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# Catalytic properties of peroxidase mimicking nanozymes

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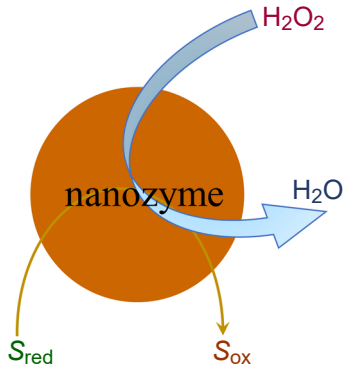
Herein is the first attempt to critically review the catalytic properties of peroxidase mimicking nanozymes. For this aim the main factors affecting nanoparticles activity are discussed, and the catalytic properties are normalized allowing true comparison of different nanomaterials. The highest catalytic activities in hydrogen peroxide ($H_2O_2$) reduction have been recorded for nitrogen-coordinated iron atoms ($FeN_4$, $FeN_5$). However, the main disadvantages of metal/metal oxide as well as 'single atom' nanozymes are their additional activities in oxygen reduction and $H_2O_2$ dismutation, impairing their application abilities. Nanoparticles catalytically synthesized from the most advantageous electrocatalyst (Prussian Blue) display enzymatic selectivity in addition to the highest catalytic activity. This may indicate simultaneous electron donation to $H_2O_2$ from different iron atoms. Accordingly, perspective synthesis of 'single atom' nanozymes can be carried out considering bi-metallic (Fe–Fe like) structures in addition to the presently synthesized ones.
The bibliography includes 121 references.
*Keywords*: peroxidase, hydrogen peroxide, nanozyme, catalytic nanoparticle, Prussian Blue.




## Contents



## 1. Introduction

The term «nanozyme» has first been introduced describing gold nanoparticle-based transphosphorylation catalysts, as written, 'in analogy to the nomenclature of catalytic polymers (synzymes)' (Ref. 1). Similar terms are known: 'abzymes' for catalytic antibodies,[2] «ribozymes» describing nucleic acids with enzyme-like activity.[3,4]

Generally, nanoparticles with catalytic activity can be referred to as nanozymes. The term, however, has not been fully accepted immediately after its introduction. As shown in Fig. 1, the number of publications (Scopus) describing the only nanoparticles with peroxidase-like activity (equation 1) up to 2020 exceeded the number of publications containing the term 'nanozyme' in their title. Moreover, the total number of publications on nanoparticles mimicking the enzymes peroxidases (Scopus) is more than 2150. As seen (see Fig. 1), the annual number of publications displays the nearly exponential growth exceeding 550 last (2022) year.

The attractive performance characteristics of nanozymes over the corresponding enzymes are the following. On the one hand, they are characterized by the dramatically improved operational stability compared to the inherently unstable biological catalysts. On the other hand, noble metal-free catalytic nanoparticles are much cheaper than biomolecules. These properties allow one to expect that nanozymes can replace the enzymes in their practical applications.

Another advantage of catalytic nanoparticles over the enzymes with, most commonly, a single active center, is operation through a large ensemble of active sites occupying

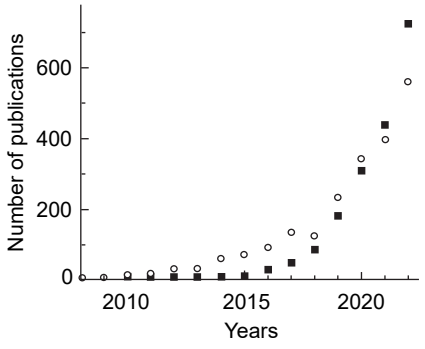



**Figure 1.** Number of publications per year on (■) nanozymes (title) and (○) describing nanoparticles with peroxidase-like activity (Scopus).

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their entire surface (or even volume). Despite more than 50 years of history, all attempts to synthesize an artificial active center with enzymatic catalytic activity are unsuccessful. However, their ensemble allows nanoparticles to achieve even over-enzymatic activity. This can be illustrated by visualization of nanoparticles through their electrocatalysis,[5,6] impossible even for the enzymes.[7] The advantage of active sites ensembles obviously does not work for the so-called 'single-atom' nanozymes. For example, the turnover number of apparently the best $FeN_5$ single-atom nanozyme in oxygen reduction is below 0.1 $s^{-1}$ (see Ref. 8), whereas for the corresponding enzyme laccase it reaches the value of 500 $s^{-1}$ (Ref. 9).

