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Nanozymes for biomedical applications: Multi-metallic systems may improve activity but at the cost of higher toxicity?

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Abstract

Nanozymes are nanomaterials with intrinsic enzyme-like activity with selected advantages over native enzymes such as simple synthesis, controllable activity, high stability, and low cost. These materials have been explored as surrogates to natural enzymes in biosensing, therapeutics, environmental protection, and many other fields. Among different nanozymes classes, metal- and metal oxide-based nanozymes are the most widely studied. In recent years, bi- and tri-metallic nanomaterials have emerged often showing improved nanozyme activity, some of which even possess multifunctional enzyme-like activity. Taking this concept even further, high-entropy nanomaterials, that is, complex multicomponent alloys and ceramics like oxides, may potentially enhance activity even further. However, the addition of various elements to increase catalytic activity may come at the cost of increased toxicity. Since many nanozyme compositions are currently being explored for in vivo biomedical applications, such as cancer therapeutics, toxicity considerations in relation to nanozyme application in biomedicine are of vital importance for translation.

This article is categorized under:

Therapeutic Approaches and Drug Discovery > Emerging Technologies

Toxicology and Regulatory Issues in Nanomedicine > Toxicology of Nanomaterials

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KEYWORDS

enzyme-like activity, high entropy nanomaterials, metal nanotoxicity, multi-metallic nanozymes

1 | INTRODUCTION

Natural enzymes play an important role in biochemical reactions in every living system and are also employed as catalytic reagents in the food industry, pharmaceuticals, diagnostics, biofuels, and bioremediation. Yet, despite their

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outstanding catalytic efficiency and substrate specificity, natural enzymes are vulnerable to extreme temperature, pH and ionic strength, resulting in protein denaturation and loss of function. Furthermore, the high cost of preparation, purification, and storage also hinders their practical applications. Therefore, attempts to develop inexpensive, stable alternatives with similar catalytic activities have gained a great deal of attention.

Nanozymes are defined as nanoparticles with enzyme-mimetic activity. Their catalytic activity is governed by factors attributed to nanocatalyst properties (chemical composition, size, morphology, surface modification) and environmental conditions (pH, temperature, stimuli, etc.). The term "nanozyme" was first coined in 2004 by Manea et al. in reference to gold-nanoparticle-based transphosphorylation catalysts (Lou-Franco et al., 2021; Manea et al., 2004). A few years later, in 2007, Gao and co-workers reported that magnetite Fe_3O_4 nanoparticles possessed intrinsic peroxidase-like activity which was 40-fold higher than horseradish peroxidase (HRP) at the same molar concentration. It was hypothesized that the abundance of surface ferrous and ferrite ions in Fe_3O_4 nanoparticles was responsible for the higher catalytic activity compared to HRP. Their investigations led to the idea that Fe^{2+} ion may play a dominant role in the catalytic activity of Fe_3O_4 (Gao et al., 2007). Following this study, the field exploded and a variety of metal and metal oxide-based nanozymes, comprised of Cu, Ce, Mn, Ag, Fe_2O_3 and Co_3O_4 were reported with peroxidase-, catalase-, oxidase-, as well as superoxide dismutase-like activity (Bhattacharjee et al., 2018; Jiang et al., 2019; Liu, Yang, et al., 2021; Masud et al., 2019; Ren et al., 2022; S. Singh, 2019). The catalytic activity of nanozymes was mainly reported to result from the generation of reactive oxygen radicals or electron transfer processes. For an overview of nanozyme classes and mechanisms of action, the authors refer readers to selected reviews (Huang et al., 2019; Liu, Yang, et al., 2021; W. Yang, Yang, et al., 2021). After the fruitful investigation of single metallic nanozymes, the next interesting development came about with the incorporation of two or more metallic elements into the metal oxide structure (Figure 1), which is thought to exploit the unique characteristics of each of the individual elements resulting in an enhanced catalytic performance via either additive or synergistic effects.

In the same year the term nanozyme was coined, high entropy oxides (Figure 1) made their debut. High entropy materials (HEMs) are defined as materials comprising five or more elements, exhibiting a configurational entropy above

