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# How to define a nanozyme?

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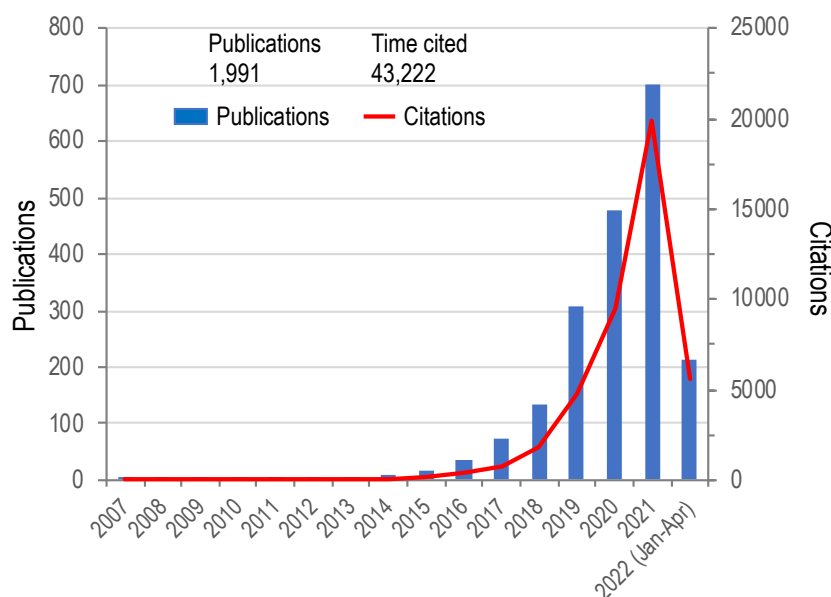
## ABSTRACT

Over the past fifteen years, many articles have considered as “nanozymes” ferromagnetic nanoparticles having an "intrinsic peroxidase-like activity" in the presence of hydrogen peroxide. However, the definition and the catalytic activity of these nanozymes have been questioned. The present Perspectives reports the main criteria that are essential to classify a nanoparticle as nanozyme. It is urgent to consider that all nanoparticles able to generate hydroxyl radicals in the presence of hydrogen peroxide without catalytic activity cannot be registered as nanozymes.

**KEYWORDS.** Nanozymes, Nanoparticles, Catalytic Activity, Turnover Number

Over the past twenty years nanoparticles have been successfully used as catalysts in many different fields.<sup>1</sup> For instance, the catalytic activities of gold nanoparticles have been discussed and compared to other nanoparticles.<sup>2</sup> These nanoparticle-based catalysts obey to the classical definitions of catalysis and occupy a significant growing part of the success of nanotechnologies. In 2004, Manea *et al.* reported a transphosphorylation reaction catalyzed by gold-nanoparticles and called such catalysts nanozymes.<sup>3</sup> This initial work rapidly triggered the use of nanoparticles as potential catalysts in many different reactions.<sup>4-7</sup>

Over the past fifteen years, the neologism "nanozyme" became highly popular and generated a large body of articles and reviews. On April 27th, 2022, using "nanozyme" as keyword, 1,991 articles can be found on the Web of Sciences



**Figure 1.** Number of articles and citations using "nanozyme" as keyword in the WoS (all fields) on 27<sup>th</sup>, 2022. Numerical data from <https://www.webofscience.com/wos/woscc/summary/6fc466b4-088b-4932-b344-42bea4023e72-341fe872/times-cited-descending/1>.

associated with 43,222 citations (and a relatively high 38% level of self-citations) (Figure 1).

Claiming nanozymes as enzyme-mimics suggests that these nanoparticles should have properties similar to enzymes. As biological catalysts, enzymes are characterized by two essential parameters: (i) they have an efficient catalytic activity and (ii) a specific mechanism for a given reaction. In addition, many enzymes are not like simple catalysts, but they exhibit a high level of substrate selectivity and are able to select a particular substrate among a family of similar chemical

structures. Since their discovery enzymes have been classified in major categories according to different classes of catalytic reactions they are performing: oxido-reductases, transferases, hydrolases, lyases, isomerases, ligases, translocases, etc...<sup>8</sup> Each of these major categories is subdivided according to the nature of substrates and chemical mechanisms involved. As example, restriction enzymes have a very high specificity in recognizing nucleotide sequences when cutting DNA.<sup>9,10</sup> The combined use of these restriction enzymes with ligases has been at the origin of the physical mapping of DNA and all advances made in the applications of molecular genetics (diagnostic tools, new biopharmaceuticals...).

