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# Investigation of the time effect on the nanozyme-mediated oxidation of 3, 3′-diaminobenzidine by hydrogen peroxide

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## Abstract

The time effect on the nanozyme-mediated oxidation of 3, 3′-diaminobenzidine by hydrogen peroxide was investigated. Silver-based nanomaterials were synthesized and then used as peroxidase mimics. The time-dependent activity of the 3, 3′-diaminobenzidine oxidation catalyzed by silver-based nanomaterials was calculated by probing the color intensity of the produced brown-colored indamine polymer during the reaction at different time intervals. The time-dependent activity curve was used as an index for evaluating the time effect on the process, showed that the concentration of the brown-colored indamine polymer was increased by increasing the oxidation time and then leveled off, revealing saturation of active nodes of nanoparticles with nanozyme-substrate.

***Keywords;*** silver nanoparticles; peroxidase-mediated oxidation; 3, 3′-diaminobenzidine; indamine polymer; time effect on oxidation

## 1. Introduction

To date, several different nanomaterials with unique properties than the bulk materials have been designed and synthesized by different protocols. The above-mentioned nanomaterials have been applied for different applications in modern life due to their unique optical characteristics [1-3], catalytic activity  [4, 5], anti-cancer, and medical properties [6, 7], as well as anti-bacterial characteristics[8, 9]. Some of the well-known nanoscale materials with a wide application range in science and technology are carbon/metal-based nanoparticles [10, 11], carbon dots and quantum dots [12, 13], metal oxides [14], and magnetic $Fe_3O_4$ nanoparticles [15], as well as some nanoscale metal-organic frameworks [16, 17]. Some of these nanomaterials exhibit characteristic enzyme-like activity and features that make them suitable as memetic materials for native enzymes. Due to the nanoscale size distribution and intrinsic enzyme-like properties of these nanomaterials, they are called "nanozymes" and have been widely applied for different practical uses in industrial, clinical, and environmental catalysis [18-21]. These nanozymes reveal some significant advantages over the native enzymes including lower cost efficiency and higher cycling stability [19, 22, 48, 49]. Due to their applicability for catalyzing enzyme-mediated reactions in harsh conditions, up to now, different nanoparticles with intrinsic peroxidase-like activity for example, $Mn_3O_4$ nanozymes [23], $Cu/CuFe_2O_4$ nanozymes [24], and BSA-modified manganese dioxide nanoparticles [25], as well as BSA-stabilized manganese phosphate nanoflower [26] had been designed synthesized. Besides, the carbon-based nanozymes [27], silica-coated- $Fe_3O_4$ magnetic nanoparticles [28], manganese dioxide ($MnO_2$) and $Fe_3O_4$ nanozymes s [29, 30], pyrite-based, metal-organic frameworks-based, gold/silver-based, S/N co-doped carbon dot-based nanozymes [31, 32, 33, 34, 35]. Among the different nanomaterials with excellent peroxidase-like activity, gold/silver-based nanozymes have been widely for developing nanozyme-based sensors [36, 37], nanozyme-based cancer treatment [38], and nanozyme-mediated dye degradation [39]. Moreover, since the first report of patients infected with the new infection disease, COVID-19 in 2019 [40, 41], nanozyme-based methods have been developed for fast clinical diagnosis of this pandemic infection [42]. Hence, due to the importance of nanozymes, their biochemical characterization is an interesting topic. In this regard, the biochemical behavior of enzyme-like nanosilver was also investigated by our research group [43]. Besides, recently, Hormozi Jangi et al. reported some research articles on the investigation of biochemical behaviors of BSA-stabilized gold nanoparticles, silver nanoparticles, and $MnO_2$ nanoparticles [44-47]. Herein, the time effect on the nanozyme-mediated oxidation of 3, 3′-diaminobenzidine by hydrogen peroxide was investigated. Silver-based nanomaterials were synthesized and then used as peroxidase mimics. The time-dependent activity of the 3, 3′-diaminobenzidine oxidation catalyzed by silver-based nanomaterials was calculated by probing the color intensity of the produced brown-colored indamine polymer during the reaction at different time intervals. The time-dependent activity curve was used as an index for evaluating the time effect on the process, showed that the concentration of the brown-colored indamine polymer was increased by increasing the oxidation time and then leveled off, revealing saturation of active nodes of nanoparticles with nanozyme-substrate.