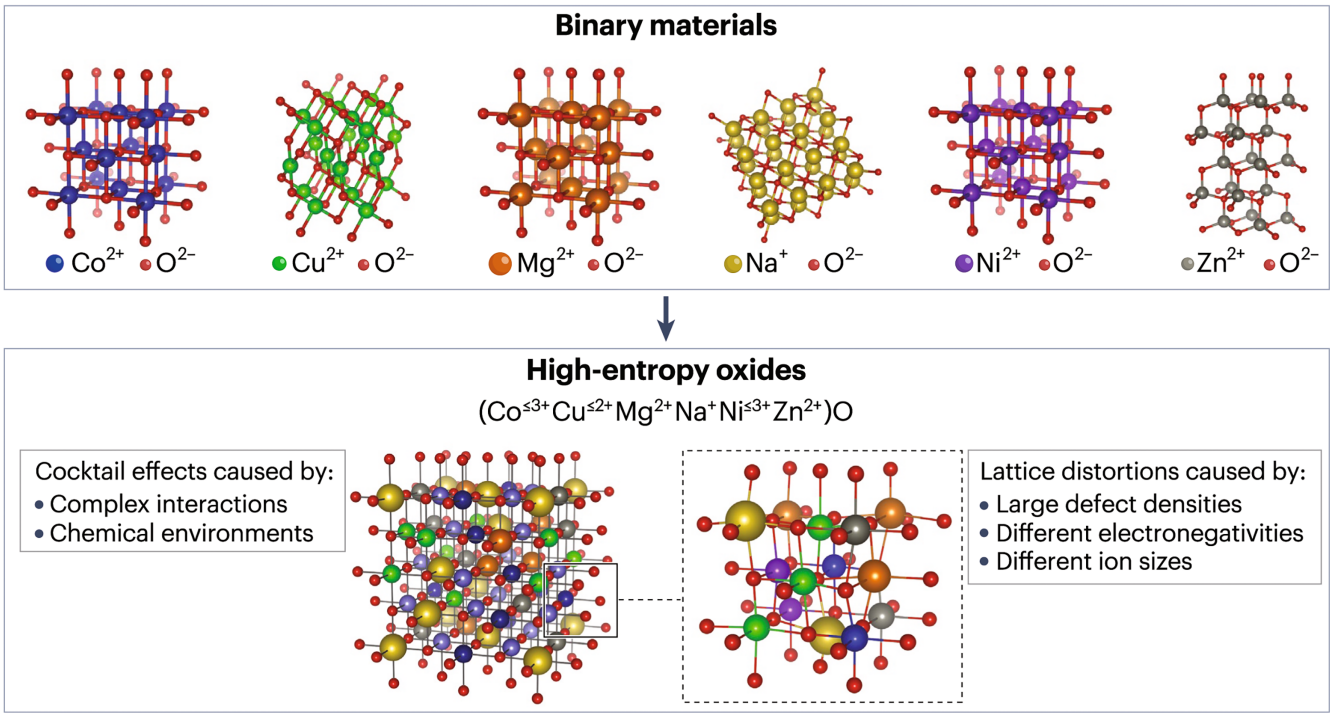

FIGURE 1 Comparison of the lattice structures of six common binary materials (top panel) to the structure of a high entropy oxide comprised of equimolar concentration of the six elements (Co, Cu, Mg, Na, Ni, Zn). The complex interactions in the high entropy structure give rise to the so-called "cocktail effect," that is, the feature that a mixture of elements results in greatly altered properties as compared with the binary materials. Further, changes in the chemical environment, crystal structure (including lattice distortions) and oxidation state have a strong effect on the material properties of HEMs. Reprinted with permission from Schweidler et al. (2024). Copyright 2024, Springer Nature.

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1.5 R (R being the ideal gas constant), or containing five different cations ranging between 5 and 35 atomic %, depending on the applied nomenclature. Selected HEMs often demonstrate structural stabilization as a result of the maximized configuration entropy (Schweidler et al., 2024; Zheng et al., 2021), although this phenomenon does not apply to all HEMs. HEMs are typically characterized by four major effects: (1) the high entropy effect, (2) the lattice distortion effect (Figure 1), (3) sluggish diffusion (decrease in diffusion rate of individual elements) and 4) the cocktail effect (Figure 1) (Xin et al., 2020). Due to large lattice distortion and the cocktail effects, HEMs demonstrate unique mechanical, magnetic, thermal, and electrical characteristics, which can find use in numerous applications across a wide variety of fields (Zhang et al., 2014). It should be noted that entropic contributions are also important driving forces in accelerating enzymatic catalysis via a decrease in the material activation energy (Åqvist et al., 2017). This is why HEMs have been studied for their use as industrial catalysts for the oxidation of methanol (Wang et al., 2014), carbon monoxide (Chen et al., 2018), ammonia (P. Xie et al., 2019), the degradation of azo dyes (Z. Y. Lv et al., 2016) and water splitting catalysis (Lin et al., 2023). However, their suitability as nanozymes has only very recently been explored. For example, only three studies to date have been published using HEMs with peroxidase-like activity for biomedical applications. (Ai et al., 2023; J. Feng et al., 2023; Sheng et al., 2024). Following the logic that synergistic effects between elements in multi-metallic nanozymes may result in dramatic increases in catalytic activity (Schweidler et al., 2024), the development of HEM-based nanozymes may be a very interesting space to watch.

2 | MORE CAN BE MORE: MULTI-METALLIC NANOZYMES FOR ENHANCED ENZYME-LIKE ACTIVITY

The first report by Gao et al. demonstrating the peroxidase-like effect of Fe_3O_4 nanoparticles kicked off a race to enhance the catalytic performance of this material (Gao et al., 2007). The earliest studies used magnetite ($Fe(II)Fe(III)_2O_4$) as a starting material and substituted the ferrous iron with a different di- or trivalent cations in the spinel lattice to create bimetallic nanozymes (Table 1). For example, the addition of Mn^{2+}, Co^{2+} or Cr^{3+} led to significantly higher catalytic activities compared to magnetite. However, both the choice of element and oxidation state were shown to be crucial (Alrozi et al., 2018), since not all substitutions led to improved activity. In particular, Ni^{2+} had a negative impact on the activity of iron oxide-based bimetallic nanozymes (Alrozi et al., 2018; X. Liang et al., 2013; Vetr et al., 2018). Later studies of bimetallic nanozymes investigated both cobalt-oxide and ceria-based nanomaterials as the starting materials, among others. This was followed by multi-metallic systems incorporating up to five different metals (Table 1).