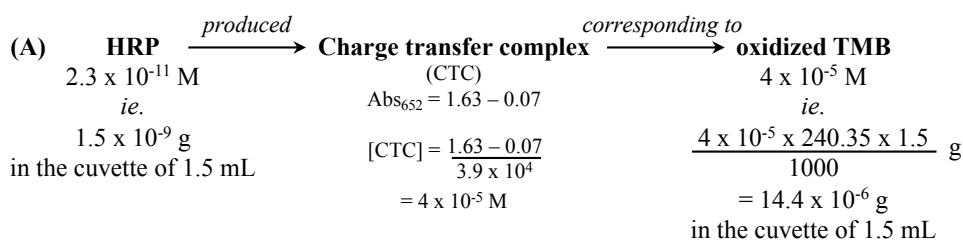
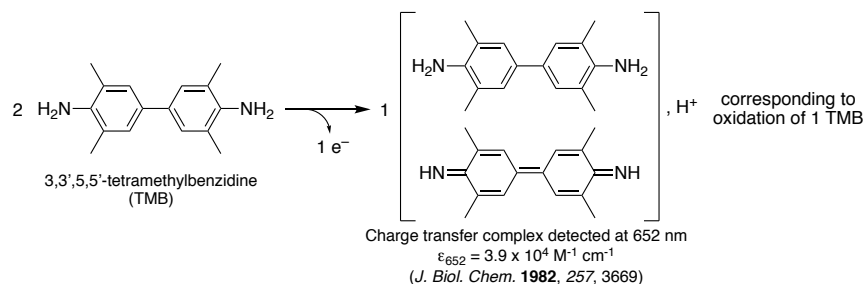
Among enzymes able to perform oxidation reactions, cytochromes P450 are an important family of monooxygenases able to selectively hydroxylate many different substrates for endogenous biosynthesis or detoxification of xenobiotics.<sup>11</sup> On the other hand, peroxidases and their biomimetic models specifically catalyze the two-electron oxidation of a large variety of substrates which have in common a redox potential compatible with the high-valent metal-oxo species generated by hydrogen peroxide associated with peroxidases.<sup>12,13</sup> As recently reminded by the Editorial of *ACS Catalysis* (December 4<sup>th</sup>, 2020) enzyme models should mimic the reactions catalyzed by enzymes,<sup>14</sup> i.e. perform the same kind of reaction (involving same substrates and same products) with similar or closely related mechanisms.

Since the article of Gao *et al.* published in 2007,<sup>15,16</sup> many articles have been devoted to nanozymes described as peroxidase models. However, these reports raised two questions: (i) are these nanoparticles real catalysts? and (ii) are they mimicking the specific reaction of peroxidase enzymes, i.e. a two-electron oxidation, or are they generating hydroxyl radicals in stoichiometric or catalytic reactions?<sup>17,18</sup> The first issue concerns the catalytic activity of peroxidase-like nanozymes. In regular textbooks, a catalyst is defined as a substance able to increase the rate of a