Current mini-review is devoted to peroxidase-mimicking nanozymes representing the dominating family among catalytic nanoparticles synthesized (see Fig. 1). Its purpose is to reconsider their kinetic peculiarities evaluated in quite different conditions. A true comparison of the reported nanomaterials concerning their catalytic properties is possible only through normalization of kinetic constants. Future perspectives for synthesis of even more catalytically active peroxidase-like nanozymes are outlined.

## 2. Towards peroxidase mimicking

Concerning their disadvantages, it is hard to expect from nanozymes the specificity often peculiar to biological catalysts. Hence, the most successful for practical applications would be mimicking of the low-specific enzymes. Among the latter is peroxidase (EC 1.11.1.7), the enzyme most widely used in practice. Peroxidases catalyze reduction of hydrogen peroxide:

$$H_2O_2 + 2\bar{e} \xrightarrow{\text{peroxidase}} 2OH^- \qquad (1)$$

Figure 2*a* displays the scheme of peroxidase action. Peroxidases are specific to hydrogen peroxide, displaying, however, broad selectivity to the reductant. Being immobilized onto the inert electrode surfaces peroxidases are involved in direct (mediator-free) bioelectrocatalysis[10,11] resulting in enzyme hydrogen peroxide sensors (Fig. 2*b*). Peroxidases are the most widely used enzyme labels; as an example the scheme of sandwich immunoassay is presented in Fig. 2*c*. Peroxidases are obviously among the first characterized enzymes.[12] Their

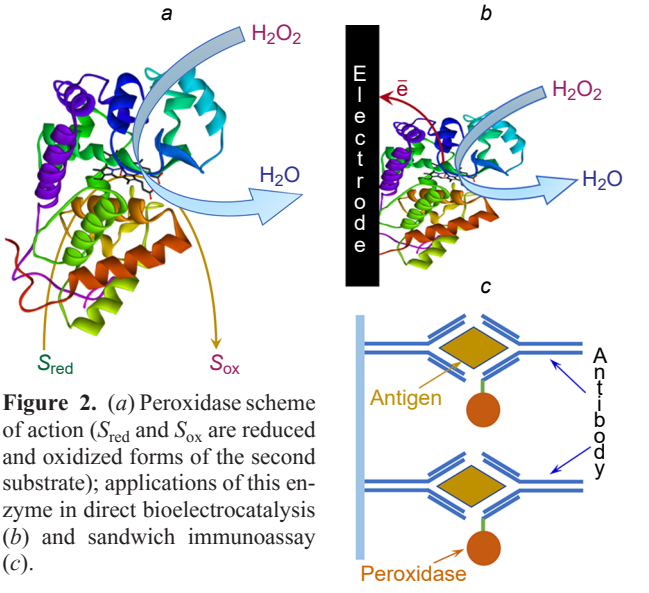


**Figure 2.** (*a*) Peroxidase scheme of action ($S_{red}$ and $S_{ox}$ are reduced and oxidized forms of the second substrate); applications of this enzyme in direct bioelectrocatalysis (*b*) and sandwich immunoassay (*c*).

active site contains the prosthetic group haem. Not surprisingly, first attempts to mimic these enzymes delt with porphyrins (their iron complexes).[13–15] However, the birth of the corresponding nanozymes has to be attributed to the discovery of intrinsic peroxidase-like activity of ferromagnetic nanoparticles.[16]

## 3. Precious metals nanoparticles with peroxidase-like activity

To mimic enzymatic activity the nanoparticles obviously should be composed of the proper material in terms of its catalytic properties. The most widely used material for hydrogen peroxide detection is platinum proposed already in 1970s (Ref. 17). In neutral aqueous solutions, however, it is characterized by low electrochemical rate constants of $H_2O_2$ oxidation ($(6–7)\times10^{-6}$ cm $s^{-1}$)[18] and reduction ($4\times10^{-6}$ cm $s^{-1}$).[19] Nevertheless, platinum nanoparticles have been investigated as catalysts in hydrogen peroxide reduction. Peroxidase-like activity has been reported for platinum nanostructures grown in ferritin,[20] porous platinum nanotubes[21] and nanoparticles,[22] DNA-based,[23] bovine serum albumin stabilized[24,25] and mesoporous silica encapsulated[26,27] Pt nanozymes. Platinum Janus nanoparticles have been reported for catalytic immunosorbent assay.[28]

