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A Comparative Study on Kinetics Performances of BSA-gold Nanozymes for Nanozyme-mediated Oxidation of 3,3',5,5'-Tetramethylbenzidine and 3,3'-Diaminobenzidine

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ABSTRACT

Commonly 3,3'-diaminobenzidine and 3,3',5,5'-thetramethylbenzidine, abbirvated as DAB and TMB, in turn, have been employed as the standard nanozyme substrates for probing the nanozme-catalyzed oxidation, and the corresponding oxidation products of these substrates have been probed as the analytical identifiers for sensing/biosensing application in lab/medial scales. However, it is well-known that the affinity of these substrates toward binding to enzymes or especially, here, nanozymes is not the same, resulting in different enzyme-like activity and different kinetics indexes including K_m and V_{max}. Since, the protein-assisted/protected gold nanozymes (BSA-gold nanozymes) are type high powerful artificial peroxidase enzymes, Therefore, the kinetics indexes of these nanozymes including both K_m and V_{max} should be changed by varying their substrate. To prove this hypotheis, in this work, a comparative study was performed on the kinetics performances of BSA-gold nanozymes for enzyme-mediated oxidations of TMB and DAB. The results showed that the K_m value of the as-mentioend nanozymes was 0.03 mM and 0.72 mM toward TMB and DAB, in order, revealing that the affinity of TMB for binding to the nanozyme active nodes is significantly higher than its alternative substrate, DAB due to its lower K_m value. In contrast, the V_{max} of the enzymatic reaction was found to be 263 nM sec^{-1} and 185 nM sec^{-1} for the nanozmye-mediated oxidation of TMB and DAB, respectively. The higher V_{max} of the nanozyme-mediated oxidation of TMB revealed that the catalytic efficiency of the as-mentioend nanozymes toward TMB oxidation is characterstically higher (about 1.5-fold) than that of the DAB oxidation. The difference between the kinetic indexes of TMB and DAB may be related to their different oxidation pathways and their different reactivity. In fact, the DAB oxidizes *via* an n-electron irreversible oxidation pathway to produce an indamine polymer. While TMB nanozyme-mediated oxidation has occurred upon a 2-electron reversible mechanism for the production of a cation radical. These different pathways resulted in different kinetic performances.

Keywords: Kinetics performances; BSA-Gold nanozymes; 3,3',5,5'-thetramethylbenzidine; 3,3'-diaminobenzidine

INTRODUCTION

It is well known that although native enzymes reveal high specificity toward their substrates and high catalytic performance, they show several disadvantages such as low stability (narrow pH and thermal range); difficult recovery, and no reusability. For overcoming these drawbacks, the development of enzyme immobilization processes has been considered as a reliable way. The enzyme immobilization permits possible increase in stability (pH, thermal, storage, and solvent performance), easy to recovery and handling *via* simple enzyme separation from the mixtures of reactants and products. Despite great advantages, the specific and relative activities of the most immobilized enzymes are found to be lower than the free enzymes. This decrease in the enzyme activity can be explained by the effect of immobilization on enzymes' conformational transition after their immobilization. To avoid the difficult

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of the enzyme immobilization and for solving its significant drawbacks especially, the enzyme inactivation during the immobilization process, development of artificial enzymes or enzyme mimics is considered. By fast development of nanotechnology, a new approach for developing artificial enzymes is developed by utilizing highly stable nanoparticles with high enzyme-like activity in the enzyme-catalyzed reactions as the artificial enzymes which commonly called as nanozymes. On the hand, the significant progress of nanochemistry and material science in recent years open a new door for design and development of the high-performance and novel nano-supports toward different applications, for instance, MOFs, catalytic materials, and nanoparticles with enzyme-like activity [1-14].

Among different artificial enzymes, nanozymes with very high enzyme-like activity and ultra-stability are recently considered the first and the best choice for proceeding enzyme-catalyzed reactions with higher efficiency compared to native enzymes. There are several different practical and medical applications for nanozymes, as reported in the literature, for instance, they have been utilized for the analytical sensing/biosensing of a verity of species. Besides, the nanozymes were used as reusable catalysts with high catalytic efficiency for biocatalysis of reactions instead of natural enzymes for instance, using in enzyme-mediated dye degradation, water treatment, food quality checking and etc [1,5,15-17].

