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Michel Nguyen, Anne Robert, Bernard Meunier. ”Nanozymes” without catalytic activity should be renamed. 2021. hal-03290323

HAL Id: hal-03290323

<https://hal.science/hal-03290323>

Preprint submitted on 19 Jul 2021

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"Nanozymes" without catalytic activity should be renamed.

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Keywords: Catalytic oxidation, Fenton reaction, magnetite, nanozymes, peroxidase.

This article was initially submitted to PNAS. The full text of the Reviewer's comments and Editor's rejection, our appeal, and the final editorial decision are published after the article.

Abstract. In the aftermath of the report by L. Gao, *et al.* (*Nature Nanotechnol.* **2007**, *2*, 577-583), ferromagnetic nanoparticles such as magnetite are considered by many authors as having an "intrinsic peroxidase-like activity" in the presence of hydrogen peroxide. These authors consequently suggest the discovery of efficient biomimetic oxidation catalysts in the rapidly expanding world of nanomaterials. However, we demonstrate that such nanoparticles have no catalytic activity, the oxidation of the substrate 3,3',5,5'-tetramethylbenzidine being far below one "catalytic cycle" with respect to iron nanoparticles. They are also unable to perform the two-electron oxidation typically catalyzed by a peroxidase, when using a specific peroxidase substrate such as the anticancer drug 9-hydroxy-*N*²-methylellipticinium, a *p*-aminophenol derivative known to produce a specific quinone-imine when oxidized by peroxidases. In these conditions, the marginal oxidation reactions observed with magnetite in the presence of hydrogen peroxide are likely due to a Fenton reaction triggered by adventitious metal impurities. The name "nanozyme" is based on a corrupted Michaelis-Menten analysis of these non-catalytic reactions. This erroneous presentation of experimental data is a misperformed research work associated to a semantic falsification. The misleading name "nanozyme" should therefore be replaced by "nano-oxidant" until future nanoparticles having accurate catalytic activities should be named nanozymes.

Significance Statement. A growing number of reports claim that iron-oxide nanoparticles such as magnetite, so-called "nanozymes", exhibit a peroxidase-like activity, suggesting efficient biomimetic catalytic nanomaterials. However, such nanoparticles have no catalytic activity, and are unable to perform the two-electron oxidation typically catalyzed by a peroxidase. The marginal oxidation reactions observed in the presence of magnetite and hydrogen peroxide are likely due to a Fenton reaction. The name "nanozyme" is based on a corrupted Michaelis-Menten analysis of non-catalytic reactions. This erroneous analysis of experimental data, associated to the fashionable name "nanozyme" is a misperformed research work described by a semantic falsification. The misleading name "nanozyme" should therefore be replaced by "nano-oxidant". The accurate scientific definitions of catalysts and enzymes must be respected.

Introduction

A growing number of reports claim that iron-oxide nanoparticles such as magnetite exhibit a peroxidase-like activity in the presence of hydrogen peroxide (1-4). These nanoparticles are named "nanozymes" despite the absence of actual catalytic activity, creating a semantic falsification. Up to now, the scientific community agrees on the fact that enzymes or enzyme-models must have a real catalytic activity (5-10). As a matter of fact, the claims on the so-called peroxidase-like activity of magnetite is based on a misconducted kinetic analysis of experimental data. In fact, several of these articles contain a Michaelis-Menten analysis of the substrate conversion (1,11), despite the fact that kinetic parameters V_{max} and K_M can be reliably obtained only when the steady-state assumption is valid in a catalytic reaction. The presentation of the Michaelis-Menten parameters for non-catalytic reactions is a corrupted presentation of the substrate conversion by these "nanozymes". It is a typical example of a misconducted research work. In order to demonstrate that these nanoparticles have no catalytic activity, we used an efficient scavenger of the hydroxyl radical $\text{HO}\cdot$ and a substrate that stamps without ambiguity an oxidation catalyzed by a peroxidase. In these conditions, we show that magnetite has no capacity to perform the two-electron oxidation catalyzed by a peroxidase, and the marginal oxidation reactions observed with these materials are likely due to a Fenton reaction. The usurped name "nanozyme" should be replaced by "nano-oxidant" until future nanoparticles having scientifically proved catalytic activities should be named nanozymes.

As starting point of the claim of the peroxidase activity of magnetite, Gao *et al.* reported in 2007 the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of magnetite Fe_3O_4 associated to hydrogen peroxide (1). Based on their published experimental data and on our own experiments with magnetite, we demonstrated that the reaction reported by Gao was not catalytic (12). The marginal amount of TMB oxidation product observed at pH 3.5 was far below one catalytic cycle with respect to magnetite, and was dependent of the treatment of the buffer solutions by the metal chelating resin Chelex-100, indicating that the observed oxidation was likely due to adventitious metal ion traces (12). In a recent article, the investigation of ascorbate oxidation in the presence of $\text{Fe}_3\text{O}_4/\text{H}_2\text{O}_2$ was carried out with magnetite concentrations equal or even higher than that of the substrate, inconsistent with catalytic conditions (13).

In all the articles about "peroxidase-like nanozymes", the experimental conditions and results did not support any catalytic activity (the product yield was lower than the initial ferrite nanoparticles iron amount) (14). In addition, none of these publications concerned the regular peroxidase activity, namely the two-electron oxidation of a substrate using H_2O_2 as oxidant (5).

Then, in order to clarify the situation concerning these "nanozymes" that have no catalytic activity, but are performing Fenton-type oxidations, we report here data to facilitate the discrimination between a peroxidase activity such that of horseradish peroxidase (HRP), and the non-catalytic oxidations observed with magnetite particles.

Results and discussion

Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by $\text{Fe}_3\text{O}_4/\text{H}_2\text{O}_2$ is not catalytic.

Hydroxyl radical is the strongest oxidizing agent after fluorine, with a standard redox potential of 2.8 V/NHE (15). At controlled-diffusion rates, $\text{HO}\cdot$ is able to perform hydrogen abstraction, hydroxylation of unsaturated bonds, one electron-oxidation, or *ipso*-substitution of many different substrates, including

recalcitrant perhalogenated hydrocarbons (15). In fact, all organic molecules can be oxidized by HO• including TMB, the substrate commonly used in articles concerning nanozymes (16).