For synthesis of nanozymes with peroxidase activity platinum has been used in combination with apparently non-catalytic materials: gold,[29–31] silver,[32] copper[33] or nickel,[34] as well as with catalytic ones: palladium,[35] ruthenium[36] and rhodium.[37] Mimetics of enzyme activity has been reported for palladium-based nanostructures.[38]

Despite the fact that gold, according to its electrochemical behavior, is not truly catalytically active in hydrogen peroxide reduction, a number of the corresponding nanoparticles with peroxidase-like activity have been reported,[39–46] including nanoparticles stabilized by bovine serum albumin,[40] DNA,[41] and also metal-organic framework.[46]

## 4. Non-precious metal/metal oxide nanoparticles with peroxidase-like activity

As mentioned, the birth of the peroxidase-like nanozymes is attributed to the discovery of intrinsic peroxidase-like activity of ferromagnetic nanoparticles.[16] Since that time the majority of peroxidase-mimicking nanozymes still use iron based compounds: iron oxide,[47–51] sulfide,[52–55] Fe-coordinated carbon nanozyme dots,[56] Fe,N-doped carbon.[57] Obviously, iron triade-mates, nickel[58] and cobalt[59,60] oxides, as well as bimetallic combinations (Ni–Co,[61] Ni–Mn[62]) have also been reported to form peroxidase-like nanozymes. Copper oxide[63] and sulfate[64] nanoparticles were reported to possess peroxidase activity. Bimetallic alloys of precious,[32,36] non-precious[65,66] metals and their combinations[33] are able to form nanoparticles with peroxidase-like activities.

## 5. Catalytic properties of metal/metal oxide nanoparticles in $H_2O_2$ reduction

Obviously, the most important parameter of peroxidase-mimicking nanozymes is their catalytic activity. We've chosen the catalytic rate constant, or turnover number, for tetramethylbenzidine (TMB) as its valuable measure,[67] because it is reported in the majority of articles. However, among an avalanche of nanozyme related papers only a few of them present recalculation of catalytic parameters per nanoparticle or

per single active site. Table 1 displays the most valuable parameters affecting catalytic activity. Only taking into account these parameters it is possible to make reliable comparison of peroxidase-like nanozymes.

First, it is solution pH. As seen, all activity studies for metal/metal oxide nanozymes have been carried out at pH values below 5.0. Only in 2022 year the reports accessing nanozyme activity at pH 5.5 (Refs 22, 78 and 6.0 (Ref. 28) appeared. They, however, relate to noble metal (platinum)[28] and metal complexes based nanozymes. Non-precious metals as well as their oxides do not seem to provide significant peroxidase-like activity close to physiological pH values.

Second, it is temperature. As known, catalytic constants are increased with temperature. For horseradish peroxidase (HRP) the activation energy at the first stage (Compound 1 forming) was reported to be 9 kJ mol$^{-1}$ (Ref. 80). For HRP and methemoglobin peroxidase activities at 86–90 °C are 2–3.6 times higher than the reaction rates observed at room temperature.[81]

Third, it is the nanozyme size. As mentioned, nanoparticles made from catalytic materials contain huge ensembles of active sites. The latter are able to provide catalytic properties, which are advantageous even as compared to the enzymes. Most commonly, nanozymes are operating through their entire surface. There are only a few reports on 'single atom' nanozymes in which peroxidase-like activity has been recalculated per active site.[70,78,79] Even concerning 'single-atom' nanozymes the review[82] mentions the turnover numbers in peroxidase-like catalysis of $4\times10^4$ s$^{-1}$ (Ref. 71) and even $5\times10^6$ s$^{-1}$ (Ref. 72). However, the sizes of the corresponding catalytic units are of 90 nm (Ref. 71) and 100 nm (Ref. 72), respectively, obviously representing large ensembles of active sites (see Table 1).

Forth, it is the hydrogen peroxide concentration. Peroxidase catalysis involves the two substrates: $H_2O_2$ and reducing agent. As it is most common in enzyme kinetics, the catalytic constant for one substrate is dependent on concentration of the second one. As seen in (see Table 1), the catalytic constants ($k_{cat}$) for TMB have been evaluated at quite different hydrogen peroxide concentrations varied by 1000 (!) times. Obviously, to estimate true catalytic ability, it is necessary to normalize the reported turnover numbers to certain $H_2O_2$ content. It is reasonable to choose 2 mM $H_2O_2$, as at this particular concentration the enzyme horseradish peroxidase apparent catalytic constant becomes substrate-saturated. Such normalization presumes the division of the reported catalytic constants by the hydrogen peroxide concentration ratio due to the lack of more detailed kinetic data. This means that the turnover numbers for recently reported Ni–Pt nanozymes[34] or ruthenium frames,[68] both evaluated at 2 M (!) of $H_2O_2$ (see Table 1), should be decreased 500–1000 times.