The first report on the enzyme-like nanomaterials was in 2007 by Gao et al. which introduced the iron oxide nanoparticles as the peroxidase mimicking materials with high catalytic efficiency and characteristic stability. After the first report of Prof. Gao as the pioneer and founder of the nanozyme field in sensing, detection, and catalysis, various nanomaterials such as noble metals, metal oxides, and carbon materials were introduced as enzyme mimics (nanozymes) [5].

Various nanomaterials exhibit peroxidase-like activity, hence, most of the nanozymes are peroxidase mimetics. For instance, there several reports on the peroxidase like activity of MnO_2 nanoparticles, bismuth oxyiodide nanoflowers (abbreviated as BiOI-NFs), different types (modified or unmodified) of silver nanoparticles [20], protein-assisted/protected gold nanoclusters (e.g., BSA-Au nanoclusters), SiO_2-Fe_3O_4 NPs, and some novel metal-organic frameworks (MOFs), e.g., NEQC-340 [18-24].

Due to the significant peroxidase-like activity of majority of nanozymes, these compounds can be used for designing catalyst-based analytical sensors, called nanozyme-based sensors. Up to now, several nanozyme-based sensors have been constructed for the quantification of a variety of analytes such as cysteine, hydrogen peroxide, speciation of mercury(II) ions, filed-detection of explosive, triacetone triperoxide, enzyme-free sensing of glucose, and glutathione bioquantification. The majority of the nanozyme-based sensors are based on probing the nanozyme-catalyzed oxidation of 3, 3´, 5, 5´-tetramethylbenzidine (TMB) to blue cation radical. However, in 2020, Hormozi Jangi et al. introduced a series of nanozyme-based sensors using the n-electron irreversible oxidation of 3,3'-diaminobenzidine (DAB) to a stable brown colored indamine polymer rather than common cation radicals resulting from TMB. Moreover, a novel class of nanozyme-based sensors called multi-nanozyme sensor was also developed by Hormozi Jangi et al. toward enhancing both sensitivity and selectivity of the sensor *via* the synergetic effect of multinanozyme system on the catalytic activity and specificity [5,15,16,18,23-28].

Recently catalytic property of gold nanozymes and their excellent peroxidase-like activity brought them up as safe, green, and high throughput alternatives of natural peroxidase enzymes to developed enzyme-mediated sensors. As we mentioned previously, commonly TMB and DAB have been applied as nanozyme substrates for probing the nanozme-catalyzed oxidation, and the corresponding oxidation products of these substrates have been probed as the analytical identifiers for sensing aims. However, it is well-known that the affinity of TMB and DAB substrates toward binding to enzymes or especially, here, nanozymes is not the same, resulting in different enzyme-like activity and different kinetics indexes including K_m and V_{max}. Since, the protein-assisted/protected gold nanozymes (BSA-gold nanozymes) are a type a high powerful artificial peroxidase enzymes, Therefore, the kinetics indexes including both K_m and V_{max} of these nanozymes should also be varied by varying their substrate [9,11,13,21-24,27].

Considering the above considerations, in this study, we aimed to perform a comparative study on the kinetics performances of protein-assisted/protected gold nanozymes for nanozyme/enzyme-mediated oxidation of TMB and DAB. To do this, initially, protein-assisted/protected gold nanozymes were synthesized *via* a protein-directed method utilizing Bovine Serum Albumin (BSA) as both reductant and stabilizer. Thereafter, the as-prepared nanozymes were characterized by recording their TEM images and DLS pattern of these nanozymes. Thereafter, the Michaelis-Menten model and the Lineweaver-Burk method were utilized for the calculation of kinetic parameters of BSA-gold including K_m and V_{max} for both DAB and TMB as the chromogen substrates.

MATERIALS AND METHODS

Materials

DAB was obtained from Sigma Aldrich Company. Deionized water was obtained from Zolal Teb chemical company (Iran). Other materials were obtained from Merck Company in their analytical or synthesis grades.

Instrumentations

The UV-Visible measurements for the investigation of the peroxidase-like activity of the as-prepared nanozymes as well as for probing nanozyme activity toward oxidation of both DAB and TMB by using an Ultrospec 4000 UV-Vis spectrophotometer manufactured by Pharmacia Biotech (Biochrom) Ltd equipped with SWIFT Software. Besides, a Metrohm 827 pH meter equipped with a combined glass electrode and a transmission electron microscope (Zeiss, model EL10C) were utilized for pH measurements and size and morphology evaluation, in order. For exploring more precise on size reporting, the DLS pattern was provided using a Shimadzu particle size analyzer (model: SALD-301V, Japan).