Cyclic voltammetry of TMB exhibits two successive 1-electron oxidation waves at 0.25 and 0.40 V/SCE, corresponding to 0.49 and 0.64 V/NHE (17). Compound I and compound II of HRP are strong oxidizing agents, able to abstract one electron at any substrate having an oxidation potential below 1.0-1.1 V/NHE (18), including TMB. Consequently, the oxidation of TMB can be performed by a Fenton- or by a peroxidase reaction. It is also the case for other electron-rich aromatic substrates such as 1,2-diaminobenzene and 3,3'-diaminobenzidine, used in Reference 1. So, we decided (i) to carry out the oxidation of TMB by magnetite in the presence of DMSO as an effective HO• trap, and (ii) to investigate the oxidation of 9-hydroxy-*N*²-methylellipticinium acetate (9-OH-NME⁺), a substrate providing a specific product in a HRP-catalyzed oxidation.

Dimethylsulfoxide (DMSO) is a potent scavenger of HO• (19). The DMSO concentration able to efficiently capture HO• is dependent on reaction conditions, and at least 1 M concentration (ca. 7.8 vol%) is required (20,21). Noteworthy, many studies of TMB oxidation by magnetite have been performed in the presence of 2-4 vol% of DMSO (1), a proportion which is necessary for TMB solubilization, but insufficient to trap HO• radicals. We therefore decided to evaluate the oxidation of TMB by magnetite in the presence of 20 vol% of DMSO in the buffered solution, compared to the 2 vol% of DMSO used in Reference 1. The oxidation product of TMB is detected at 652 nm (diamine/diimine charge transfer complex, $\epsilon_{652} = 3.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (22). After 30 min of reaction time, the oxidation of TMB was 66 % lower in the presence of 20 vol% of DMSO, compared to 2 vol% of DMSO (Figure 1a). It should be noted that the control experiment carried out in the absence of Fe₃O₄ resulted in a *ca.* 10 times lower TMB oxidation, and exhibited also a similar inhibition when the DMSO concentration was increased from 2 vol% to 20 vol% (– 57%, Figure 1b). This control experiment confirms that adventitious low-valent metal traces are able to oxidize TMB. These results indicate that HO• radicals were significantly scavenged by 20 vol% of DMSO in the reaction mixture, and confirmed that Fe₃O₄ generates HO• in the presence of H₂O₂.

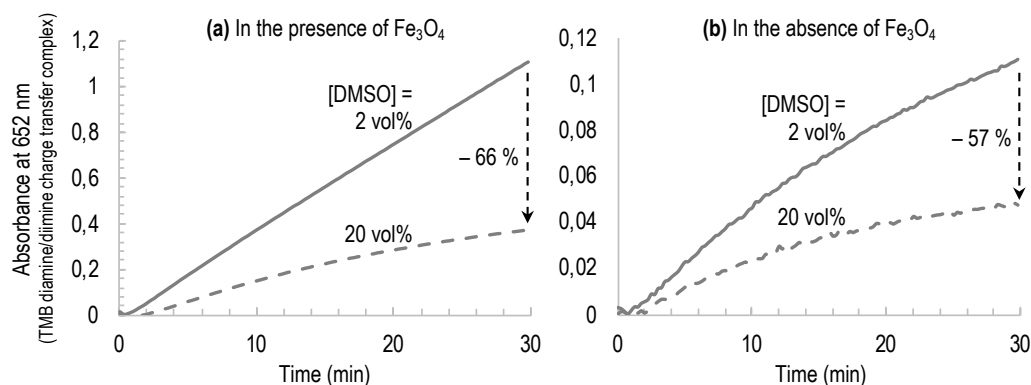


Figure 1. **(a)** Oxidation of TMB by Fe₃O₄/H₂O₂ in the presence of 2 vol% or 20 vol% of DMSO in acetate buffer 200 mM, pH 3.5, 37 °C ([TMB] = 816 μM, [Fe₃O₄] = 0.04 mg/mL = 173 μL, [H₂O₂] = 98 mM); For oxidation of TMB in 2 vol% of DMSO by Fe₃O₄ associated to variable concentration of H₂O₂, see Supporting Information, Figure S1. **(b)** As control, oxidation of TMB by H₂O₂ alone (98 mM) in the presence of 2 vol% or 20 vol% of DMSO. The diamine/diimine oxidation product is detected at 652 nm. All solutions were treated with Chelex resin.

On a quantitative point of view, the Fe₃O₄/H₂O₂ mediated oxidation of TMB in the presence of 2 vol% of DMSO (insufficient proportion to efficiently scavenge HO• radicals) resulted in a 28 μM concentration of the diamine/diimine charge transfer complex (yield = 3.4 %). The Fe₃O₄ concentration being 0.04 mg/mL = 173 μM [corresponding to a 173 μM concentration of Fe(II)], this oxidation reaction was not catalytic with respect to the Fe(II) contained in magnetite (which was six time the

quantity of oxidized TMB). Conversely, the concentration of TMB oxidation product in the control experiment containing 2 vol% of DMSO (and H₂O₂ alone) was ten times lower (2.8 μM, yield = 0.34 %) (Figure 1). This oxidation reaction was therefore very marginal, as expected when using solutions demetalated by Chelex treatment, but underlines the difficulty to fully remove redox metal traces from aqueous solutions and glassware. These results confirmed that the very low oxidation of TMB in the presence of Fe₃O₄/H₂O₂ was not catalytic with respect to magnetite particles, and is mediated by HO• generated by adventitious metal traces. This result is consistent with a recent report indicating that pure magnetite does not significantly contribute to ROS production (13).

Fe₃O₄ associated to H₂O₂ is unable to oxidize the HRP substrate 9-OH-NME⁺.

The peroxidase catalytic oxidation of 9-hydroxy-*N*²-methyllellipticinium acetate (9-OH-NME⁺, Figure 2), a drug developed as anticancer agent in the 1980s, generates the quinone-imine derivative 9-oxo-NME⁺ (Figure 2) (23,24). Such quinone-imine, resulting of a 2-electron oxidation of a *p*-aminophenol structure, is the typical signature of a reaction catalyzed by a peroxidase. The electrophilic C10 position of this reactive intermediate can further react with nucleophiles such as H₂O or H₂O₂ to provide 9,10-dioxo-NME⁺, or with the substrate itself to generate oligomers (25).

The quinone-imine derivative 9-oxo-NME⁺ has a specific absorbance at λ_{max} = 490 nm. So, monitoring the oxidation of 9-OH-NME⁺ at λ = 515 nm allows the detection of 9-oxo-NME⁺ without contribution of the substrate or of any other oxidation product [ε₅₁₅ (9-oxo-NME⁺) = 8.4 x 10³ M⁻¹ cm⁻¹] (26). In addition, since 9-OH-NME⁺ is easily soluble in aqueous solution, this peroxidase substrate does not require DMSO as a co-solvent, contrarily to TMB which must be added in reaction mixtures after a preliminary solubilization in DMSO, a solvent that partly interferes with HO• in TMB oxidation as discussed above.