For comparison, Table 1 includes HRP turnover number, evaluated at the wavelength $\lambda$ = 450 nm, which corresponds to the fully oxidized form of TMB. The corresponding catalytic constant at $\lambda$ = 652 nm is an order of magnitude higher. The fact that the turnover number for HRP encounters thousands of s$^{-1}$ has been confirmed in [16].

True comparison with the enzyme catalytic efficiency is possible only for 'single atom' nanozymes, which activity is recalculated per active site.[70,78,79] However, their catalytic constants encountering units of s$^{-1}$ are (considering the HRP turnover number) in fact three orders of magnitude (1000 times) lower rather than 'surpassing', as declared in,[79] those of natural enzyme. Additionally, taking into account that catalytic constants of the nanozymes under discussion[70,78,79] are evaluated at 25–50 times higher $H_2O_2$ concentrations (see Table 1), it is possible to consider their catalytic efficiency to be five (!) orders of magnitude (100 000 times) lower.

Table 1, composed in chronological order, presents general trend in improving of peroxidase-like activity of metal/metal

**Table 1** TMB turnover number (catalytic rate constant) of peroxidase-like catalytic nanoparticles.

| Nanoparticle | pH (temp) | [$H_2O_2$], mM | Size, nm | $k_{cat}$, s$^{-1}$ | Ref. |
|---|---|---|---|---|---|
| $Fe_3O_4$ | 3.5 (40 °C) | 530 | 300 | $3\times10^4$ | 16 |
| Au@Pt-nanorods | 4.5 (30 °C) | 20 | 10×70[a] | $1\times10^4$ | 29 |
| Au@Pt-nanorods | 4.5 (37 °C) | 2 | 20×80[a] | $3\times10^3$ | 30 |
| Pt@m-$SiO_2$ | 4.7 (rt) | 200 | 10 | $2\times10^4$ | 26 |
| Pt@Pd | 4.5 (rt) | 10 | 40 | $3\times10^4$ | 38 |
| Ru (frames) | 4.0 (rt) | 2000 | 2×6 | $1\times10^4$ | 68 |
| $Fe_3O_4$@imprinted polymer | 4.0 (25 °C) | 10 | 30 | 56 | 69 |
| Fe–N–C 'single atom' | 3.0 (–) | 100 | per Fe | 0.8 | 70 |
| Fe–$N_x$ 'single atom' | 4.0 (–) | – | 90 | $3\times10^6$ | 71 |
| Zn–$N_4$ (porphyrin) | 4.5 (rt) | 150 | 100 | $5\times10^6$ | 72 |
| Fe–$N_4$ (heme) | 3.8 (37 °C) | 6 | 200 | 0.5 | 73 |
| Fe–N–Carbon nanotube | 3.5 (–) | 530 | – | 0.5 | 74 |
| Fe–N–rGO | 4.0 (–) | 100 | – | $1.5\times10^5$ | 75 |
| $FeN_3P$ | 3.6 (37 °C) | 3000 | per Fe | 1.1 | 76 |
| Au-nanorods@Cys | 4.6 (25 °C) | 13 | 87×12 | 11 | 44 |
| Ru@G | 4.0 (rt) | 600 | 45 | $3\times10^7$ | 77 |
| Ni–Pt | 4.0 (–) | 2000 | 15 | $4.5\times10^7$ | 34 |
| Pt Janus | 6.0 (–) | 15 | 200 | $5\times10^5$ | 28 |
| Pt mesoporous | 5.5 (25 °C) | 7 | 70 | $5\times10^6$ | 22 |
| Fe–$N_5$ 'single atom' | 5.5 (25 °C) | 100 | per Fe | 4 | 78 |
| Rh–$N_4$ 'single atom' | 4.5 (–) | 50 | per Rh | 1.7 | 79 |
| HRP[b] (450 nm) | 5.0 (rt) | 2 | per Enz. | 220 | 67 |

**Note**. rGO — reduced Graphene Oxide, m-$SiO_2$ — mesoporous $SiO_2$, G — graphen, rt — room temperature. [a] Estimated from SEM images. [b] Horseradish peroxidase.

oxide based nanozymes. Synthesis of metal complexes with iron as central atom coordinated with 4–5 tertiary nitrogen atoms ($Fe–N_4$, $Fe–N_5$)[70,71,73,78] and even with both nitrogen and phosphorus ($FeN_3P$)[76] seems to be the best way. Substitutions of the central atom to zink (Zn)[72] and rhodium (Rh)[79] are also reported.