Green Synthesis of BSA-Gold Nanozymes

Protein protected-gold nanozymes were synthesized *via* a simple, high throughput and green method at physiological temperature *via* incubation of 10 mL mixed solution (pH=10.5-11.0) of a 1:1 volume ratio of $HAuCl_4.4H_2O$ solution and 50 mg mL^{-1} at 37.0° C for 12 hours. The as-prepared nanozymes were then collected and stored at 4°C [21].

Nanozyme Activity Assay and Kinetics Studies

For the DAB assay, 80.0 µL BSA-gold nanozymes were added into 1.3 mL of phosphate buffer solution (pH 7.0, 0.4 M) containing 200.0 µL of DAB (different concentrations) and 40.0 µL of HP (with a final concentration of 0.24 M) and thoroughly mixed at ambient temperature. The oxidation was followed for 25.0 min to complete the production of the corresponding indamine polymer (i.e., poly-DAB). Thereafter, the nanozyme activity (nM sec^{-1}) was measured by probing the absorbance of the resulting polyDAB at 460 nm considering a molecular extinction coefficient ε=5500 molar cm^{-1}.

Regarding the TMB assay, 40 µL hydrogen peroxide solution (final concentrations of 0.24 M), 200 µL of TMB (different concentrations), and 80 µL of BSA-gold nanozymes were introduced to 1.3 mL of acetate buffer (0.3 M; pH, 0.4), followed by incubation for about 10 minutes at ambient temperature. After that, the absorbance of the blue-colored TMB-ox at 658 nm was used for nanozyme activity (nM sec^{-1}) calculation considering a ε of 39000 M cm^{-1}. Finally, the kinetic parameters of the as-prepared BSA-gold nanozymes for both DAB and TMB were investigated based on Michaelis-Menten equation and the Lineweaver-Burk method as the standard models for evaluation of the enzyme kinetics performances.

RESULTS AND DISCUSSION

Characterization of as-Mentioned Nanozymes

Protein-assisted/protected gold nanozymes called as BSA-gold nanozymes were synthesized by using the native bovine serum albumin (BSA) as both reductant and stabilizer in physiological temperature and basic pH utilizing a protein-directed method as a green, high throughput, and one-pot approach. The morphological characterization of the as-prepared nanozymes was performed *via* recording their TEM image with a transmission electron microscope. The results of this analysis are shown in **Figure 1**, the as-prepared BSA-gold nanozymes reveal semi-spherical morphological properties with an approximately narrow size distribution which makes them suitable for enzyme-mimicking applications. More precisely, the results showed that the as-mentioned nanozymes have a very narrow size distribution over 7.7 nm-18.3 nm. The narrow size distribution of these nanozyme can be considered as a significant advantage for them because the variation of the size of nanozymes can significantly affect their enzymatic activity, for instance, increasing the size of the nanozymes leads to decreasing the active surface area. Consequently their active enzyme-like nodes for binding the enzyme substrate have decreased and the enzyme-like activity of the nanozymes was decreased, as previously reported. It is notable that the mean size of the as-prepared nanozymes was estimated at about 13.2 nm [21] (**Figure 1**).

However, for exploring more precise size reporting of the as-mentioned nanozymes, the DLS analysis of the nanozymes was performed. The results are shown in **Figure 2**, as shown, the as-mentioned nanozymes show a size distribution over 7.3 nm-31.3 nm with an average diameter of 13.16 nm. However, the maximum of particles has a size in the range of 10.5 nm-17.2 nm. Besides, the mode of the particles has a size of about 13.0 nm. It is notable that the results obtained from the DLS analysis (mean size of about 13.16 nm) are close to those provided from the TEM imaging method (mean size of about 13.2 nm) (**Figure 2**).