## 2. Experimental section

### 2.1. Synthesis of AgNPs

The synthesis was performed based on the process reported [27]. To do this, silver ions were reduced by $NaBH_4$ in the presence of sodium citrate as a stabilizer within 3 hours. After this time, the AgNPs were collected and stored at 4 °C.

### 2.2. Oxidation reactions

To do the oxidation reactions, a suitable amount of DAB was introduced into the buffer solutions containing silver nanoparticles and hydrogen peroxide with a fixed pH of 7.0. The reaction proceeded for about 0.0000-25.0 min for DAB oxidation. Thereafter the colored products were analyzed by UV-Vis spectrophotometer at 460.0 nm for detecting the DAB-ox.

## 3. Results and discussion

### 3.1. Characterization of silver nanozymes

The silver-based nanoparticles were synthesized using citrate as the capping and stabilizer. The as-synthesized silver nanoparticles were then characterized via the TEM imaging method for calculation of their mean size as well as for the morphological properties. The TEM image of these nanoparticles shown in Figure 1 exhibited that the as-prepared nanozymes have a spherical morphology and uniform particles. Besides, the mean size of these nanoparticles was estimated at about 11.0 nm for the TEM image.

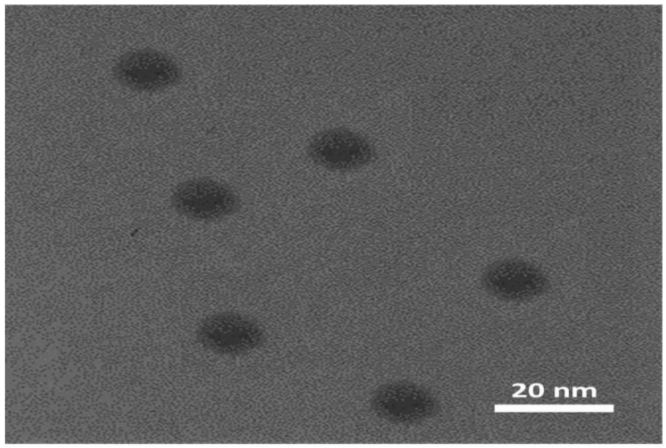


**Figure 1.** TEM image of as-prepared silver nanoparticles.

### 3.2. Time-course studies toward DAB oxidation

the peroxidase-like activity of silver nanoparticles toward DAB oxidation was also evaluated. To evaluate the peroxidase-like activity of the as-prepared AgNPs against DAB, the oxidation of DAB was performed by hydrogen peroxide in the presence of AgNPs as peroxidase mimics. In this regard, the time course studies were performed by probing the brown-colored product via spectrophotometric detection at 460.0 nm. Afterward, the plot of oxidation of DAB in the presence of AgNPs as a function of time was constructed by plotting the absorbance at 460.0 nm as a function of reaction time. The results are shown in Figure 2. As can be seen from this figure, the AgNPs can catalyze the oxidation of DAB to form a brown-colored product with a maximum absorbance at 460.0 nm.