Considering potential applications, the main disadvantage of the metal/metal oxides nanozymes is their poor selectivity. Certainly, there are methods for improving of the latter, such as molecular imprinting.[83,84] This approach was successfully applied for peroxidase-like nanozymes.[33,69] However, the selectivity improvement concerns only the reducing substrate. For nearly all systems including 'single atom' nanozymes the authors bravely report on oxidase-like (oxygen reduction) and catalase-like (hydrogen peroxide dismutation) activities in addition. However, true mimicking of the peroxidase enzyme is possible only with selective catalytic material.

## 6. Selective electrocatalyst for $H_2O_2$ reduction: Prussian Blue

Selective detection of hydrogen peroxide in the presence of oxygen is apparently the key problem of biosensorics. Since $H_2O_2$ is the side product of the enzymes oxidases, monitoring of its concentration, as shown back in the 1970s (Refs 17, 85), is the most progressive way to record a signal of the corresponding biosensors. However, detection of hydrogen peroxide through its electrochemical oxidation as initially suggested[17,85] suffers from false-positive biosensor responses generated by easily oxidizable compounds.[86]

As a perspective catalytic material for selective hydrogen peroxide reduction we discovered Prussian Blue, or ferric hexacyanoferrate[87,88] ($Fe_4[Fe(CN)_6]_3$). Prussian Blue is among the most ancient coordination compounds known, first mentioned in the beginning of XVII century.[89,90] Its electroactivity was discovered only two and a half centuries later.[91] Electrochemical synthesis of this material is carried out by reduction of ferric ions ($Fe^{III}$) from their mixture with ferricyanide ions ($[Fe(CN)_6]^{3-}$)[91–93] resulting in Turnbull's Blue. The latter was shown to be identical to Prussian Blue.[94] The mentioned mixture accumulates one-to-one complex $Fe^{III}[Fe^{III}(CN)_6]$,[95] which reduction also results in Prussian Blue.[93]

Cyclic voltammograms of Prussian Blue modified electrodes contain two sets of peaks (Fig. 3). The cathodic one corresponds to reduction of Prussian Blue into Prussian White. Sharp peaks with separation from 15 to 30 mV indicate regular structure of inorganic polycrystal.[96] Prussian Blue oxidation into Berlin Green causes appearance of the anodic set of peaks.

Steady-state current of oxygen reduction from air-saturated solution ($[O_2]$ = 0.2 mM) on Prussian Blue modified electrodes is rather low (see Fig. 3). As we found already almost 30 years ago,[87,88] an addition of twice lower concentration of $H_2O_2$ (0.1 mM) causes both cathodic current in Prussian White potential range and anodic current in Berlin Green potential range (see Fig. 3). Prussian Blue, thus, operates as true redox catalyst: its reduced form (Prussian White) reduces $H_2O_2$, and its oxidized form (Berlin Green) oxidizes it. Activities in hydrogen peroxide reduction and oxidation are comparable.[87,88]

The performance characteristics of Prussian Blue making it advantageous over all known electrocatalysts of $H_2O_2$ reduction are both dramatically higher activity and selectivity. Electrochemical rate constant ($k_s$), characterizing activity of Prussian Blue, for 4–6 nmol $cm^{-2}$ of the electrocatalyst exceeds

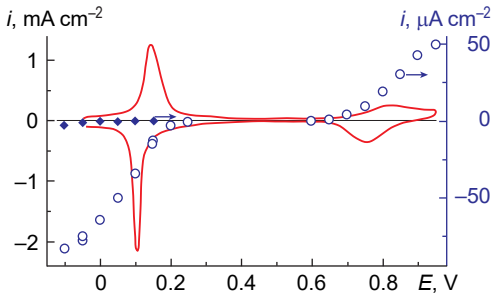


**Figure 3.** Cyclic voltammogram of Prussian Blue (solid, left axis) and steady-state currents in air saturated (0.2 mM $O_2$) solution (♦) and after addition of 0.1 mM $H_2O_2$ (○).