The oxidation of 9-OH-NME⁺ was performed with HRP/H₂O₂ or with Fe₃O₄/H₂O₂ and the substrate conversion was monitored by HPLC, with UV-visible and mass spectrometry detection. In the presence of HRP/H₂O₂ [concentrations at t₀ of 9-OH-NME⁺, H₂O₂ and HRP = 200 μM, 400 μM and 100 nM, respectively], the substrate [t_R (9-OH-NME⁺) = 9.8 min] totally disappeared after 15 min of reaction time (conversion > 99 %). In these conditions, the transient quinone-imine 9-oxo-NME⁺ was not detected, the main oxidation products were the orthoquinone 9,10-dioxo-NME⁺ [*m/z* = 291.1 (M⁺), Figure 2], and dimeric [*m/z* = 283.1 (M²⁺)] and trimeric [*m/z* = 280.5 (M³⁺)] derivatives (for structure proposals, see Supporting Information, Figure S2).

When the concentration of HRP was ten times lower (10 nM, all other parameters being unchanged), the conversion of 9-OH-NME⁺ was 85 % after one minute, corresponding to a turnover rate higher than 17,000 cycles/min, and the product distribution was identical.

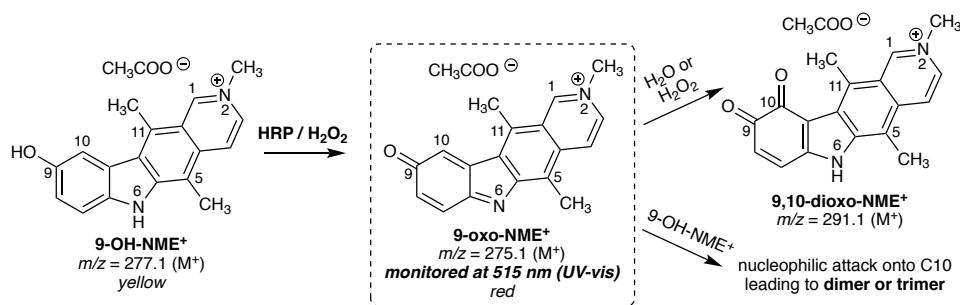


Figure 2. Oxidation of 9-hydroxy-*N*²-methyl-ellipticinium acetate (9-OH-NME⁺) in the presence of HRP/H₂O₂, according to a 2-electron peroxidase pathway.

By comparison, the oxidation of 9-OH-NME⁺ (200 μM) by H₂O₂ (130 mM) in the presence of magnetite particles {0.01 mg/mL = 43 μM, [Fe(II)] = 43 μM} resulted in less than 3 % of substrate conversion, even after 1 h of reaction time, corresponding to 0.14 mole of substrate converted per mole of Fe(II) present in magnetite, far below even one catalytic cycle. When the concentrations of H₂O₂ and magnetite were 4-fold increased {[H₂O₂ = 530 mM, [Fe₃O₄] = 0.04 mg/mL corresponding to [Fe(II)] = 173 μM}], the substrate conversion was 6 % after 1 h of reaction time, corresponding to 0.07 mole of substrate converted per mole of Fe(II) present in magnetite. Noteworthy, the control reaction carried out in the presence of the same concentration of H₂O₂ (530 mM) but in the absence of magnetite resulted in the same conversion of 9-OH-NME⁺, indicating that this marginal reaction was not dependent on magnetite particles, but was due to unidentified low-valent metal traces.

The formation of the transient quinone-imine 9-oxo-NME⁺ product was monitored by UV-visible spectroscopy at 515 nm, as a function of time (Figure 3) (26). Due to the high absorbance of 9-OH-NME⁺ at 313 nm, the reaction mixtures were diluted by a factor of 5, the 9-OH-NME⁺/HRP or Fe₃O₄/H₂O₂ molar ratio being kept the same than above. Initial mixtures containing 9-OH-NME⁺ and HRP or Fe₃O₄ were prepared at 37 °C. Three min later, the oxidation was triggered by addition of H₂O₂.

In a reaction medium composed of 9-OH-NME⁺/HRP/H₂O₂ (40 μM, 20 nM, and 80 μM, respectively), the absorbance at 515 nm quickly increased after addition of H₂O₂, to reach a maximum value of Abs₅₁₅ = 0.27 after 30 sec [Figure 3, trace (a)]. This result, fully consistent with already reported data (25), corresponds to a quinone-imine concentration of 32 μM [(0.27 × 10⁻⁶)/8400], and a yield of 80 % with respect to the starting amount of 9-OH-NME⁺ (40 μM). On the opposite, no absorbance at 515 nm was observed for the reaction medium 9-OH-NME⁺/Fe₃O₄/H₂O₂ [40 μM, 8.5 μM, and 26 mM, respectively, trace (b)], confirming the absence of significant amount of quinone-imine. The overlay of the spectra recorded in the range 220-700 nm, confirmed that the substrate was not significantly oxidized in these conditions (Supporting Information, Figure S3). Even in the presence of 20-fold amounts of Fe₃O₄ and H₂O₂, 9-oxo-NME⁺ was not detectable and 9-OH-NME⁺ remained unchanged [9-OH-NME⁺/Fe₃O₄/H₂O₂ = 40 μM/173 μM/530 mM, respectively, Figure 3, trace (c)]. The control experiment in the presence of H₂O₂ alone (530 mM) indicated that 9-OH-NME⁺ was not oxidized in these conditions [trace (d)].