Since nanozyme activity follows Michaelis–Menten kinetics, most studies evaluate and report the Michaelis–Menten constant (K_m; [mM]) and the maximum velocity (V_{max}; [M/s]) of the catalyst for a defined substrate to enable comparisons with the literature. As a general rule, a lower K_m and higher V_{max} indicate a better performance of the nanozyme material. A comparison of the reported values for the peroxidase-like activity of the materials in Table 1 when using TMB as a substrate provides some interesting insights (Figure 2). First, it should be noted that K_m values reported in the literature can vary greatly, sometimes over 2–3 magnitudes of order, making relative comparisons between literature values difficult to interpret. Secondly, it is also essential to note that the reaction temperatures used to determine K_m can also vary substantially (typically between 20 and 40°C) (Cai et al., 2018; Gao et al., 2007; B. Jiang et al., 2018; Yu et al., 2009), which will have a distinct impact on the K_m and V_{max}. Higher reaction temperatures typically result in lower K_m values (Box 1). One of the reasons for the variation in reaction temperatures is that many nanozyme systems may be designed for use in cell culture or in vivo models, where body temperature (37°C) represents the relevant physiological condition. In other studies, ambient room temperature may be chosen as relevant. Within this selected data set, the iron oxide-based bimetallic nanozymes generally showed a lower overall performance compared to other classes. However, this observation should be taken with caution, since Table 1 does not include a fully comprehensive list of all nanozyme studies published in the literature.

3 | A SUMMARY OF MECHANISMS RELEVANT FOR MULTI-METALLIC CATALYTIC ACTIVITY

Researchers investigating the effects of increasing the diversity of nanozyme composition have listed a variety of putative mechanisms which may explain enhanced catalytic performance. These reported mechanisms are listed below, citing the original studies in which the mechanisms were investigated or postulated.

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TABLE 1 Summary of the catalytic properties of selected bimetallic/trimetallic alloys/oxides and high entropy nanomaterials with enzyme-like activities.

Materials	Enzyme-like activity	Substrate	K_m (mM)	V_{max} (10^{-8} M/s)	Reference(s)
Iron oxide-based NPs					
$CoFe_2O_4$	POD	TMB	0.007	0.083	(Zhang et al., 2015)
		H_2O_2	0.036	0.502	
$CoFe_2O_4$	POD	H_2O_2	0.075	-	(Shi et al., 2011)
$ZnFe_2O_4$	POD	TMB	0.509	9.18	(Sahoo et al., 2016)
		H_2O_2	490.72	6.32	
$CuFe_2O_4$	Protease	BSA	0.380	-	(Chen et al., 2022)
		Casein	0.74	-	
$CuFe_2O_4$	POD	TMB	2.26	2.07	(F. Xia et al., 2020)
		H_2O_2	0.5	2.61	
Cu-$CuFe_2O_4$		TMB	0.69	16.87	
		H_2O_2	0.087	32.18	
$MnFe_2O_4$	POD	OPD	27.5	10.4	(Vetr et al., 2018)
$CoFe_2O_4$			32.7	7.8	
$NiFe_2O_4$			8.4	0.86	
$ZnFe_2O_4$			22.6	0.82	
$MgFe_2O_4$	POD CAT	TMB	0.67	2.09	(Su et al., 2015)
		ABTS	0.14	12.54	
		H_2O_2	4.61	13.46	
$NiFe_2O_4$		TMB	0.55	4.57	
		ABTS	0.46	17.48	
		H_2O_2	2.6	14.11	
Co@Fe_3O_4	POD	TMB	1.17	37.9	(Wang, Huang, et al., 2019)
		H_2O_2	0.19	71.5	
Fe_3O_4-MnO_2	POD	TMB	0.101	0.57	(Wang, Li, et al., 2019)
		H_2O_2	0.041	2.94	
PtFe@ Fe_3O_4	POD CAT	TMB	0.213	5.477	(S. Li et al., 2019)
		H_2O_2	53.55	10.78	
Fe_3O_4@SiO_2@CuO	POD	TMB	3.69	47.6	(Tao et al., 2022)
		H_2O_2	9.89	202.2	
MnFeO	POD	TMB	0.11	-	(Lu et al., 2021)
		H_2O_2	0.01	-	
	OXD	TMB	0.04		
Cobalt oxide-based NPs					
Mo-Co_3O_4	POD	TMB	0.0558	9.54	(Gao et al., 2018)
		H_2O_2	22.48	5.245	
Co_3O_4/MoO_3	POD	TMB	0.0352	2.83	(Zhang et al., 2020)
		H_2O_2	0.134	2.91	
Mn@ Co_3O_4	POD	TMB	0.15	5.0	(Isho et al., 2022)
		H_2O_2	0.025	2.5	
Pt@ Co_3O_4	POD	TMB	0.0705	-	(Cao et al., 2022)
$ZnCo_2O_4$	POD	ODP	0.4	5.9	(Chen et al., 2021)

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TABLE 1 (Continued)