0.01 cm $s^{-1}$ (Ref. 97). Platinum in neutral media is characterized by 1000 times lower $k_s$ for $H_2O_2$ oxidation ($7 \times 10^{-6}$ cm $s^{-1}$)[18]; for its reduction it is even lower.[19] Prussian Blue is also 1000 times more selective to $H_2O_2$ reduction than Pt: at 0.00 V (Ag|AgCl) the corresponding modified electrodes generate 400–600 fold higher current to $H_2O_2$ than to $O_2$ (see also Fig. 3).[98] Such enzymatic selectivity has not been reported for any other catalytic material.

The discovery of Prussian Blue as the most advantageous electrocatalyst for hydrogen peroxide reduction stimulated repots on catalytic properties for non-iron transition metal hexacyanoferrates.[99–107] Our special investigation, however, has shown that non-iron transition metal hexacyanoferrates are catalytically inactive; their minor activity is due to the presence of Prussian Blue structural units as defects.[108]

Let us consider structure of Prussian Blue (Fig. 4). Hexacyanoferrate ion forms C-coordinated iron atoms. On the contrary, ion atoms originated from its salt are coordinated with tertiary nitrogen. As mentioned in the previous paragraph, substitution of these, $Fe–N_6$ atoms leads to a complete lost of electrocatalytic activity. Moreover, in catalytically silent, Prussian Blue redox state of the electrocatalyst these iron atoms are in oxidized ($Fe^{III}$) state. Only reduction of these iron atoms to $Fe^{II}$ state in Prussian White stimulates reduction of hydrogen peroxide. Hence, nitrogen-coordinated iron ($Fe–N_6$) forms catalytic sites in Prussian Blue. A confirmation of this hypothesis can be found considering recently reported 'single atom' nanozymes (see Table 1). The most active of them are based on Fe–N–C (Refs 70, 74, 75), $Fe–N_4$ and $Fe–N_5$ (Refs 71, 73, 78) reactive centers.

## 7. Catalytically synthesized Prussian Blue nanoparticles

Knowing that hydrogen peroxide is able to reduce the one-to-one complex $Fe^{III}[Fe^{III}(CN)_6]$ formed through conditioning of ferricyanide and ferric ions,[109] we proposed[67] catalytic synthesis of Prussian Blue nanoparticles. Since the driving force for such

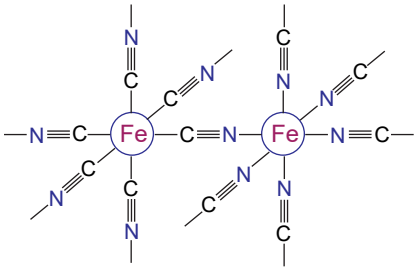


**Figure 4**. Structural subunit of Prussian Blue (ferric hexacyanoferrate).

deposition of the inorganic polycrystal would be $H_2O_2$ oxidation, we believed that this way the most catalytically active structures would be synthesized. Taking into account that Prussian Blue is similarly active in $H_2O_2$ oxidation and its reduction (see Fig. 3), we have expected the higher peroxidase-like activity of the resulting nanoparticles.

Indeed, activity of the catalytically synthesized Prussian Blue nanoparticles is more than 10 times higher as compared to conventionally synthesized colloid.[67] Their catalytic rate constants (evaluated in conditions favorable for the enzyme, see Table 1) always exceed the turnover number of horseradish peroxidase.[67] Size dependence of catalytic constants for catalytically synthesized Prussian Blue nanoparticles display the slope of 2.7 in double logarithmic plots indicating that hydrogen peroxide penetrates the bulk of nanoparticles. For large Prussian Blue nanozymes with a diameter of 570 nm the turnover number becomes 4 orders of magnitude larger than that of the enzyme peroxidase.[67]

As mentioned (see Table 1), the highest catalytic constants for TMB recalculated per active site of 'single atom' nanozymes are 0.8 $s^{-1}$ (Ref. 70), 1.7 $s^{-1}$ (Ref. 79) and 4 $s^{-1}$ (Ref. 78), evaluated, however, at 100, 50 and 100 mM of hydrogen peroxide, respectively. These concentrations are 50 and 25 times higher than one useful for horseradish peroxidase (2 mM $H_2O_2$, above). For Prussian Blue nanozymes we've registered linear dependence of catalytic constants for TMB on $H_2O_2$ concentration.[67,110] Hence, the turnover number evaluated at 100 mM has to be reduced by at least 20–25 times to recalculate it to 2 mM.