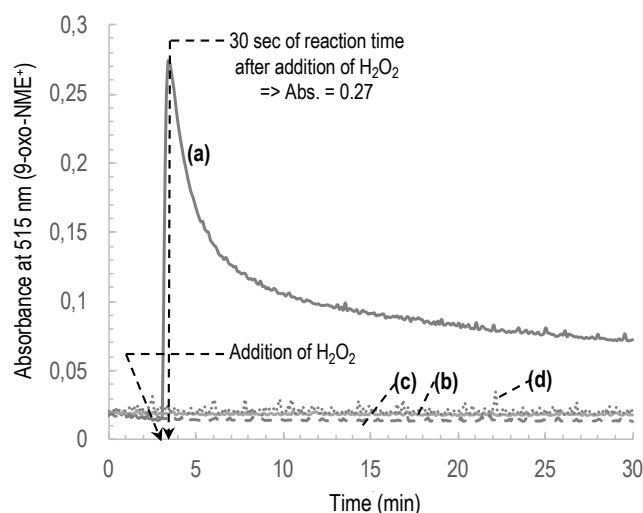


Figure 3. Kinetic UV-vis spectra of the oxidation of 9-hydroxy-*N*²-methyl-ellipticinium acetate (9-OH-NME⁺) in the presence of HRP, (a) or Fe₃O₄ [(b) and (c)] associated to H₂O₂, in 200 mM acetate buffer, pH 3.5, at 37 °C. Control oxidation of 9-OH-NME⁺ with H₂O₂ alone (d). [9-OH-NME⁺] = 40 μM (all traces); [H₂O₂] = 80 μM (a), or 26 mM (b), or 530 mM (c, d); [Fe₃O₄] = 8.5 μM or 173 μM. For the overlaid spectra in the 220-620 nm range, see Supporting Information, Figure S3.

It is noticeable that, when H_2O_2 was added in the reaction mixture containing 9-OH-NME⁺ and HRP, the reaction immediately turned red, the color of 9-oxo-NME⁺. Conversely, the mixture containing 9-OH-NME⁺ and Fe_3O_4 did not change color upon addition of H_2O_2 (Supporting Information, Figure S4).

So, using 9-OH-NME⁺, a substrate that stamps without ambiguity an oxidation catalyzed by a peroxidase, we clearly confirmed that magnetite has no significant oxidative ability in the presence of hydrogen peroxide. All claims concerning the enzymatic activity of nanoparticles like magnetite should be reconsidered until someone will be able to obtain a real catalytic activity using new nanoparticle-based catalysts. At the moment, to the best of our knowledge, all works on these "peroxidase-like nanozymes" correspond to the detection of an oxidation close to the noise background, very far from any catalytic activity. The claims of peroxidase-like activity of magnetite are in fact an erroneous presentation of experimental data.

Michaelis-Menten analysis is invalid for non-catalytic reactions.

Several reports on the TMB oxidation by magnetite and hydrogen peroxide are reporting a Michaelis-Menten analysis of the substrate conversion (1,11). First of all, nobody up to the recent years, ventured to apply a Michaelis-Menten kinetic analysis to *non-catalytic reactions*. In addition, such kinetic analyses of enzymatic catalysis (or enzyme-like catalysis) is reliable only when the substrate concentration is high enough to saturate the enzyme. The kinetic parameters V_{max} and K_M can be obtained only when the steady-state assumption is valid. The pseudo-saturation curve reported in Reference 1 is due to the fact that the conversion of the substrate is reaching a plateau because of the limited non-catalytic oxidation of the substrate, not because the "nanozyme" is saturated by the substrate. The substrate stationary assumption is strictly required to obtain Michaelis-Menten parameters in valid conditions (27,28). Outside of these requirements, any attempt to extract Michaelis-Menten parameters are meaningless and, in any case, are not the proof of an enzyme-like catalysis. The invalid use of the Michaelis-Menten method is a corrupted presentation of the substrate conversion by these so-called "nanozymes". This is a typical example of a misconducted research work.

In conclusion, the misuse of "nanozymes" in many articles dealing with so-called "peroxidase-like nanoparticles" suggests that the authors did not resist to create a fashionable word, trying to describe a new category of biomimetic catalysts in the rapid expanding world of nanomaterials. In the case of magnetite-mediated oxidations, it is clear that these reactions are far away from any catalytic reaction. They are due to the formation of hydroxyl radicals *via* a Fenton-type reaction triggered by traces of low-valent transition metals that are very difficult to efficiently remove from reaction media and from nanoparticle surface itself.

The titles and the presentation of articles devoted to "nanozymes" with enzyme-like catalytic activities are not scientifically acceptable because they are overriding the accurate scientific definitions. Non-catalytic oxidants cannot be classified as "nanoenzymes", they should be renamed as "nano-oxidants". The established definitions of catalysts and enzymes should be respected. In the present case, the misuse of the ending -zyme attached to nano- is a *semantic falsification*, creating damage for the future generation of students interested in learning true definitions in enzymology and catalysis. Real "nanozymes" may exist in the future, and that will be a new step in the field of biomimetic catalysis. At the moment, this field is not open.

In addition, despite the deleterious effect of $\text{HO}\cdot$ is well-known, the detection of free radical generation by nanozymes models should not be uncritically extrapolated to living organisms, as well as the relevancy of these models to neurodegenerative diseases (29). The hydrogen peroxide concentrations

typically used in nanozyme reactions are in the millimolar range, about 10,000 times higher than that present physiologically (30). Many pathological conditions due to the dyshomeostasis of endogenous metal ions are known to produce HO• more efficiently than magnetite nanoparticles (31).

Materials and Methods

Materials and equipments. 9-Hydroxy-*N*²-methyllellopticinium acetate (9-OH-NME⁺, LT 50100-S.2729) was a gift from Sanofi. No impurity was detectable by ¹H NMR. The purity determined by HPLC was > 98.8 % (detection at λ = 313 nm); impurities did not significantly absorb at 515 nm. All other reagents were purchased from Sigma-Aldrich, except otherwise stated. Commercial Fe₃O₄ (purity of 97% trace metals basis, size 50-100 nm), horseradish peroxidase (type VI, ≥ 250 units/mg solid), hydrogen peroxide (H₂O₂, 30% w/w), dimethyl sulfoxide (DMSO, ACS reagent, purity 99.8%), 3,3',5,5'-tetramethylbenzidine (TMB) were used as received. Chelex 100 resin (sodium form, 100-200 mesh) was supplied by BioRad. Sodium acetate buffer and all aqueous solutions were prepared in milliQ water previously treated with Chelex 100 to reduce traces of metal ion contaminants. The Chelex treatment consisted to gently shake milliQ water with resin (10 g resin/100 mL water) overnight, then decantation and filtration through a Chromafil GF/PET-45/25 filter.