chemical reaction when added in *small quantity* with respect to the substrate engaged in a reaction. A catalyst is characterized by its turnover number (or TON) and its catalytic activity by the turnover frequency (TOF), which is the TON per time unit. Such basic definitions are expected to be also valid for nanoparticles, as they are for chemical catalysts, enzymes or biomimetic catalysts. To calculate the catalytic activity of nanozymes, evaluation of the number of active sites is obviously a crucial issue. It has been recently emphasized that "higher activities for nanomaterials relative to enzymes should not be claimed when the number of active sites in the nanomaterial is unknown".<sup>14</sup> In 2018, we mentioned that the "peroxidase-like activity of Fe<sub>3</sub>O<sub>4</sub>" reported by Gao *et al.*<sup>15</sup>, based on the amount of iron atoms, was only marginal.<sup>17-19</sup> In fact, while the turnover number of HRP was  $1.8 \times 10^6$  cycles in 5 min (turnover frequency =  $6 \times 10^3 \text{ s}^{-1}$ ), the activity of magnetite (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles was below one catalytic cycle in the conditions reported by Gao *et al.* Recently, Zandieh and Liu reinvestigated the calculations of Gao *et al.* using a different method to evaluate the number of *surface* iron atoms considered as the only active catalytic sites, and concluded that the TON was 2.7 catalytic cycles, far below the high catalytic activity originally reported.<sup>20</sup> Moreover, these active sites are part of a structure that includes inactive sites contributing to the weight of nanoparticles as ballast. Consequently, it is essential to consider the total weight of nanoparticles engaged in the reaction mixture. In the conditions reported by Gao *et al.*<sup>15</sup> and reproduced by Gumpelmayer *et al.*<sup>17</sup>, the lack of catalytic activity of magnetite nanoparticles can be illustrated by comparing the amount of horseradish peroxidase enzyme (HRP) or magnetite required to achieve the oxidation of a substrate such as TMB (3,3',5,5'-tetramethylbenzidine). To oxidize 1 gram of TMB, only 0.1 *milligram* of HRP is required (corresponding to 0.01 wt% with respect to the substrate),<sup>21</sup> whereas 36.2 *grams* of magnetite nanoparticles are necessary (calculations are provided in the Table). These two numbers are

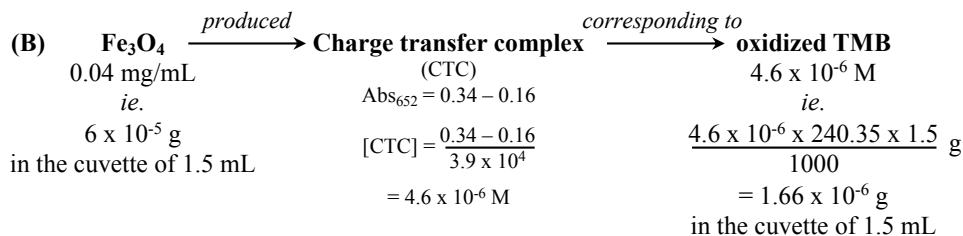
separated by 6 orders of magnitude, clearly indicating that HRP has a real catalytic activity, whereas magnetite is only a ballast with a very small amount acting as oxidant. The weight of magnetite is more than 36-fold the weight of substrate, far away from the “catalytic world”.

**Table.** Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by horseradish peroxidase (HRP, panel A) or Fe<sub>3</sub>O<sub>4</sub> nanoparticles (panel B). Calculations are from data of Figure 1, Reference 17.



So,  $14.4 \times 10^{-6}$  g of TMB were oxidized by  $1.5 \times 10^{-9}$  g of HRP

Consequently,  $\frac{1.5 \times 10^{-9}}{14.4 \times 10^{-6}} \text{ g} = 0.1 \times 10^{-3} \text{ g}$  of HRP is required for oxidation of 1 g of TMB, *ie.* 0.01 wt% of enzyme with respect to substrate.



So,  $1.66 \times 10^{-6}$  g of TMB were oxidized by  $6 \times 10^{-5}$  g of  $\text{Fe}_3\text{O}_4$

Consequently,  $\frac{6 \times 10^{-5}}{1.66 \times 10^{-6}} \text{ g} = 36.2 \text{ g of Fe}_3\text{O}_4$  is required for oxidation of 1 g of TMB, *ie.* 3.620 wt% of  $\text{Fe}_3\text{O}_4$  nanoparticles with respect to substrate.