Materials	Enzyme-like activity	Substrate	K_m (mM)	V_{max} (10^{-8} M/s)	Reference(s)
		H_2O_2	0.23	4.75	
Ce-$ZnCo_2O_4$	POD OXD	TMB	0.0886	18.796	(Yin et al., 2020)
		H_2O_2	0.553	2.618	
$CdCo_2O_4$	POD, OXD, CAT	TMB	0.317	2.19	(Wei, Chen, et al., 2020)
		H_2O_2	0.325	3.75	
$NiCo_2O_4$	POD OXD	TMB	0.23	59.665	(Q. Mu et al., 2021)
		H_2O_2	28.33	26.773	
$NiCo_2O_4$-Au		TMB	0.1523	56.163	
		H_2O_2	13.17	29.25	
Ceria-based NPs					
Au@CeO_2	POD	TMB	0.29	3.9	(Bhagat et al., 2018)
		H_2O_2	44.69	2.23	
Pr-doped CeO_2	OXD Phosphatase	-	-	-	(L. Jiang et al., 2021)
Bi-doped CeO_2	Haloperoxidase	Phenol red	0.177	1.76	(Frerichs et al., 2020)
Cr-doped CeO_2	POD	TMB	0.08	13.8	(Zhang et al., 2021)
		H_2O_2	0.867	15.9	
Fe-doped CeO_2	POD	TMB	0.087	14.36	(Yue et al., 2021)
		H_2O_2	29.37	14.05	
Co-doped CeO_2		TMB	0.0554	29.29	
		H_2O_2	52.88	25.06	
Mn-doped CeO_2		TMB	0.1048	21.60	
		H_2O_2	59.61	12.32	
Other bimetallic and trimetallic NPs					
$Co_{1.5}Mn_{1.5}O_4$	POD	TMB	0.016	1.88	(Liu, Yang, et al., 2021)
		H_2O_2	14.46	8.76	
	OXD	TMB	0.0029	1.894	
	CAT	H_2O_2	210		
	Laccase	Dopamine	0.0128	7.92	
$Cu_{1.5}Mn_{1.5}O_4$	POD	H_2O_2	1.453	714.09	(Wu et al., 2022)
Co-MnO_2	Uricase	Uric acid	0.022	0.0148	(Parmekar & Salker, 2020)
$LaNiO_3$	POD	TMB	0.105	36.2	(Wang et al., 2017)
		H_2O_2	90.05	260	
PtPdCu	POD	OPD	0.091	7.53	(Mao et al., 2021)
		H_2O_2	1	23.18	
	CAT	H_2O_2	50.172	9.62×10^{-4}	
CoAlCe	POD	TMB	0.273		(Chen et al., 2020)
		H_2O_2	32.9		
PtRuTe	POD	TMB	0.037	58.1	(Shang et al., 2022)
		H_2O_2	2.272	116.7	
PdIr	POD	TMB	0.4	89	(Xi et al., 2020)
PdPtIr	POD	TMB	0.08	31.7	(He et al., 2022)
		H_2O_2	4.08	85.9	

(Continues)

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TABLE 1 (Continued)

Materials	Enzyme-like activity	Substrate	K_m (mM)	V_{max} (10^{-8} M/s)	Reference(s)
PdPt	OXD	TMB	0.058	11.4	(Jin et al., 2019)
AgPt	POD	OPD	0.129	8971	(Gharib et al., 2019)
		H_2O_2	76.05	12849	
	CAT	H_2O_2	62.98	610	
FePt	OXD	TMB	0.03	1.42	(Shah et al., 2018)
Au@Pt	POD	TMB	2.431×10^{-3}	4.425	(Wei, Zhang, et al., 2020)
		H_2O_2	4.076×10^{-3}	6.013	
Au@Pd@Pt	POD	TMB	0.065	24.78	(Sun et al., 2022)
		H_2O_2	4.59	19.82	
Pd@Pt	POD	TMB	0.0865	6.228	(Wei et al., 2015)
		H_2O_2	2.231	5.0	
RuCu	POD	H_2O_2	0.25	0.146	(Hu, Xiao, et al., 2022)
CuFeMn-ATP	POD	TMB	0.046	4.3	(Xu et al., 2021)
		H_2O_2	0.14	10	
Fe_xCu_ySe	POD	TMB	1.837	12.75	(Hu, Shuai, et al., 2022)
		H_2O_2	8.525	4.738	
PdCuAu	POD	TMB	0.54	17	(Nie et al., 2020)
		H_2O_2	0.16	12	
High entropy NPs					
FeCuAgCeGd	POD	TMB	6.6	16.9	(Sheng et al., 2024)
		H_2O_2	765	9.65	
MnFeCoNiCu	POD	TMB	0.07	6.26	(J. Feng et al., 2023)
		H_2O_2	0.6	16.62	
PtPdRuRhIr	POD	TMB	0.034	24.57	(Ai et al., 2023)
		H_2O_2	4094	18.82	

Note: The Michaelis–Menten constant (K_m) and the maximum velocity of an enzymatically catalyzed reaction (V_{max}) are listed for various substrates (TMB, 3,3′,5,5′-tetramentylbenzidine; H_2O_2, hydrogen peroxide; OPD, O-phenylenediamine dihydrochloride; ABTS, 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; BSA, Bovine serum albumin). The class of enzyme-like activity is abbreviated as peroxidase-(POD), catalase-(CAT) and oxidase-(OXD)-like, respectively.

1. Increases in the specific surface area and the number of surface hydroxyl groups via doping with different transition metal cations (Ramankutty & Sugunan, 2001; Vetr et al., 2018; Zhong et al., 2013)
2. Oxidation state of the doped cations (Mei et al., 2020)
3. Distribution of metal ions among tetrahedral and octahedral sites of spinel lattice (Goyal et al., 2014; X. Liang et al., 2014)
4. Interactions (e.g., electrostatic, π-π and coordinate interactions) and molar ratios between individual metal components (B. Hu, Xiao, et al., 2022; C. Liu et al., 2020; Tseng et al., 2012; J. Wu et al., 2018)
5. Number of oxygen vacancies on the surface, which act as active sites for effective heterogeneous catalytic reactions (D. Feng et al., 2020; L. Jiang et al., 2021; Lee et al., 2018; Parmekar & Salker, 2020; Polarz et al., 2006; Shu et al., 2020; L. Xu et al., 2016; Yin et al., 2020; X. Zhang et al., 2020)
6. Changes in the electronic structure (e.g., changes to d-band center position as a result of incorporating transition metals) (Kong et al., 2021; Luo & Guo, 2017; Shao et al., 2019; B. Wang, Yao, et al., 2021; Z. Xia & Guo, 2019; Xin et al., 2020; Y. Yang et al., 2018)
7. Changes in the phase structure (e.g., crystalline to amorphous transitions) (Shang et al., 2022)