UV-visible spectra were recorded using Agilent 8453 or Cary 3500 spectrophotometers, both equipped with a magnetic stirrer and a Peltier temperature controller to maintain the reaction mixtures at 37°C. For kinetic experiments, the full spectra were acquired from 220 to 700 nm and absorbance at 652 nm or 515 nm was extracted to study the oxidation of TMB or 9-OH-NME⁺ respectively, as a function of time. ¹H and ¹³C-NMR spectra of 9-OH-NME⁺ were recorded at 293 K on Bruker Avance 400 spectrometer using TMS as the external standard. HPLC analyses were carried out using an Agilent 1200 system equipped with a diode array detector and a Waters XBridge C18 column (4.6 x 150 mm, 5 μm), eluted with a linear gradient acetonitrile/water/formic acid from 5/95/0.1, v/v/v to 100/0/0.1, v/v/v, in 24 min; flow rate: 1.0 mL/min, UV detection: 313 and 515 nm; injection volume: 10 μL. Blank DMSO injections were made between each sample injection. LC-MS analyses were performed on a Waters Xevo-G2TOF mass spectrometer with the same column and the same gradient as above mentioned; spectra acquisition was in positive mode; *m/z* range 100-1200 amu, flow rate 0.5 mL/min; injection volume 7 μL, and UV detection at 313 nm.

Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of DMSO 2 vol% or 20 vol%. A TMB stock solution was daily prepared in DMSO (41 mM) and kept in the dark. A Fe₃O₄ stock solution was prepared as a suspension of black powder in ultrapure water (5 mg/mL) and vigorous mixing was applied before each sample collection. Kinetic UV-visible measurements were carried out at 37°C under stirring at 800 rpm as followed: In a UV-vis cuvette, a solution containing TMB, Fe₃O₄ and eventually an appropriate volume of DMSO was prepared in sodium acetate buffer 0.2 M, pH 3.5. H₂O₂ was then added, and the absorbance of the oxidized TMB was immediately monitored at 652 nm for 30 min. Final concentrations in the cuvette were: [TMB] = 816 μM, [Fe₃O₄] = 173 μM (0.04 mg/mL) and [H₂O₂] = 98 mM, and final volume in the cuvette was 1.5 mL. The DMSO concentration was 2 vol% or 20 vol% taking in account the DMSO brought by the TMB stock solution. Control experiments in the absence of Fe₃O₄ were also performed, as oxidations with different concentrations of H₂O₂ (Figure S1).

Oxidation of 9-OH-NME⁺ by HRP/H₂O₂ or Fe₃O₄/H₂O₂. HPLC and LC-MS monitoring. The usual oxidation reactions were carried out in sodium acetate buffer 200 mM pH 3.5 with 9-OH-NME⁺ (200 μM) in presence of HRP/H₂O₂ (10 nM or 100 nM/400 μM) (23) or Fe₃O₄/H₂O₂ (0.01 mg/mL = 43.2 μM/130 mM) (1), under stirring at 37°C. The reaction started by addition of H₂O₂. Final volume was 1 mL. Reaction mixtures were analyzed by HPLC and LC-MS injections after 3, 15, 30 or 60 min. Experiments in presence of 4-fold increased concentrations of Fe₃O₄/H₂O₂ (0.04 mg/mL = 173 μM/530 mM) were carried out with the same substrate concentration (200 μM). Control experiments in the absence of HRP or Fe₃O₄ and with 9-OH-NME⁺ alone were also performed.

Oxidation of 9-OH-NME⁺ by HRP/H₂O₂ or Fe₃O₄/H₂O₂. Kinetics of formation of 9-oxo-NME⁺ measured by UV-visible spectrophotometry. The formation of 9-oxo-NME⁺ upon oxidation of 9-OH-NME⁺, was monitored by UV-visible at 515 nm as a function of time. Due to the high absorbance of 9-OH-NME⁺, the reaction mixtures were diluted compared to conditions above mentioned for HPLC and LC-MS monitoring. The oxidation reactions were carried out in sodium acetate buffer 200 mM pH 3.5 with 9-OH-NME⁺ (40 μM) in presence of HRP/H₂O₂ (20 nM/80 μM) or Fe₃O₄/H₂O₂ (0.002 mg/mL = 8.5 μM/26 mM), under stirring (800 rpm), at 37 °C. The oxidation started by H₂O₂ addition at 3 min of the run. Final volume in the cuvette was 1.5 mL. Experiments in the presence of 20-fold increase of Fe₃O₄/H₂O₂ (0.04 mg/mL = 173 μM/530 mM) were carried out for the same substrate concentration (40 μM). Controls experiments without HRP or Fe₃O₄ and with 9-OH-NME⁺ alone were also performed.

Oxidation of 9-OH-NME⁺ by HRP/H₂O₂ or Fe₃O₄/H₂O₂. Photographs of oxidation mixtures. The oxidation of 9-OH-NME⁺ produces a color change from yellow to red, observable with the naked eye. The oxidation reactions were carried out in sodium acetate buffer 200 mM, pH 3.5, with 9-OH-NME⁺ (200 μM or 816 μM) in presence of HRP/H₂O₂ (100 nM/400 μM) or Fe₃O₄/H₂O₂ (0.04 mg/mL = 173 μM/530 mM), respectively, under stirring at 37 °C. Photographs of mixtures were taken after 1 min, 3 min, 15 min, 1 h and 2 h. The TMB oxidation produced a color change from colorless to blue. TMB oxidations were carried out in sodium acetate buffer 200 mM, pH 3.5, with TMB (816 μM) in presence of Fe₃O₄/H₂O₂ (0.04 mg/mL/530 mM) or H₂O₂ alone (530 mM) under stirring at 37 °C.

Acknowledgments

This work was supported by the CNRS and partially by GDUT (BM). The authors thank Dr. Nathalie Martins (Institut de chimie de Toulouse) for LC-MS analyses.

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Supplementary Information for

"Nanozymes" without catalytic activity should be renamed.

Contains Figures S1-S4.

Figure S1. Oxidation of TMB by Fe₃O₄ associated to different concentrations of H₂O₂ in acetate buffer 200 mM, pH 3.5, 37 °C.

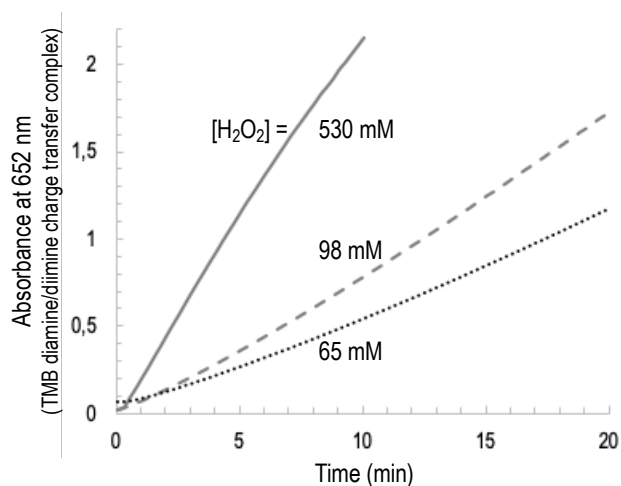


Figure S2. Proposed structures of dimeric and trimeric compounds resulting from reaction of 9-oxo-NME⁺ in the presence of HRP/H₂O₂, and detected by LC-MS.