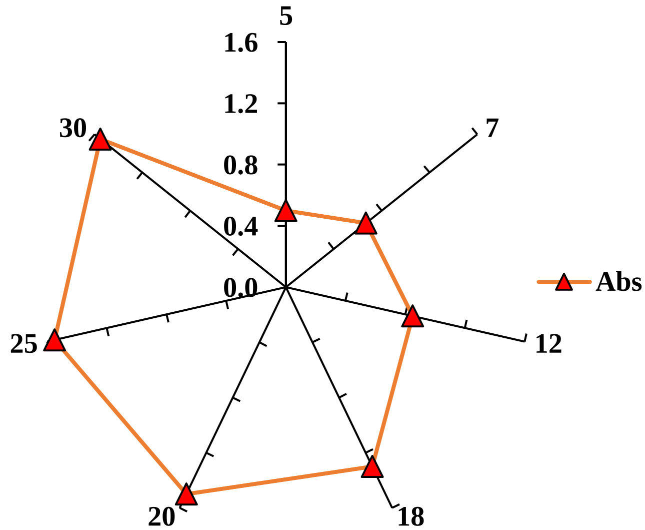


**Figure 2.** Time course radar plot of oxidation of DAB in the presence of silver nanozymes as a function of time

To explore more precise on the nanozymatic activity of the as-prepared silver nanozymes toward oxidation of DAB at different incubation times, the relative activity of nanozymes was also calculated and used as an index for investigating the time effect on the oxidation process of DAB for producing the corresponding poly(DAB). The results are shown in Figure 3. The results showed that after a long oxidation time as long as five minutes, the nanozyme activity reached about 32% of its maximal activity toward DAB oxidation. The oxidation process slowly proceeded and the nanozyme activity reached about 54% after 12.0 min. The maximal activity of silver nanozymes was obtained after 20 min toward DAB oxidation. After this time, the incubation time could not affect the production of the poly(DAB), and therefore the relative activity of the nanozymes was leveled off. The results reveal that an incubation time over 25.0 min was enough for active nodes presented on the surface of the silver nanoparticles, to be blocked by the substrate (DAB) molecules. In fact, the active nodes on the surface of the silver nanoparticles were completely saturated in 25.0 min by the DAB molecules. Considering this fact that the active nodes (the binding sites) on the surface of the nanozymes are limited, the saturation of the DAB molecules leads to leveling off of the relative activity of silver nanzymes.

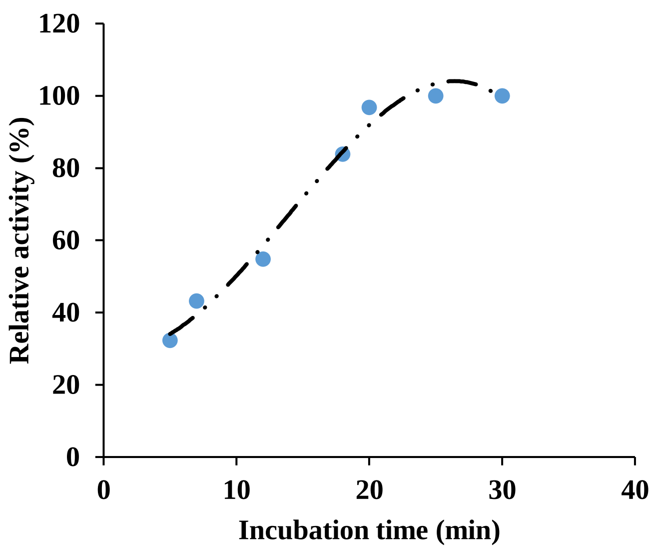


**Figure 3.** Relative activity of silver nanozymes for oxidation of DAB as a function of time

## 4. Conclusions

Time effect on the nanozyme-mediated oxidation of 3, 3′-diaminobenzidine by hydrogen peroxide was investigated. Silver-based nanomaterials were synthesized and then used as peroxidase mimics. The time-dependent activity of the 3, 3′-diaminobenzidine oxidation catalyzed by silver-based nanomaterials was calculated by probing the color intensity of the produced brown-colored indamine polymer during the reaction at different time intervals. The time-dependent activity curve was used as an index for evaluating the time effect on the process, showed that the concentration of the brown-colored indamine polymer was increased by increasing the oxidation time and then leveled off, revealing saturation of active nodes of nanoparticles with nanozyme-substrate.

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**Conflict of interest**

None.

## 5. References

[1] Dehghani Z., Akhond M., Hormozi Jangi S.R., Absalan G. (2024) Highly sensitive enantioselective spectrofluorimetric determination of R-/S-mandelic acid using l-tryptophan-modified amino-functional silica-coated N-doped carbon dots as novel high-throughput chiral nanoprobes, Talanta, 266, 1, 124977

[2] Kelly, K. L., Coronado, E., Zhao, L. L., & Schatz, G. C. (2003). The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. *The Journal of Physical Chemistry B*, *107*(3), 668-677.

[3] Hormozi Jangi, S. R., & Gholamhosseinzadeh, E. (2023). Developing an ultra-reproducible and ultrasensitive label-free nanoassay for L-methionine quantification in biological samples toward application in homocystinuria diagnosis. Chemical Papers, 1-13.

