systematic mechanistic research.

Exploring New Bio- and Biomimetic Applications. With improved activity, bioconjugation, and specificity, it is possible to achieve more sophisticated applications. Some recent examples include the monitoring of biological activities in vivo,[49,50] and supramolecular regulation of intracellular catalysis.[22] We constructed a cell-like structure with an iron oxide nanozyme "nucleus", a molecularly imprinted hydrogel "cytoplasma", and a lipid bilayer envelope showing a burst response to ionic strength.[51] With future engineering of nanozymes, we expect to see more impactful and insightful applications in the near future.

■ CONCLUSIONS

In summary, the nanozyme field has experienced rapid growth in the past decade in synthesis, characterization, and application. It is now a good time to think about the progress made and to formulate plans for the next stage of development. We speculate that by learning from protein enzymes, the same chemical strategies can be applied to nanozymes to achieve even better activity and specificity. By systematic synthesis and combinatorial screening for activity, more insights can be obtained. Such studies can in turn improve our understanding of bio/nano interfaces and enable applications in analytical chemistry and nanomedicine.

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Notes

The authors declare no competing financial interest.

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