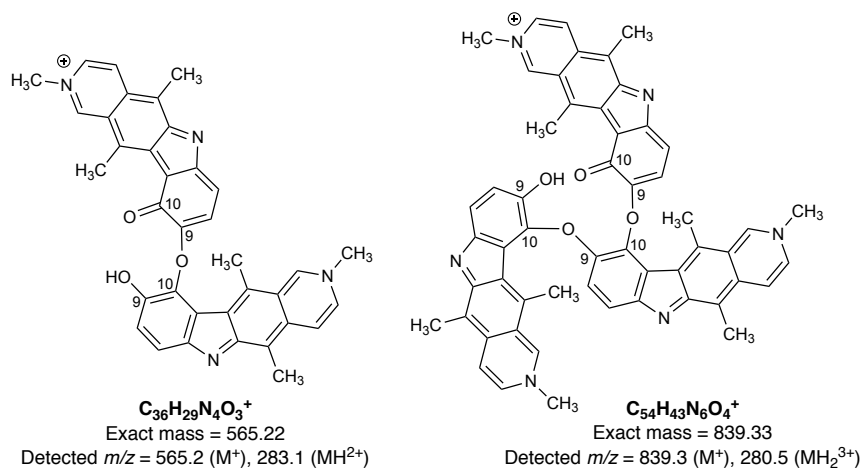


Figure S3. Overlaid UV-visible spectra of the oxidation of 9-OH-NME⁺ (40 μM) by (a) HRP/H₂O₂ (20 nM/40 μM) or by (b) Fe₃O₄/H₂O₂ (8.5 μM/26 mM). In (b), the high absorption below 300 nm (grey trace) is due to concentrated H₂O₂

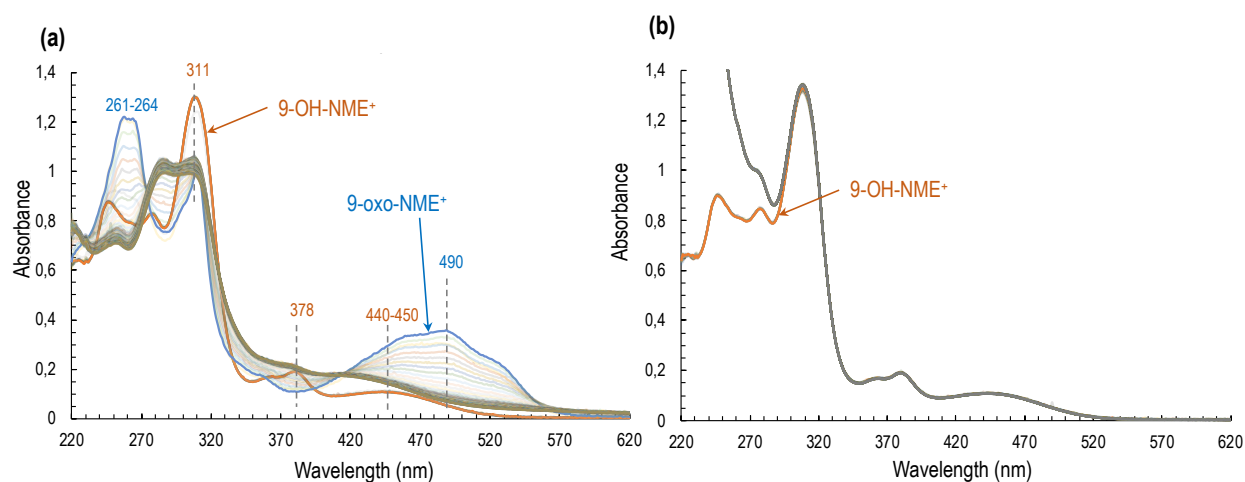
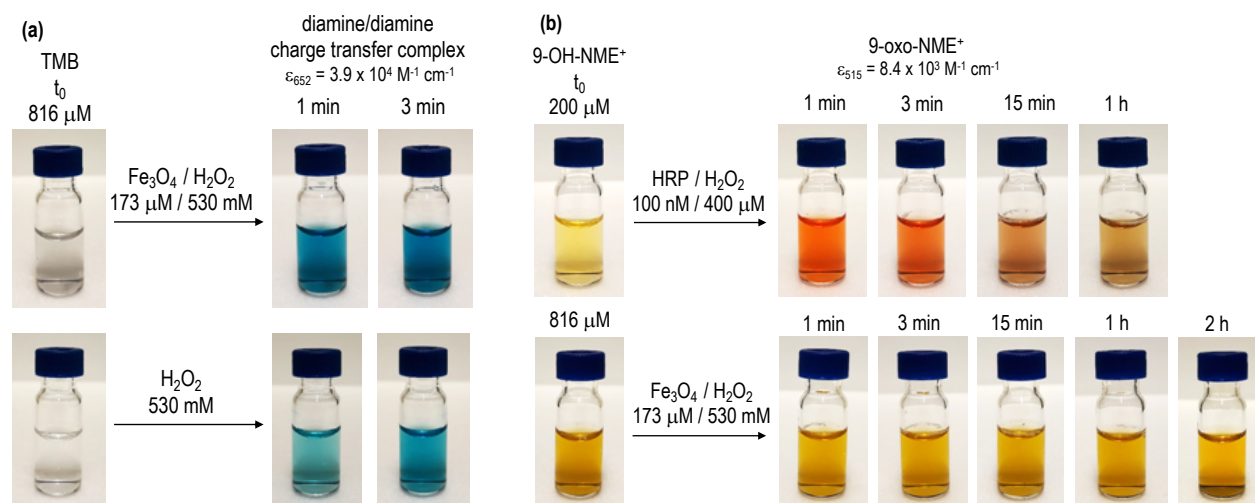


Figure S4. (a) Oxidation of TMB by H₂O₂ to diamine/diimine charge transfer complex in the presence or in the absence of magnetite Fe₃O₄; (b) Oxidation of 9-OH-NME⁺ by HRP/H₂O₂, and lack of reactivity in the presence of Fe₃O₄/H₂O₂.