[4] Hormozi Jangi S. R.; Akhond M. (2020). High throughput green reduction of tris (p-nitrophenyl) amine at ambient temperature over homogenous AgNPs as H-transfer catalyst. Journal of Chemical Sciences, 132, 1-8.

[5] Hormozi Jangi, S. R. (2023). Low-temperature destructive hydrodechlorination of long-chain chlorinated paraffins to diesel and gasoline range hydrocarbons over a novel low-cost reusable ZSM-5@ Al-MCM nanocatalyst: a new approach toward reuse instead of common mineralization. Chemical Papers, 1-15.

[6] Shi, Y., Shan, S., Li, C., Song, X., Zhang, C., Chen, J., ... & Xiong, J. (2020). Application of the tumor site recognizable and dual-responsive nanoparticles for combinational treatment of the drug-resistant colorectal cancer. *Pharmaceutical Research*, *37*, 1-14.

[7] Singh, R., & Nalwa, H. S. (2011). Medical applications of nanoparticles in biological imaging, cell labeling, antimicrobial agents, and anticancer nanodrugs. *Journal of biomedical nanotechnology*, *7*(4), 489-503.

[8] Rajasekaran, J., & Viswanathan, P. (2023). Anti-bacterial and antibiofilm properties of seaweed polysaccharide-based nanoparticles. *Aquaculture International*, 1-25.

[9] Amany, A., El-Rab, S. F. G., & Gad, F. (2012). Effect of reducing and protecting agents on size of silver nanoparticles and their anti-bacterial activity. *Der Pharma Chemica*, *4*(1), 53-65.

[10] Hormozi Jangi, S. R. (2023). Effect of daylight and air oxygen on nanozymatic activity of unmodified silver nanoparticles: Shelf-stability. Qeios.

[11] Jangi, S. R. H. (2023). Determining kinetics parameters of bovine serum albumin-protected gold nanozymes toward different substrates. Qeios.

[12] Hormozi Jangi, S. R., & Akhond, M. (2021). Ultrasensitive label-free enantioselective quantification of d-/l-leucine enantiomers with a novel detection mechanism using an ultra-small high-quantum yield N-doped CDs prepared by a novel highly fast solvent-free method. Sensors and Actuators B: Chemical, 339, 129901.

[13] Li, G., Liu, Z., Gao, W., & Tang, B. (2023). Recent advancement in graphene quantum dots based fluorescent sensor: Design, construction and bio-medical applications. *Coordination Chemistry Reviews*, *478*, 214966.

[14] Carrapiço, A., Martins, M. R., Caldeira, A. T., Mirão, J., & Dias, L. (2023). Biosynthesis of metal and metal oxide nanoparticles using microbial cultures: Mechanisms, antimicrobial activity and applications to cultural heritage. *Microorganisms*, *11*(2), 378.

[15] Dongsar, T. T., Dongsar, T. S., Abourehab, M. A., Gupta, N., & Kesharwani, P. (2023). Emerging application of magnetic nanoparticles for breast cancer therapy. *European Polymer Journal*, 111898.

[16] Hormozi Jangi, S. R., & Akhond, M. (2021). High throughput urease immobilization onto a new metal-organic framework called nanosized electroactive quasi-coral-340 (NEQC-340) for water treatment and safe blood cleaning. Process Biochemistry, 105, 79-90.

[17] Hormozi Jangi, S. R. (2023). Synthesis and characterization of magnesium-based metal-organic frameworks and investigating the effect of coordination solvent on their biocompatibility. Chemical Research and Nanomaterials, 1(4), 1-9.

[18] Li, W., Chen, B., Zhang, H., Sun, Y., Wang, J., Zhang, J., & Fu, Y. (2015). BSA-stabilized Pt nanozyme for peroxidase mimetics and its application on colorimetric detection of mercury (II) ions. Biosensors and Bioelectronics, 66, 251-258.

[19] Hormozi Jangi, A. R., Hormozi Jangi, M. R., & Hormozi Jangi, S. R. (2020). Detection mechanism and classification of design principles of peroxidase mimic based colorimetric sensors: A brief overview. Chinese Journal of Chemical Engineering, 28(6), 1492-1503.