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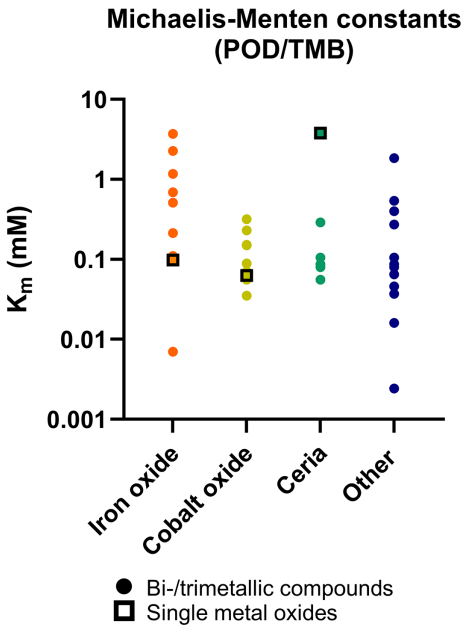

FIGURE 2 Comparison of the Michaelis–Menten constants reported for selected multi-metallic nanozymes when investigated for POD-like activity using TMB as a substrate. Values are presented according to the respective groups listed in Table 1 (Box 1).

BOX 1 Michaelis–Menten kinetics: A hot topic!

Although it is useful to compare nanozyme performance using Michaelis–Menten kinetic parameters, it is important to realize that such studies are conducted over a variety of temperatures in the literature. Since catalysis reactions are highly temperature-dependent processes, direct comparisons of Michaelis–Menten parameters between studies reported in the literature are difficult, unless the conditions are comparable. Therefore, it is good practice to control and report the reaction conditions, such as temperature and pH, at which studies are conducted. Including an appropriate reference material, such as commonly studied nanozymes like magnetite (Fe_3O_4), to act as a standard can also be useful for comparison with literature values.

The reported mechanisms provide us important insights into the impact of multi-metal doping which can be used as guide to prepare multi-metallic nanoparticles with enhanced catalytic activity. For example, cerium can be considered as a metal of choice due to its high capacity to form oxygen vacancies. Variation of elemental concentration may also be a promising strategy to modify the affinity towards a certain substrate (Kong et al., 2021).

4 | HIGH ENTROPY NANOMATERIALS—AN UPGRADE TO THE MULTI-METALLIC DOPING STRATEGY

As stated above, HEMs consist of five or more cations in equimolar ratios with maximized configurational entropy, $S_{config} \geq 1.5R$ (where R is the universal gas constant). What makes these materials interesting are their unique effects including catalytic activity (Wang, Guo, & Fu, 2021; Wang, Yao, et al., 2021). The high entropy effect can play an important role in the phase stability of these materials, which as reported above can improve catalytic activity. Generally, the thermodynamic potential of multi-metallic systems can be determined through the Gibbs free energy equation. The difference in Gibbs free energy between reactants and products (ΔG) often allows conclusions to be drawn about the stability of the formed material. Tphase stability of multi-metallic systems can be determined through the Gibbs free energy equation:

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$$\Delta G = \Delta H - T\Delta S$$

where ΔG, ΔH and ΔS are changes in the Gibbs free energy, mixing enthalpy and mixing entropy, respectively, and T is the thermodynamic temperature (George et al., 2019). The major part of the entropy, when including many different elements in one single phase crystal structure, is the configurational entropy (ΔS), which can become the driving factor for the phase stability of the multi-element system at sufficiently high temperatures, thereby enabling the incorporation of a higher number of components into a lattice structure. From a catalysis point of view, maintaining a single-phase state may be vital since the formation of phase impurities with different surface properties may interfere with the active sites which are necessary for the catalytic reaction (Sun & Dai, 2021). This effect was demonstrated by Chao et al, who produced quinary CoMoFeNiCu nanoparticles in a single solid-solution phase with 20-fold higher catalytic activity (of ammonia decomposition) compared to the standard Ru catalyst, the most active metal for ammonia decomposition (Xie et al., 2019).

Importantly, the incorporation of multiple components with differing atomic sizes results in an inevitable lattice distortion in HEMs (Figure 1). In addition to the atomic size differences, different bonding energy and crystal structure tendencies among constituent components are also believed to cause even higher lattice distortion because of asymmetrical binding and electronic structure between an atom and its first neighbors (Yeh, 2013). This deformation behavior can induce a thermodynamic nonequilibrium state (Xin et al., 2020), which may reduce the energy barrier for the adsorption, activation, and conversion of molecules (Sun & Dai, 2021). The distortion also has a significant impact on modifying the energy levels of bound intermediates (Khorshidi et al., 2018) or changes the mechanical, electrical, thermal, optical and chemical properties of materials (Sun & Dai, 2021). In high entropy oxides, this effect also produces oxygen defects which have been shown to improve the catalytic performances in various oxidation reactions (D. Feng et al., 2020; Shu et al., 2020).

Lastly, the "cocktail effect" (Figure 1) may be an essential property of the nanozyme activity of HEMs. The concept was first proposed by Ranganathan (2003) to emphasize that the performance of multi-component systems does not simply result from the properties of individual primary components but the totality of their inter-element interactions. In other words, mixing multiple elements can offer unexpected properties which are not manifested by using a single independent component. The cocktail effect can be considered as a complex synergistic mechanism that is attributed to the outstanding catalytic performance of HEMs. However, due to the complicated multicomponent context, the mechanism of inter-element interactions is still unknown. More in-depth investigations into electronic properties and lattice structures may be useful to determine the underlying mechanism.