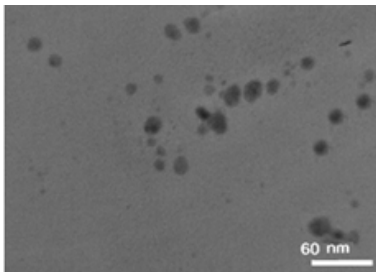

Figure 1: TEM image of as-prepared BSA-gold nanozymes

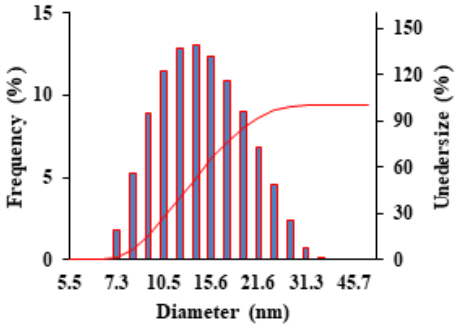

Figure 2: DLS analysis of as-mentioned BSA-gold nanozymes

Proving Nanozymatic Activity of the as-Prepared BSA-Gold Nanozymes

The nanozymatic (peroxidase-like) activity of the as-synthesized BSA-gold nanozymes was evaluated by nanozyme-catalyzed 3,3',5,5'-tetramethylbenzidine (TMB) oxidation. The activity was evaluated by probing the absorbance of the blue-colored oxidation product (i.e., TMB-ox) at 655 nm based on the standard enzyme assay. It is notable that to prove the oxidation catalysis by the as-prepared nanozymes, the oxidation process was performed in the presence and the absence of the as-prepared nanozymes. The results are shown in **Figure 3**, as seen in this figure, the oxidation of TMB to its corresponding blue-colored product cannot efficiently proceed in the absence of the as-prepared nanozymes, revealing very slow kinetics of the oxidation process of TMB by hydrogen peroxide. In contrast by introducing the as-prepared nanozymes into the mixture of TMB and hydrogen peroxide, the oxidation quickly proceeded and a significant absorbance was observed at 658 nm which is related to the oxidation product of TMB. Based on these results, it can be concluded that the as-prepared BSA-Au nanozymes have a great nanozymatic activity (**Figure 3**).

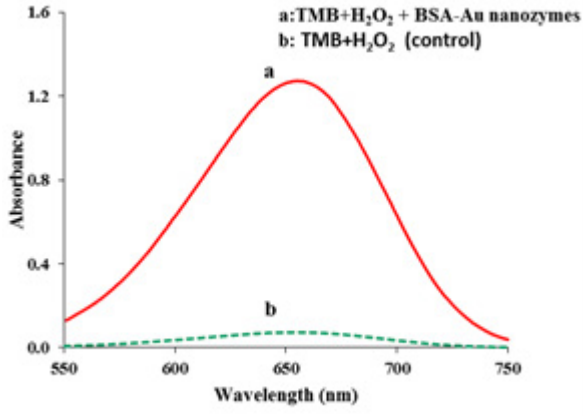

Figure 3: Nanozymatic activity of the as-prepared BSA-Au nanozymes

compared to the control sample

Kinetics Parameters of BSA-gold nanozyme for TMB Oxidation

To explore more precise on the peroxidase-like activity and enzymatic power of the as-prepared nanozymes, the kinetics studies were carried out by estimating the nanozyme activity as a function of TMB concentration and then estimating the nanozymatic kinetics parameters, the standard Lineweaver–Burk plot was provided by plotting the inverse of the velocity of the nanozymatic reaction (V^{-1}) as a function of [TMB]-1. It is notable that the velocity of the reaction was calculated based on the estimation of the concertation of the produced TMB-ox per one second of the reaction (μM sec^{-1}), considering this fact that the molar absorption efficient of TMB-ox at 655 nm was 39000 μM^{-1} cm^{-1}. The kinetic parameters of the as-mentioned nanozymes including V_{max} and K_m were then calculated by plotting the steady-state Michaelis-Menten curve and the linear plot of Lineweaver-Burk for both substrates.The Michaelis-Menten plot for the enzymatic oxidation of TMB catalyzed by the as-mentioned nanozymes was shown in **Figure 4A**, revealing that the rate of nanozyme-mediated oxidation of TMB was increased by increasing the substrate concertation and then leveled off. To estimate the kinetic parameters of BSA-gold nanozymes toward TMB oxidation, the Lineweaver–Burk plot was constructed (**Figure 4B**). Considering the results obtained in **Figure 3B**, the V_{max} and K_m of the as-mentioned nanoymes toward TMB oxidation were calculated at about 263 nM sec^{-1} and 0.03 mM, in order. The high value of the V_{max} of the as-prepared nanozymes for TMB oxidation compared to the V_{max} of the natural horseradish peroxidase (V_{max}=20.1 nM sec^{-1}) revealed that the as-prepared nanozymes have a greater catalytic efficiency toward the oxidation of TMB compared of the natural enzyme which make them as high powerful alternative for the enzyme [29]. Besides, the very lower K_m of these nanozymes compared the natural enzyme (K_m=0.43 mM) reveal that the very higher affinity of the TMB toward the as-prepared nanozymes compared to the HRP enzyme. In fact, the ratio of V_{max} (nanozyme/V_{max} (HRP)) was calculated at about 13, revealed that the catalytic efficiency of the as-mentioned nanozymes for TMB oxidation. It is 13-fold higher than that of natural enzyme [2-4,29] (**Figure 4**).