[20] Hormozi Jangi, S. R., & Dehghani, Z. (2023). Spectrophotometric quantification of hydrogen peroxide utilizing silver nanozyme. Chemical Research and Nanomaterials 2 (1), 15-23

[21] Jangi, S. R. H. (2023). Introducing a High Throughput Nanozymatic Method for Eco-Friendly Nanozyme-Mediated Degradation of Methylene Blue in Real Water Media. Sustainable Chemical Engineering, 90-99.

[22] Huang, Y., Ren, J., & Qu, X. (2019). Nanozymes: classification, catalytic mechanisms, activity regulation, and applications. Chemical reviews, 119(6), 4357-4412.

[23] Lu, L., Huang, M., Huang, Y., Corvini, P. F. X., Ji, R., & Zhao, L. (2020). Mn 3 O 4 nanozymes boost endogenous antioxidant metabolites in cucumber (Cucumis sativus) plant and enhance resistance to salinity stress. *Environmental Science: Nano*, *7*(6), 1692-1703.

[24] Xia, F., Shi, Q., & Nan, Z. (2020). Facile synthesis of Cu-CuFe 2 O 4 nanozymes for sensitive assay of H 2 O 2 and GSH. *Dalton Transactions*, *49*(36), 12780-12792.

[25] Liu, J., Gao, J., Zhang, A., Guo, Y., Fan, S., He, Y., ... & Cheng, Y. (2020). Carbon nanocage-based nanozyme as an endogenous H 2 O 2-activated oxygenerator for real-time bimodal imaging and enhanced phototherapy of esophageal cancer. *Nanoscale*, *12*(42), 21674-21686.

[26] Dega, N. K., Ganganboina, A. B., Tran, H. L., Kuncoro, E. P., & Doong, R. A. (2022). BSA-stabilized manganese phosphate nanoflower with enhanced nanozyme activity for highly sensitive and rapid detection of glutathione. *Talanta*, *237*, 122957.

[27] Sun, H., Zhou, Y., Ren, J., & Qu, X. (2018). Carbon nanozymes: enzymatic properties, catalytic mechanism, and applications. *Angewandte Chemie International Edition*, *57*(30), 9224-9237.

[28] Huang, Y., Ren, J., & Qu, X. (2019). Nanozymes: classification, catalytic mechanisms, activity regulation, and applications. Chemical reviews, 119(6), 4357-4412.

[29] Hormozi Jangi, S. R., Akhond, M., & Absalan, G. (2020). A field-applicable colorimetric assay for notorious explosive triacetone triperoxide through nanozyme-catalyzed irreversible oxidation of 3, 3′-diaminobenzidine. Microchimica Acta, 187, 431.

[30] Dong, H., Du, W., Dong, J., Che, R., Kong, F., Cheng, W., ... & Zhang, Y. (2022). Depletable peroxidase-like activity of Fe3O4 nanozymes accompanied with separate migration of electrons and iron ions. *Nature Communications*, *13*(1), 5365.

[31] Meng, X., Li, D., Chen, L., He, H., Wang, Q., Hong, C., ... & Fan, K. (2021). High-performance self-cascade pyrite nanozymes for apoptosis–ferroptosis synergistic tumor therapy. *ACS nano*, *15*(3), 5735-5751.

[32] Hormozi Jangi, S. R., & Akhond, M. (2020). Synthesis and characterization of a novel metal-organic framework called nanosized electroactive quasi-coral-340 (NEQC-340) and its application for constructing a reusable nanozyme-based sensor for selective and sensitive glutathione quantification. Microchemical Journal, 158, 105328.

[33] Hormozi Jangi, S. R., Akhond, M., & Absalan, G. (2020). A novel selective and sensitive multinanozyme colorimetric method for glutathione detection by using an indamine polymer. Analytica Chimica Acta, 1127, 1-8.

[34] Chen, Y., Jiao, L., Yan, H., Xu, W., Wu, Y., Wang, H., ... & Zhu, C. (2020). Hierarchically porous S/N codoped carbon nanozymes with enhanced peroxidase-like activity for total antioxidant capacity biosensing. *Analytical Chemistry*, *92*(19), 13518-13524.