5 | MULTI-METALLIC NANOZYMES UNDER INVESTIGATION FOR BIOMEDICAL APPLICATIONS

Despite a variety of interesting potential biomedical applications of novel metallic nanozymes with enhanced catalytic activity, care must be taken when approaching the question of the suitability of such systems for different biomedical applications. Applications, such as the use of metallic nanozymes in biosensors or ex vivo diagnostic assays, where the materials do not interact with living systems may pose a very low risk in terms of toxicity concerns. In contrast, the implementation of nanozymes in cell-based assays or in vivo studies, either as diagnostic or therapeutic agents, are associated with higher risks of toxicity and adverse reactions. Paradoxically, increasing the catalytic performance of multi-metallic nanozymes via the incorporation of an increasing variety of metal species may in turn increase the risk of toxicity.

The use of multi-metallic nanozymes for a variety of biomedical applications is increasing rapidly. Table 2 provides selected examples of different applications highlighting the variety of uses of nanozymes both in ex vivo and in vivo scenarios. Further applications of bimetallic and trimetallic nanozymes can be found in the selected review articles (Cui et al., 2024; Q. Liu, Yang, et al., 2021; Pietrzak & Ivanova, 2021).

6 | WHAT IS KNOWN ABOUT METAL NANOPARTICLE TOXICITY?

Nanomaterial toxicity is complicated and governed not only by material properties such as size, shape, coatings, surface charge, surface reactivity and bio persistence, but also the administration/exposure route, dose concentration, as well as

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TABLE 2 Selected examples of ex vivo and proposed in vivo applications of multi-metallic nanozymes.

Ex vivo applications as biosensors or diagnostics	Nanozyme composition	Reference (s)
Lateral flow immunoassay strip to detect SAR-CoV-2 nucleocapsid protein (detection limit = 0.207 ng/mL)	Au@Pd@Pt nanozymes with peroxidase-like activity	(Sun et al., 2022)
Point-of-care detection of human dopamine (detection limit = 0.77 μM)	FeCuAgCeGd high entropy nanozymes with peroxidase-like activity	(Sheng et al., 2024)
ELISA-based detection of prostate specific antigen (detection limit = 38 fg/mL which is ~1500-fold lower than conventional ELISA)	Pd-Ir NPs@GVs nanozymes with peroxidase-like activity	(Ye et al., 2017)
Colorimetric and amperometric detection of p53 autoantibody (detection limit = 0.08 which is ~1500-fold lower than p53-ELISA kit)	Au-NPFe2O3NC nanozymes with peroxidase-like activity	(Masud et al., 2017)
Detection of alcohol (detection limit = 0.11 mM which is comparable to commercial alcohol meters)	Au@PtRu nanozyme with peroxidase-like activity	(F. Lv et al., 2020)
Colorimetric detection of glucose in urine (detection limit = 3 × 10−7 M/L)	$ZnFe_2O_4$ nanozymes with peroxidase-like activity	(Su et al., 2012)
Colorimetric detection of glucose, H_2O_2 and ascorbic acid (detection limit = 4 μM, 15.29 μM and 28.59 μM, respectively).	MnFeCoNiCu high entropy nanozymes with peroxidase-like activity	(J. Feng et al., 2023)
Proposed in vivo applications as therapeutics (examples from the literature with in vivo proof-of-concept data)	**Nanozyme composition**	**Reference (s)**
Brain injury repair	PtPdMo nanozymes with multi-enzyme-mimetic activity	(X. Mu et al., 2019)
Combination of chemodynamic therapy, magnetic hyperthermia therapy and magnetic resonance imaging against tumor	Ir@$MnFe_2O_4$ nanozyme with peroxidase-like activity	(Shen et al., 2020)
Chemodynamic anti-tumor therapy	CoO@AuPt nanozymes with multiple catalytic activities	(Fu et al., 2020)
Chemodynamic anti-tumor therapy	$AuPt_3Cu$ nanozymes with triple catalytic activity	(L. Li et al., 2023)
Photodynamic anti-tumor therapy	MnCo-Pt nanozymes with catalase-like activity	(D. Wang et al., 2023)
Photothermal anti-tumor therapy	PtPdRuRhIr high entropy nanozymes with peroxidase-like activity	(Ai et al., 2023)
Synergistic therapy of chemodynamic-, photodynamic- and photothermal therapy on osteosarcoma	RhRu/$Ti_3C_2T_x$ with double enzyme-like activity	(Y. Liang et al., 2023)

biodistribution and elimination kinetics (Gubala et al., 2018). The generation of reactive oxygen species (ROS) such as singlet oxygen, superoxide anion radicals, peroxide ions, etc. is consider the main toxicity mechanism of metal nanoparticles, in addition to the interaction of nanoparticles with cell membranes or cellular components. These mechanisms were thoroughly discussed in several review articles (Attarilar et al., 2020; Sengul & Asmatulu, 2020; W. Yang, Wang, et al., 2021). Furthermore, it should be highlighted that the toxicity may be the result of crosstalk between different mechanisms rather than a single one, which make the metal nanotoxicity more complicated (N. Zhang et al., 2022). Many of the proposed therapeutic applications of metal oxide nanozymes listed above (e.g., in tissue engineering, wound healing, and cancer therapy) would have internal targets in the body, requiring either intravenous or intraoperative administration (Bottagisio et al., 2019; Pan et al., 2021). Therapeutic agents administered via such routes require very high product quality standards and have very special biodistribution profiles, meaning that they are likely to distribute throughout the body following administration and have the potential for accumulation in other organs, where they may have off-target adverse effects (Gubala et al., 2018). Currently, the only regulatory approved metal oxide-based medicine/diagnostic agent is ferumoxytol (Feraheme® or Rienso®), which is comprised of an aqueous colloidal suspension (30 mg/mL) of ferric superparamagnetic iron oxide particles coated with a polyglucose sorbitol carboxymethylether shell for isolation (5–15 nm) pH of 6–8 (CHMP, 2012). It is used clinically as an intravenous infusion