In fact, calculation of the activity of magnetite should not be biased by considering only a tiny number of "catalytic sites" compared to the total mass of particles. Noteworthy, enzymes are usually high molecular weight entities with diluted catalytic centers, and a major part of the enzyme weight can be considered as ballast. Nobody will calculate the activity of HRP just by using the weight of the ferri-protoporphyrin-IX without taking in consideration the protein surrounding the active site of this enzyme. The high activity of HRP is not an isolated case in enzymology. For examples, dismutation of  $\text{H}_2\text{O}_2$  by catalase occurs with a turnover frequency of ca.  $3.6 \times 10^5 \text{ s}^{-1}$  (equivalent to  $9 \times 10^4 \text{ s}^{-1}$  by catalase heme residue of beef liver catalase) and the activity of cytochrome P450, which is considered as a "slow enzyme" is close to  $1 \text{ min}^{-1}$ .<sup>22</sup> It should be noted that unambiguous catalytic activities have also been observed using nanoparticles. As example, one can cite the catalytic Fenton oxidation of phenol using gold nanoparticles (Au/DNP, 1 wt% Au; TON = 26500, turnover frequency =  $5300 \text{ h}^{-1}$ ).<sup>23</sup> In addition, after washing, Au/DNP nanoparticles were reusable with a very similar conversion profile of phenol decomposition, indicating that the catalyst was not destroyed during the reaction and remain unchanged after it. As another example,  $(\text{RuO}_2)_{0.038}(\text{SiO}_2)_{0.962}$  nanoparticles were used to achieve the reduction of glucose to sorbitol.<sup>24</sup> Even with a relatively modest efficiency (30% of glucose conversion after 5 h, see Figure 3 of Reference 24), the catalyst achieved 20 cycles in 5 h, and a turnover frequency  $\text{TOF} = 4 \text{ h}^{-1}$  ( $11 \times 10^{-4} \text{ s}^{-1}$ ). The re-use of the catalyst was also possible without loss of activity or selectivity. There is no reason why "nanozymes" should escape from the basic definition of catalytic activity.

The second question raised about "peroxidase-like" nanoparticles concerns their oxidizing mechanism: are they mimicking the specific reaction of peroxidase enzymes, i.e. a two-electron oxidation, or are they generating hydroxyl radicals in stoichiometric or catalytic reactions?<sup>17,18</sup> It

is well-known that hydroxyl radical ( $\text{HO}\bullet$ ) is easily generated by one-electron oxidation of hydrogen peroxide in the presence of low-valent metal traces (Fenton reaction), and that it is one of the strongest oxidizing agents in chemistry.  $\text{HO}\bullet$  is able to abstract hydrogen atoms or to hydroxylate unsaturated bonds at controlled-diffusion rates. Substrate oxidation by a Fenton reaction is different than a peroxidase-type oxidation, each reaction having its own mechanism. Inaccurate naming is detrimental for the correct understanding and accurate description of reactions and processes. Since all organic chemicals are potential targets of Fenton reaction, the difference between Fenton oxidation and a peroxidase mimicking oxidation should be done by using a substrate which provides a specific product in an HRP-catalyzed oxidation. By using a 5-hydroxyindole derivative (9-hydroxy-*N*<sup>2</sup>-methyl-ellipticinium acetate) that provides a quinone-imine derivative upon HRP-catalyzed oxidation, we have demonstrated that such oxidation did not occur with "peroxidase-like" magnetite nanoparticles. Conversely, marginal oxidation triggered by these nanoparticles were mediated by  $\text{HO}\bullet$  generated by adventitious metal traces.<sup>18,19</sup>

Moreover, several reports on TMB oxidation by magnetite-based nanozymes are reporting Michaelis-Menten data to support an enzyme-like behavior.<sup>4,15</sup> However, one should keep in mind that such kinetic analyses are only reliable in two conditions: (i) the reaction should be catalytic and (ii) the substrate concentration should be high enough to saturate the enzyme active sites. These conditions are required for a valid steady-state approximation, and to allow calculation of the kinetic parameters  $V_{max}$  and  $K_M$ .<sup>25</sup> The pseudo-saturation curves reported in the literature for magnetite nanoparticles do not reflect saturation of an enzyme like catalyst, but are only due to the fact that the substrate conversion reaches a plateau because of the limited non-catalytic oxidation of the substrate.<sup>19</sup>



In conclusion, until now, nearly all "peroxidase-like nanozymes" are nanoparticles that are generating hydroxyl radicals in a non-catalytic fashion, far away from the typical two-electron oxidation catalyzed by peroxidases. These nanoparticles without catalytic activity should be considered for what they are and not for what there are not. We are confident that it will be possible to create, in the future, true nanozymes able to mimic most of the properties of enzymes, as it has been done in the chemical modeling of heme enzymes, few decades ago.<sup>26</sup>

## AUTHOR INFORMATION

### **Author Contributions**

The manuscript was written through equal contributions of all authors. All authors have given approval to the final version of the manuscript.

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### **Conflict of Interest**

The authors declare no conflict of interest.

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### Graphical Table Entry