Since Prussian Blue is a material, it is not obvious, how many iron atoms compose its active site. Based on crystalline structure,[111] the unit cell of Prussian Blue contains 8 iron atoms. Recalculation of the Prussian Blue nanozymes turnover number to the unit cell results in the rate constant of 0.06 $s^{-1}$. We, however, note that due to their intrinsic absorption the catalytic activity of Prussian Blue nanozymes cannot be evaluated at 652 nm, accordingly the reaction rate has been monitored at 450 nm peculiar to fully oxidized TMB. Comparing catalytic activities of the enzyme peroxidase, we've found that the reaction rate determined at 652 nm is an order of magnitude higher than that at 450 nm. Hence, the activity of Prussian Blue catalytic unit would be still higher than for the most active 'single atom' nanozyme last reported.

A few more words about Prussian Blue as catalytic material. As seen in the structure of its subunit (see Fig. 4), coordination spheres of iron atoms are fully occupied. Despite outer-sphere electron transfer is known, it is hard to believe in outer-sphere catalysis. Hence, catalytic units of Prussian Blue are formed as defects, most probably, bringing N-bounded iron atoms with uncompleted coordination spheres in a proximity ($N_5Fe-FeN_5$). This means that Prussian Blue nanozymes turnover number has to be recalculated to the unit cell containing much larger number of iron atoms than 4 or 8. This allows a conclusion that activity of catalytically synthesized Prussian Blue nanoparticles even if recalculated per active site is unreachable for all reported peroxidase-like nanozymes.

## 8. Prussian Blue based nanozymes 'artificial peroxidase': selectivity and mechanism of action

As mentioned, the main disadvantage of the reported peroxidase-like nanozymes is their poor selectivity. For nearly all systems including the so-called 'single atom' ones the authors bravely report on oxidase-like and catalase-like activities in addition. Poor selectivity, however, affects negatively their potential applications, particularly analytical ones.

Accordingly, among the main motivations of using Prussian Blue as catalytic material for synthesis of nanoparticles with peroxidase-like activity was its selectivity. As indicated, in a certain potential range the current of hydrogen peroxide reduction on Prussian Blue modified electrodes is 400–600 times higher than it of oxygen reduction (see Fig. 3). The achieved selectivity is more than 1000 times higher as compared to platinum.[19]

Conventionally synthesized Prussian Blue nanoparticles have been optimized for multienzymes-like activity catalyzing in addition hydrogen peroxide dismutation observable by oxygen generation from high $H_2O_2$ concentrations.[112] The proposed by us catalytic synthesis of Prussian Blue nanoparticles resulted in at least an order of magnitude improved their peroxidase-like activity.[67] Perhaps, it is because of the synthesis in favor of peroxidase activity, that for these nanoparticles we haven't registered $H_2O_2$ consumption in the absence of reductants (catalase-like activity).

As mentioned, for applications it is important to achieve the selectivity in hydrogen peroxide reduction relatively to oxygen. Indeed, for catalytically synthesized Prussian Blue nanoparticles we haven't noticed TMB oxidation without hydrogen peroxide.[67] However, since Prussian Blue selectivity in electrocatalysis is potential dependent, one would expect an appearance of oxidase-like activity for low-potential substrates. Further study has shown some oxidase-like activity for ferrocyanide, *o*-phenylinediamine, hydroquinone, being still 5–6 times lower as compared to peroxidase-like one.[110] However, in addition to TMB for pyrogallol, catechol, guaiacol, *o*-dianisidine Prussian Blue based nanozymes 'artificial peroxidase' show a complete absence of oxidase-like activity.[110]

It was of great interest to investigate the kinetic mechanism of catalytically synthesized Prussian Blue nanoparticles as the only nanozymes really mimicking the enzyme peroxidase. For that matter as first shown by B.Chance[113–115] and became generally accepted[116–118] at the first stage the peroxidase active site reacts with hydrogen peroxide forming the so-called 'compound I' (Table 2). On the contrary, $H_2O_2$ does not react with Prussian Blue in the oxidation state of the ferric ferrocyanide (see Fig. 3). The ground state (Prussian Blue) has to be first reduced itself to carry out $H_2O_2$ reduction.