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for the treatment of severe iron deficiency. In many countries, the product has been withdrawn from the market or restricted in use following toxicity concerns due to severe allergic reactions and unpredictable pharmacokinetics (Suciu et al., 2020). Topical applications of metal oxides include hafnium oxide nanoparticles (Hensify®) for advanced squamous cell carcinoma, as well as ZnO and TiO_2 in skin care products including sunscreens (Pan et al., 2021). The risk of off-target toxicity with topical administration is very different to intravenous administration. Therefore, it is important to think about and understand how different administration routes will be associated with different toxicity risks during the development of nanozymes for therapeutic use. Over the past three decades, a great wealth of knowledge has accumulated on the key toxicity paradigms of nanomaterials in the human body. This body of literature is too broad to discuss here, therefore we refer the reader to selected review articles (highlight in bold in the bibliopgraphy) which are recommended as essential literature for understanding the approach to toxicity testing of multi-metallic nanozymes (Box 2).

7 | PROTECTING ANIMALS AND DATA INTEGRITY: TIPS FOR PREPARING MATERIALS FOR IN VIVO STUDIES

Generating in vivo proof-of-concept data on nanozyme activity is often a pre-requisite for publication in many journals these days. Before starting in vivo studies, whether they are designed to test safety or efficacy, it is important to think about lessons learned from the nanotoxicology field. In an *Angewandte Chemie* review article from 2014, H. Krug examined >10,000 papers studying environmental and health effects of nanomaterials and concluded that "we are left with a plethora of low-value results due to the lack of harmonized experimental protocols, poor or nonexistent characterization of the nanomaterials, a lack of reference materials, ..., and so on" (Krug, 2014). Similar conclusions were drawn in the biomedical sector, where a majority of novel nanomaterials for development as cancer therapeutics faced serious translational issues due to the complexity of nanomaterial characterization and the difficult interpretation of preclinical studies. This latter observation led to the foundation of the Nanotechnology Characterization Lab (NCL) by the National Cancer Institute in the United States (with a satellite institute in Europe).

One of the key issues faced in nanotoxicology and nanomedicine development is a lack of awareness of how sample quality should be designed into the system from the very first stages of basic research. To address this, the NCL have compiled clear, easy-to-use guidelines for the preparation and characterization of high-quality nanomaterials for biomedical uses, and specifically for colloidal metal-based nanoparticle systems (Nanotechnology Characterization Lab, n. d.). Despite availability of this information, many basic research publications do not routinely incorporate the full spectrum of characterization techniques prior to commencement of in vivo studies (Figure 3). While some quality parameters may not significantly alter the outcomes of in vivo studies, issues with poor material quality could cause significant and avoidable harm to test animals as well as confound study results. For example, excipients or impurities in excess may cause toxicity which is incorrectly attributed to the nanozyme materials. Similarly, unexpectedly high dissolution of multi-metallic nanomaterials within cellular compartments, such as phagosomes, could be responsible for unexpected toxicity events. Aggregation of nanomaterials in plasma could lead to blockage of capillaries and embolism in test animals. High batch-to-batch variations in critical material properties may require higher numbers of animals

BOX 2 Common myths about metal toxicity (...at least for oral exposure)

According to Egorova and Ananikov, so-called heavy metals (e.g., palladium, platinum, rhodium, chromium etc.) are commonly thought to be significantly more harmful than lighter metals (nickel, copper, cobalt, etc.) (Egorova & Ananikov, 2017). However, the comparison of oral toxicity of different chromium compounds in rats paints a more nuanced picture. Depending on the oxidation state of the metal and compound composition, the oral toxicity (expressed by the half maximal lethal dose; LD50) can differ greatly. For example, chromium compounds can range from low to moderately toxic in the following order: Cr_2O_3 (LD50: 15000 mg/kg) << $Cr(NO_3)_3{\cdot}9H_2O$ (3250 mg/kg) < $CrCl_2$ (1870 mg/kg) < $CrCl_3$ (440 mg/kg) < CrO_3 (52 mg/kg). However, remember that toxicity is dependent on the administration or exposure route, therefore compounds that are "safe" for oral exposure may not necessarily be well-tolerated after intravenous administration.

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in test groups to achieve sufficient power in the in vivo study. Microorganism or endotoxin contamination, due to improper preparation and storage, may trigger immune responses in vivo that are also falsely attributed to the nanomaterial. These are just examples of common errors observed in nanotoxicology studies (Krug, 2014). However, awareness of important material characteristics, well-designed nanomaterials and good laboratory practices (including good documentation) can protect the welfare of study animals and improve the quality of data of in vivo studies.

8 | CONCLUDING REMARKS AND OUTLOOK

The development of multi-metallic nanozymes for use as promising alternatives to native enzymes in various biomedical applications is an exciting and rapidly moving field. The incorporation of two or more metals in a nanozyme structure can lead to favorable alterations in electronic structure, oxygen vacancies or phase properties, thus an enhancing catalytic activity in an unprecedented manner. The emergence of HEMs in the nanozyme field has provided a broadened scope. The unique core effects offer these materials remarkable mechanical, thermal strengths, phase stability as well as uniform dispersion of catalytically active species on the surface, thereby achieving an outstanding catalytic performance. In addition, the high entropy approach offers the potential of a larger chemical space for the individually tailored design of potential catalysts by fine-tuning their chemical compositions.