Annex for the manuscript

"Nanozymes" without catalytic activity should be renamed.

Michel Nguyen¹, Anne Robert^{1*}, Bernard Meunier^{1,2*}.

This article was initially submitted to PNAS. The full text of the Reviewer's comments and Editor's rejection, our appeal, and the final editorial decision are published below.

1. Notification of rejection by PNAS Editorial Office, April 26, 2021.

journalstaff@pnascentral.org

26 avril 2021 à 22:59

PNAS MS# 2021-04697 Decision Notification

À : Anne Robert

April 26, 2021

Title: ""Nanozymes" without catalytic activity should be renamed."

Tracking #: 2021-04697

Authors: Nguyen et al.

Dear Dr. Robert,

After an initial evaluation of its general suitability for PNAS, your manuscript was assigned to an editor with expertise in the subject area of your work, who then examined the manuscript and obtained reviews from 3 authorities in the field. These reviews, which appear below, were then considered by the editor, who determined the comments were of sufficient significance to recommend rejection.

Because we receive many more manuscripts for consideration than we can accept for publication, critical comments from expert reviewers that cannot be easily and rapidly addressed in a revision are sufficient cause for rejection; our goal to publish important research rapidly prevents us from undertaking multiple rounds of revision and re-review. A resubmission of the article will not be permitted.

Although we expect that this outcome is disappointing, we hope that the rationale for our decision process is clear and that you will find the comments of the reviewers helpful when revising your work for submission elsewhere. We thank you for choosing PNAS as a potential venue for publishing your work and we hope that you'll continue to consider PNAS for future submissions.

Yours,
May R. Berenbaum
Editor-in-Chief

Editor Comments:

The manuscript by Nguyen, Meunier and Robert argues that the term "nanozymes" should be abolished, at least until there are examples of nanoparticles that display proper Michaelis-Menten kinetics. Meunier and Robert have published on this topic twice before, once in *Angew. Chem.* in 2018 (ref. 11) and then a 'comment' in *J. Phys. Chem. C* in 2019 (ref. 13). The present work extends the argument against magnetite nanoparticles as nanozymes by including new experiments that show Fenton-like chemistry and no M-M kinetic behavior. Similarly, reference 2 (Maldonado, et al. 2019) evaluated magnetite and found hydroxyl radical as the major reactive species being produced.

The three reviewers all agree that the present work makes some very valid points, and they also make some suggestions for minor revision that would improve the manuscript. Notably, the tone of the writing is very aggressive, perhaps because the recent papers have not gathered a great deal of traction in the literature since publication. It is therefore understandable that the authors wish to make a strong statement.

On the other hand, it is not clear to this editor that PNAS is the right venue for this work. The research reported does not really report a new frontier but rather seeks, again, to correct work that was originally published 14 years ago in Nature Biotechnology. I agree with Reviewer 2 that the work should be published in the journal in which the original work appeared.

Reviewer Comments:

Reviewer #1:

Suitable Quality?: Yes
Sufficient General Interest?: Yes
Conclusions Justified?: Yes
Clearly Written?: Yes
Procedures Described?: Yes

Comments on Significance Statement:

It would be advantageous to the authors' arguments if the Significance Statement were somewhat less polemic. While I am sympathetic to the authors' conclusions, the phrase "misconducted research work described by a semantic falsification" is a little over the top. Something along the lines of "misinterpreted prior results and an inaccurate and unneeded neologism" might be more persuasive.

Comments:

This is a thorough and important evaluation of a topical and controversial area. I think that the manuscript is worthy of a broad audience that extends beyond just the chemical community and is appropriate for PNAS. The authors are certainly right that comparisons between iron-oxide nanoparticles and peroxidases are way overblown and I agree that "nanozyme" is certainly an inappropriate piece of hype that the scientific community would be best avoid (until such time as a nanoparticle actually exhibits real enzymatic characteristics, and perhaps even AFTER such a time!). The evidence that the authors provide is overwhelming convincing that Gao et al. merely are observing Fenton chemistry (i.e., a radical chain reactions produced from the reaction of H₂O₂ and probably Fe(II)/Fe(III) ions) and not in any way related to the chemistry shown by peroxidases.

I will say, however, that the Authors' would be more persuasive and will better serve the scientific community if they were to trim back the polemic nature of some of their conclusion statements. In both the Abstract and in the Significant Statement, the phrase "This erroneous presentation of experimental data is a misconducted research work associated to a semantic falsification. " is over the top. Mind you, the authors' are quite right, but it would be more persuasive if they used a phrasing more along the lines of "misinterpreted prior results and an inaccurate and unneeded neologism". Similarly in the concluding paragraphs, rephrase "the misuse of "nanozymes" in many articles dealing with so-called "peroxidase-like nanoparticles" suggests that the authors did not resist to create a fashionable word, trying to describe a new category of biomimetic catalysts" Likewise, "...'nanozymes' ... are not scientifically acceptable because they are overriding the accurate scientific definitions" would be better stated "In light of the errors in prior analysis, the use of the term nanozymes is simply inappropriate for scientific communication and reflects poorly on the apparent motivations of those who would use such a term."

Reviewer #2:

Suitable Quality?: No
Sufficient General Interest?: No
Conclusions Justified?: Yes
Clearly Written?: Yes
Procedures Described?: Yes

Comments:

This article addresses the role of magnetic nanoparticles (MNPs, specifically Fe₃O₄) in the oxidation of organics dyes. It has been claimed by others that the Fe₃O₄ MNP mimics horse radish peroxidase, carrying out catalytic conversion of reduced to oxidized organic substrates in the presence of hydrogen peroxide.

In the present paper the authors continue their analysis of the Fe₃O₄ reactivity, with the key questions being

whether Fe₃O₄ MNPs are capable of high turnover numbers and whether they proceed via 2 electron oxidation reactions (as occurs with horse radish peroxidase, HRP).

A number of experimental strategies are employed in the present work. These include the use of an alternate organic substrate that does not require the presence of the free radical quencher DMSO for solubilization and which allows for the observation of the 2 electron oxidized intermediate and product, respectively, seen in the presence of HRP. They show that Fe₃O₄ fails to produce the spectral signatures characteristic of HRP and, further, that the addition of the radical quencher DMSO greatly reduces the observed yield. The authors conclude that the actual reaction pathway is quite different from HRP, taking place via a reaction of trace metals with added H₂O₂ to produce hydroxyl radical as oxidant. They also show similar chemistry in the complete absence of the added MNP.