[35] Hormozi Jangi, S. R., & Dehghani, Z. (2023). Kinetics and biochemical characterization of silver nanozymes and investigating impact of storage

conditions on their activity and shelf-life. Chemical Research and Nanomaterials, 1(4), 25-33.

[36] Zhou, X., Wang, M., Chen, J., & Su, X. (2022). Cascade reaction biosensor based on Cu/N co-doped two-dimensional carbon-based nanozyme for the detection of lactose and β-galactosidase. *Talanta*, *245*, 123451.

[37] Akhond, M., Hormozi Jangi, S. R., Barzegar, S., & Absalan, G. (2020). Introducing a nanozyme-based sensor for selective and sensitive detection of mercury (II) using its inhibiting effect on production of an indamine polymer through a stable n-electron irreversible system. *Chemical Papers*, *74*, 1321-1330.blood

[38] Zeng, X., Ruan, Y., Chen, Q., Yan, S., & Huang, W. (2023). Biocatalytic cascade in tumor microenvironment with a $Fe_2O_3$/Au hybrid nanozyme for synergistic treatment of triple negative breast cancer. Chemical Engineering Journal, 452, 138422.

[39] Ahmadi-Leilakouhi, B., Hormozi Jangi, S. R., & Khorshidi, A. (2023). Introducing a novel photo-induced nanozymatic method for high throughput reusable biodegradation of organic dyes. Chemical Papers, 77(2), 1033-1046.

[40] Jangi, S. R. H. (2023). Natural Polyphenols of Pomegranate and Black Tea Juices can Combat COVID-19 through their SARS-CoV-2 3C-like Protease-inhibitory Activity. Qeios.

[41] Hormozi Jangi, S. R. (2023). A Brief Overview on Clinical and Epidemiological Features, Mechanism of Action, and Diagnosis of Novel Global Pandemic Infectious Disease, Covid-19, And its Comparison with Sars, Mers, And H1n1. World J Clin Med Img, 2(1), 45-52.

[42] Meng, X., Zou, S., Li, D., He, J., Fang, L., Wang, H., ... & Gao, L. (2022). Nanozyme-strip for rapid and ultrasensitive nucleic acid detection of SARS-CoV-2. Biosensors and Bioelectronics, 217, 114739.

[43] Hormozi Jangi, S. R. (2023). Biochemical characterization of enzyme-like silver nanoparticles toward nanozyme-catalysed oxidation reactions. Micromaterials and Interfaces, 1(1), 2170.

[44] Hormozi Jangi, S. R. (2023). A comparative study on kinetics performances of BSA-gold nanozymes for nanozyme-mediated oxidation of 3,3',5,5'-tetramethylbenzidine and 3,3'-diaminobenzidine. Biochem Mol Biol J. 9:21.

[45] Hormozi Jangi, S. R. (2023). Evaluation of Biochemical Behavior and Stability of Gold Nanoparticles with High Intrinsic Peroxidase-Like Activity. *Petro Chem Indus Intern*, *6*(4), 234-239.

[46] Hormozi Jangi, S. R. (2023) Experimental evaluation of kinetics and biochemical characteristics of MnO2 nanoparticles as high throughput peroxidase-mimetic nanomaterials, Micromaterials and Interfaces 1 (1) 2234.

[47] Hormozi Jangi, S. R. (2023). Biochemical characterization of enzyme-like silver nanoparticles toward nanozyme-catalysed oxidation reactions. Micromaterials and Interfaces, 1(1), 2170.

[48] Hormozi Jangi, S. R., Akhond, M., & Dehghani, Z. (2020). High throughput covalent immobilization process for improvement of shelf-life, operational cycles, relative activity in organic media and enzymatic kinetics of urease and its application for urea removal from water samples. Process Biochemistry, 90, 102-112.

[49] Jangi, S. R. H., & Akhond, M. (2022). Introducing a covalent thiol-based protected immobilized acetylcholinesterase with enhanced enzymatic performances for biosynthesis of esters. *Process Biochemistry*, *120*, 138-155.