However, despite a rapidly increasing number of publications where multi-metallic nanozymes are investigated for biomedical applications (e.g., chemotherapeutic agents and biosensors), toxicity concerns remain one of the main hurdles hampering their clinical translation. The evaluation of nanozyme toxicity is challenging due to the complicated nature of nanomaterials and their interactions with the human body. This complexity becomes even more apparent in the context of HEMs where multiple elements with different intrinsic properties for example atomic radius, electronegativity, redox potential, etc. coexist. Unexpected interactions between these components may have a significant impact on physicochemical properties, thus altering the toxicity profile. Therefore, the rational design of multi-metallic

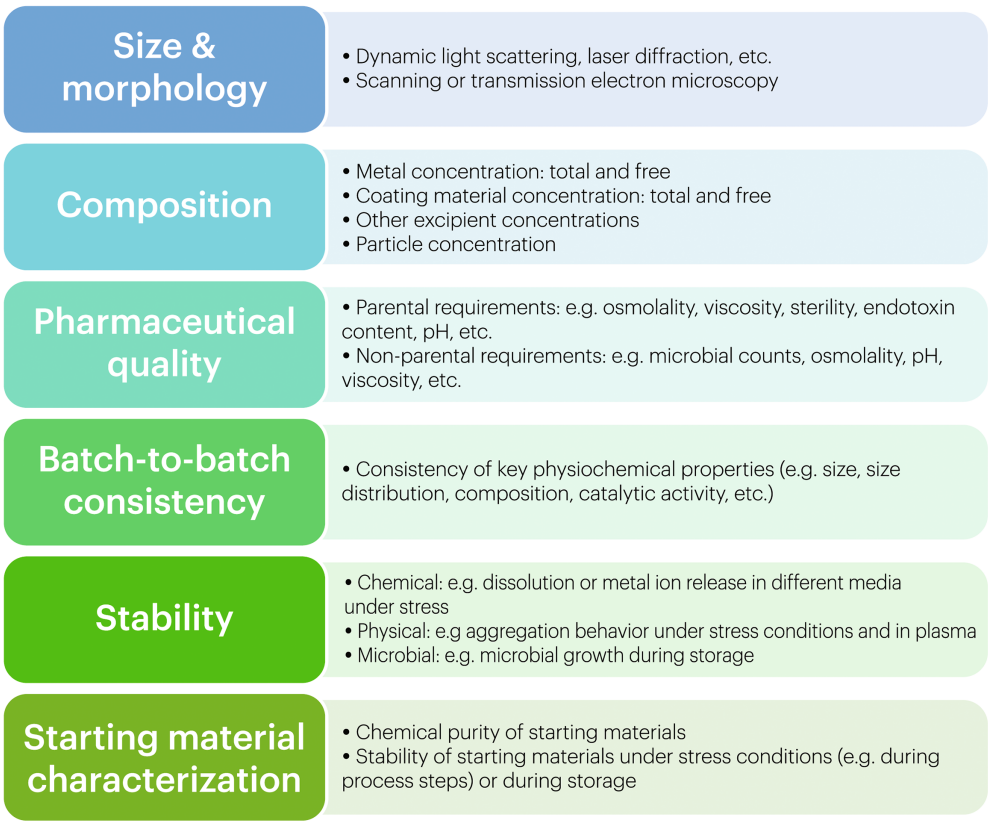

FIGURE 3 List of important material characteristics which should ideally be assessed prior to in vivo investigations of nanozymes (cited from https://www.cancer.gov/nano/research/ncl/protocols-capabilities/physicochemical-characterizations-colloidal-metal-nanoparticle.pdf; accessed on 27 March 2024).

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nanozymes is of the highest importance, not only to obtain nanozymes with superior catalytic activity but also with sufficient quality for subsequent proof-of-concept studies.

Outlook: Owing to the advanced manufacturing methods and characterization techniques of nanomaterials, more complicated metal-based nanoparticles, especially multi-metallic platforms with superior enzyme-mimetic catalytic activity will appear. These nanozymes hold promising prospects in a wide range of biomedical applications, such as biosensors, imaging agents, anti-bacterial and anti-tumor therapeutics. However, toxicity evaluation of these materials must be assessed thoroughly for clinical translation. Recently, computational approaches for material design and in silico toxicology predications have been attracting a great deal of attention. The purpose of these models is to correlate structural or physicochemical features (presented as descriptors which can be achieved from experimental or computational methods) with toxic effects. Furthermore, integrating machine learning algorithms into computational models allows analysis and interpretation of large amounts of data, providing a better understanding of toxicity mechanisms. However, to create robust and highly predictive models, high-quality data is pivotal (A. V. Singh et al., 2023). Standardization and high-quality documentation would help to improve quality of input data, reducing duplicated experiments and inter-laboratory variations, contributing to create uniform, sufficient and diverse database which is extremely beneficial to toxicity evaluation of metal nanozymes.

AUTHOR CONTRIBUTIONS

Thuong Phan-Xuan: Conceptualization (equal); data curation (lead); formal analysis (equal); writing – original draft (equal). **Ben Breitung:** Conceptualization (equal); writing – review and editing (equal). **Lea Ann Dailey:** Conceptualization (equal); formal analysis (supporting); supervision (lead); writing – original draft (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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