One important aspect of the initial characterization of the Fe₃O₄ MNP was an appearance of apparent Michaelis-Menten behavior, with regard to added substrate and/or hydrogen peroxide. As discussed herein, these former observations may be attributable simply to a limiting concentration for the actual oxidant

All of the above data and conclusions appear quite reasonable, as does a published correction of the original reports on the properties of Fe₃O₄ particles. However, the authors of this work have already addressed some of the issues regarding a lack of true peroxidase activity and the likely involvement of a free radical as the active oxidizing agent in the behavior of the Fe₃O₄ NP. I very much agree with the authors about "wanting to get this right in the literature", but do not think that the present work is appropriate for PNAS. This study may be better published in one of the more specialized journals where the original studies appeared.

Reviewer #3:

Suitable Quality?: Yes
Sufficient General Interest?: Yes
Conclusions Justified?: Yes
Clearly Written?: Yes
Procedures Described?: Yes

Comments:

This work presents experimental findings and analysis demonstrate that ferromagnetic nanoparticles, such as magnetite, do not have an "intrinsic peroxidase-like activity" in the presence of hydrogen peroxide. Therefore, it is incorrect to refer to this particles as "nanozyme", but rather as "non-oxidant".

The experimental protocols to demonstrate that ferromagnetic nanoparticles have no catalytic activity is well-thought and rigorous. It is clear that the oxidation of the substrate 3,3',5,5'-tetramethylbenzidine being far below one "catalytic cycle" with respect to iron nanoparticles. The authors shown that the "nanoenzymes" cannot perform the two-electron oxidation typically catalyzed by a peroxidase, when using a specific peroxidase substrate such as the anticancer drug 9-hydroxy-N²-methylellopticinium, a p-aminophenol derivative known to produce a specific quinone-imine when oxidize by peroxidases. Interestingly, this work demonstrates that the marginal oxidation reactions observed with magnetite in the presence of hydrogen peroxide is likely due to a Fenton reaction triggered by adventitious metal impurities.

In the paper, the authors point out to two specific manuscripts (1 and 11), where TMB oxidation by magnetite and hydrogen peroxide is analyzed Michaelis-Menten equation. This reviewer could not see any Michaelis-Menten analysis in the paper 11; this should be checked by the authors. However, there was a very questionable Michaelis-Menten analysis in paper 1. Personally, I am shocked that paper 1 has been cited over 3,000 times (according to Google Scholar), and the poorly performed Michaelis-Menten shown in Figure 4 passed the peer-review process of Nature Biotechnology. I took the liberty of carrying out a double reciprocal plot for Figures 4a, b, c and d. There is no clear saturation in the Michaelis-Menten process, and the form of the curves do not show the typical rectangular hyperbola form. As a result of this, it is questionable that it passes the linear transformation test for the Michaelis-Menten data. Moreover, the origin of the double reciprocal plots shown in Figures 4 e and f is questionable. The reported double reciprocal plots in Figures 4 e and f have only four points, while the Michaelis-Menten plots have 7 or 10 points.

The arguments about the questionable Michaelis-Menten analysis will be strengthened by making the above points.

It is straightforward to extract the original data for Figures 4 a, b, c and d of paper 1 by using software tools, and plotting the double reciprocal plots.

Needless to say there is sufficient experimental data to demonstrate that there is no catalytic conversation in the reaction, and the Michaelis-Menten analysis captures a pseudo-saturation process triggered by substrate is reaching a plateau due to the limited non-catalytic oxidation of the substrate.

I have a couple of minor observations. First, in the introduction, the authors write "...several of these articles contain a Michaelis-Menten analysis of the substrate conversion..." but only cite two papers, and one of them doesn't have a Michaelis-Menten analysis. If there are several, they should cite several; two is not sufficient. Second, the statement "such kinetic analyses of enzymatic catalysis (or enzyme-like catalysis) is reliable only when the substrate concentration is high enough to saturate the enzyme." This is not correct. The steady-state analysis for Michaelis-Menten type reactions can be carried out under different conditions, including for enzyme and substrate concentrations with an equal order of magnitude as long as the K_m is significantly larger than the substrate concentration. This is illustrated in the review paper (27), which you cite and other papers by the Schnell lab.

2. Our appeal, April 27, 2021.

↳ Bernard MEUNIER

27 avril 2021 à 10:16

Rép : PNAS MS# 2021-04697 Decision Notification

[Détails](#)

À : journalstaff@pnascentral.org, Cc : Anne Robert

Dear Editor-in-Chief, Dear Pr Berenbaum,

Frankly speaking, I am rather surprised that you decided to reject our manuscript.

Referee 1 and 2 answered yes to all questions about the manuscript and Referee 2 had some remarks concerning the wording of the few sentences, but he answered yes to the questions concerning the conclusions, clarity and procedures.

In a revised version, it would be easy to make the corrections and to adjust the tone of some sentences since Referee 2 kindly to us the way to do it. (He also understands that English is not our mother language).

Referee 3 stated : " **Personally, I am shocked that paper 1 has been cited over 3,000 times** (according to Google Scholar), **and the poorly performed Michaelis-Menten shown in Figure 4 passed the peer-review process of Nature Biotechnology** ».

We are also shocked by such situation.

I had the feeling that PNAS would have been the best journal to stop such « poor science », but in fact not, despite two very positive reports and a third one asking for wording modifications.

As senior author, strongly attached to the quality of experimental sciences, I wish to tell you that I am more than disappointed by the rejection decision.

I had the feeling that the Journal of the National Academy of Sciences, because of its long tradition with high quality research was the suitable medium. Obviously, I was wrong ...

I am sorry to insist, but can you consider a revised manuscript taking in consideration the different corrections kindly proposed by the Referees ?

With my best and sincere regards.

B. Meunier

3. Appeal rejection, May 6, 2021.

journalstaff@pnascentral.org

6 mai 2021 à 23:04

PNAS Decision Notification: Appeal Request

À : Anne Robert

Title: ""Nanozymes" without catalytic activity should be renamed."

Tracking #: 2021-04697

Author(s): Nguyen et al.

Dear Dr. Robert,

Thank you for submitting your appeal. I am very sorry to say that, after careful deliberation by the Editorial Board, your appeal has been declined. The Editor's decision to decline your submission remains unchanged, and we are therefore unable to consider a resubmission of this paper.

We hope you will consider submitting future work to PNAS.

Yours,
May R. Berenbaum
Editor-in-Chief