Kinetics Parameters of BSA-gold Nanozyme for DAB oxidation

To evaluate the kinetics performances of the as-mentioned BSA-gold nanozymes for the enzyme-catalyzed DAB oxidation, the steady-state Michaelis–Menten plot was constructed for the DAB oxidation by hydrogen peroxide in the presence of the as-mentioned nanozymes as the peroxidase-mimicking agents, and the results are represented in **Figure 5A**, revealed that the rate of nanozymatic DAB oxidation was increased by increasing the substrate concertation and then leveled off which is same of the TMB oxidation but the TMB oxidation rate was found to be higher than that of the DAB oxidation by the as-mentioned nanozymes. To explore more precise on the calculation of the kinetic indexes of the BSA-gold nanozymes toward DAB oxidation, the linear Lineweaver-Burk plot was constructed for the more accurate estimation of K_m and V_{max} of BSA-gold enzymes-mediated oxidation of DAB (**Figure 5B**), revealing a V_{max} and K_m of 185 nM sec^{-1} and 0.72 mM, in order, for the DAB oxidation. The value of the V_{max} of the as-prepared nanozymes for DAB oxidation was found to be higher than that of the natural, revealing its greater catalytic efficiency compared of the natural enzyme (**Figure 5**).

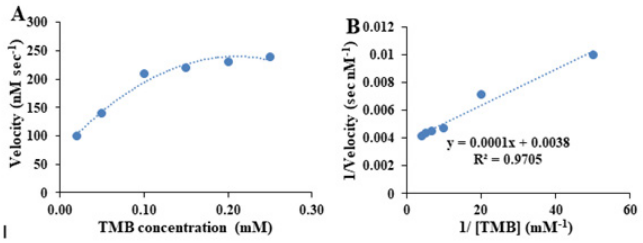

Figure 4: Kinetic performances of BSA-Au nanozymes toward TMB oxidation, (A) Michaelis-Menten plot and (B) Lineweaver-Burk plot

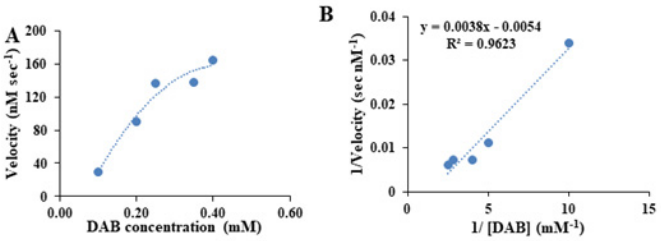

Figure 5: Kinetic performances of BSA-Au nanozymes toward DAB oxidation, (A) Michaelis-Menten plot and (B) Lineweaver-Burk plot

Comparison of Kinetic Performances of BSA-Gold Nanozymes for Different Substrates