It was a challenge to apply the steady state kinetic approach accepted for the enzymes. Before our publication[110] the only hyperbolic dependencies of the initial reaction rates on the concentration of one of the substrates have been reported. Successful application of the enzyme kinetics to the action nanozymes including not only Michaelis-type dependencies, but also the linearization of whole kinetic curves in Walker-Schmidt plots. In summary, the catalytic pathway of nanozymes 'artificial peroxidase' includes three stages: interaction with reducing

**Table 2** Comparative mechanisms of hydrogen peroxide reduction by the enzyme peroxidase and Prussian Blue based nanozymes 'artificial peroxidase'.

| Enzyme peroxidase | Nanozymes 'artificial peroxidase' |
|---|---|
| $E + H_2O_2 \rightarrow$ Compound I | $N + S \rightleftarrows NS$ |
| Compound I + $S \rightarrow$ Compound II | $NS + H_2O_2 \rightarrow NP$ |
| Compound II + $S \rightarrow E$ | $NP \rightleftarrows N + P$ |

**Note**. E — enzyme, N — nanozyme, S — reducing substrate, P — product of substrate oxidation.

substrate, irreversible oxidation by hydrogen peroxide and release of the product (oxidized form of reducing substrate) (see Table 2).[110]

A comprehensive kinetic investigation made it possible for the first time to estimate the rate constants of elementary stages. For nanozymes the rate limiting stage is the second one. The corresponding bimolecular constants for the fastest substrates pyrogallol and ferrocyanide are above $1 \times 10^8$ $M^{-1}$ $s^{-1}$ (Ref. 110). This value is 10 times higher than the constant of $H_2O_2$ interaction with peroxidases.[115] However, for enzymes the rate limiting stage is the last one characterized by the 10 times lower constant.[115,119] Thus, nanozymes 'artificial peroxidase' are characterized by the 100 times higher rate limiting constants.

Steady-state kinetics also allows estimation of the rate constant for the first stage: nanozyme interaction with reducing substrate. For TMB its lower limit ($3 \times 10^9$ $M^{-1}$ $s^{-1}$) is 100 times higher than the corresponding constants of peroxidases.[110]

Once again, comparing enzymes with nanozymes one can conclude that an entire surface of the latter is reactive, whereas in case of the enzymes substrate has to hit their active site. The uniformly accessible surface avoiding effect of rotation on the diffusion-controlled rate[120,121] can be the reason for the novel advantage of nanozymes: their up to two orders of magnitude improved bimolecular rate constants.

## 9. Conclusion

The great interest to nanoparticles mimicking the enzymes appeared after the discovery of peroxidase-like nanozymes. The generally accepted advantages of nanozymes over their biological predecessors are (i) high stability, (ii) low cost if they do not contain noble metals, and (iii) even higher activity due to operation through the huge ensemble of active sites. Highly active catalytically synthesized Prussian Blue nanoparticles have made it possible to discover the novel advantage of nanozymes: their up to two orders of magnitude higher bimolecular rate constants. The latter is most probably due to avoiding rotation limits on the diffusion controlled rate.

The most active catalytic centers contain iron coordinated with four — five tertiary nitrogen atoms ($Fe-N_4$, $Fe-N_5$). Haem in the active site of peroxidases has similar structure.

In view of the mentioned ultra-high activity of catalytically synthesized Prussian Blue, it is also essential to consider mimicking of this material, as this would result in nanozymes with improved catalytic characteristics. On the one hand, an involvement of the only nitrogen coordinated iron in catalysis of hydrogen peroxide reduction explains, why Prussian Blue oxidation state does not reduce $H_2O_2$. Indeed, in Prussian Blue the reduced ($Fe^{2+}$) iron atoms are C-coordinated, but N-coordinated ones are in an oxidized ($Fe^{3+}$) state. Reduction of Prussian Blue to Prussian White forms reduced ($Fe^{2+}$) N-coordinated iron centers, which rapidly react with $H_2O_2$ reducing it. On the other hand, in the fully reduced state (Prussian White) the iron(II) atoms ($Fe^{2+}$) come to the close proximity to each other with the distance of just one $CN^-$ ligand. Hence, it is possible to assume that oxygen atoms in hydrogen peroxide become able to accept electrons simultaneously from different iron centers. Accordingly, for synthesis of 'single atom' nanozymes it is essential to consider bi-metallic ($Fe-Fe$) structures in addition to the presently synthesized single-metal ones.

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