Table 1 shows the kinetic parameters of the as-mentioned BSA-gold nanozymes including K_m and V_{max} for the nanozyme-medaited oxidation of both DAB and TMB, as seen in this table, the V_{max} of DAB oxidation was found to be lower than that of the TMB oxidation which pointed to the fact that the catalytic efficiency of the as-mentioned nanozymes toward TMB is significantly higher than their efficiency for the DAB oxidation. The ratio of V_{max} (TMB)/V_{max} (DAB) was estimated as high as 1.42, revealing that the catalytic efficiency of the as-prepared BSA-gold nanozymes for TMB oxidation is about 1.5-fold higher than their catalytic performances for the DAB oxidation. The difference between the kinetic indexes of TMB and DAB may be related to their different oxidation pathways and their different reactivity. In fact, the DAB oxidizes *via* an n-electron irreversible oxidation pathway to produce an indamine polymer. While TMB nanozyme-mediated oxidation has occurred upon a 2-electron reversible mechanism for the production of a cation radical. These different pathways resulted in different kinetic performances. Besides, the K_m value for DAB was found to be 24-fold higher than that for TMB. It is well-known that the K_m shows the affinity of an enzyme to its substrate which its lower value assigned to a higher affinity [2-4]. Hence, the very higher K_m for DAB reveals the very lower affinity of DAB for binding to BSA-gold nanozyme active nodes compared to its alternative substrate, TMB. This difference can be related to the different reactivity of DAB and TMB. For exploring more precise on the different affinity and reaction kinetics of the nanozyme-mediated oxidation of TMB and DAB, the schematic representation of TMB oxidation pathway compared of DAB oxidation mechanism on the surface of BSA-gold nanozyme was provided and represented in Scheme 1. As can be seen from this scheme, the TMB oxidation on the surface of the as-prepared nanozymes occurs *via* a reversible two-electron ox-

idation pathway by the produced active hydroxyl radicals generated form reaction of hydrogen peroxide over the as-mentioned nanozymes, as previously reported. In contrast, the oxidation of DAB occurs on the surface of the as-mentioned nanozymes by reacting the DAB molecule with hydroxyl radicals, followed by successive polymerization to produce an indamine polymer *via* an irreversible n-electron pathway. Considering these facts and different reactivity of TMB and DAB, the difference between the kinetics indexes of BSA-gold nanozymes for their oxidation is clearly acceptable [2-5,8,23,24,27] (**Table 1**) (**Figure 6**).

Table 1: Kinetic parameters of BSA-gold nanozymes for TMB oxidation compared to those for DAB oxidation.

Substrate	Oxidation pathway	K_m (mM)	V_{max} (nM sec^{-1})
DAB	n-electron irreversible	0.72	185
TMB	2-electron reversible	0.03	263

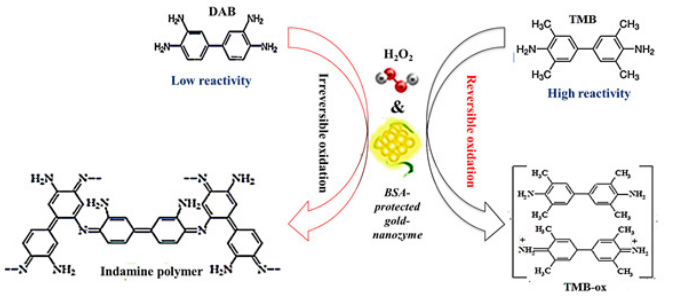

Figure 6: DAB oxidation pathway against TMB oxidation pathway on surface of BSA-gold nanozymes in the presence of hydrogen peroxide as oxidizing agent

CONCLUSION

Herein, a comparative study was performed on the kinetics performances of BSA-gold nanozymes for enzyme-mediated oxidations of 3,3',5,5'-thetramethylbenzidine, and 3,3'-diaminobenzidine. The results showed that the K_m value of BSA-gold nanozymes was 0.03 mM and 0.72 mM toward TMB and DAB, in order, to reveal the higher affinity (lower K_m) of TMB for binding to nanozyme active nodes compared to its alternative substrate, DAB. In contrast, the V_{max} was found to be 263 nM sec^{-1} and 185 nM sec^{-1} for nanozmye-mediated oxidation of TMB and DAB, respectively. The higher V_{max} of the nanozyme-mediated oxidation of TMB revealed that the catalytic efficiency of BSA-Au nanozymes toward TMB oxidation is higher (about 1.5-fold) than that of the DAB oxidation. The difference between the kinetic indexes of TMB and DAB may be related to their different oxidation pathways and their different reactivity. In fact, the DAB oxidizes *via* an n-electron irreversible oxidation pathway to produce an indamine polymer. While TMB nanozyme-mediated oxidation has occurred upon a 2-electron reversible mechanism for the production of a cation radical. These different pathways resulted in different kinetic performances.

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CONFLICT OF INTEREST

None.

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