Introducing a High Throughput Nanozymatic Method for Eco-Friendly Nanozyme-Mediated Degradation of Methylene Blue in Real Water Media

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Received: 11 June 2023; Revised: 7 July 2023; Accepted: 10 July 2023

Abstract: A high throughput nanozyme-based catalytic system was developed for eco-friendly nanozyme-mediated biodegradation of organic dye, methylene blue, in real water media. The above-mentioned nanozymes were synthesized and then characterized for their morphology, composition, and size by Transmission Electron Microscopy (TEM), elemental mapping, and Dynamic Light Scattering (DLS) analysis, in order. The nanozymatic properties of the as-prepared nanomaterials were evaluated by standard enzyme activity assay, revealing high peroxidase-like performances for the as-prepared nanozymes. Therefore, for more precise reporting of the peroxidase-like performances of the nanozymes, the kinetics behavior of the as-prepared nanozymes was evaluated by the standard Lineweaver-Burk method, revealing a $V_{\text{max}}$ as high as 0.263 µM sec$^{-1}$ and a $K_m$ as low as 0.03 mM, revealing high catalytic efficiency and affinity of the as-prepared nanozymes. Hence, the as-prepared nanozymes were utilized for the degradation of organic dye in real water utilizing methylene blue as a model dye. The factors affecting the dye degradation including pH, degradation time, ionic strength, nanozyme amount, etc. were optimized utilizing the one-factor-at-a-time optimization method. At optimal experimental conditions, the as-prepared nanozymes can degrade about 99.0% of the organic dye within a degradation time as low as 7 min. The developed method was finally employed for the nanozyme-mediated degradation of methylene blue from real water media such as pool water, mineral water, river water, and tap water samples. The results of this study revealed an excellent biodegradation yield of over 94.3%-99.0% for the different real samples, proving the suitability of the developed method for dye degradation in real media.

Keywords: nanozyme-mediated dye degradation, organic dye, nanozyme, methylene blue, real water media

1. Introduction

Industrial wastewaters commonly contain several toxic wastes with high water solubility, for instance, toxic organic dyes, phenols, and pharmaceutically active compounds which are non-biodegradable. Hence, entering these non-biodegradable into the environment makes several serious environmental damages. Considering these issues, the high throughput removal of wastes is necessary from an environmental safety view. There are several different methods for the removal of contaminants (e.g., dyes) from water media including simple adsorption-based systems or catalytic degradation of dyes to mineral and safe materials. In the case of catalytic degradation, up to now, several
methods are introduced for dye removal such as chemo-degradation, photo-degradation, biodegradation, photo-induced nanozymatic degradation, multienzyme-based degradation, and chemo-bio degradation. Among different methods, nanozyme-based dye degradation recently attracted more attention due to their high catalytic efficiency, fast degradation, green properties, and high stability against environmental harsh conditions compared to natural enzymes. In fact, despite the high specificity and catalytic efficiency of the native enzymes, they suffer several drawbacks such as low stability against environmental changes. Hence, replacing native enzymes with high stable nanozymes can be considered a new point in the biocatalysis field. The fast development of material science and nanotechnology led to introduce several novel nanoscale materials such as carbon dots, nanoporous MOFs, and ZSM-5@ Al-MCM nanocatalysts, noble metal nanoparticles, and magnetic nanoparticles. Among different nanoparticles, some of them show high enzyme-like activity, for instance, silver nanoparticles, Fe$_2$O$_3$/Au hybrid nanozyme, Fe/Cu single-atom nanozymes, manganese dioxide nanoparticles, BSA-Au nanoclusters, iron single-atom nanoparticles with Fe-N nodes, N-doped metal-free carbon nanoparticles, cerium oxide@ nanoclusters, and BiOI-NFs. As it is well-known, nanozymes are defined as nanomaterials with intrinsic enzyme-like features. In fact, they can be used to simulate enzymatic reactions in harsh environmental conditions for example, higher temperature or wider pH range (e.g. in highly acidic or basic conditions). As we cited previously, natural enzymes show several weaknesses as follows; (I) low stability (narrow thermal range and pH range), (II) difficulty in recovery, and (III) inability to reuse the enzyme in reactions, especially industrial reactions. Traditionally, to overcome these problems, the enzyme immobilization process has been considered while a newly opened door for solving the enzymes’ drawbacks is the use of nanozymes with high stability and high quasi-enzymatic activity in enzyme-catalyzed reactions. Considering the high enzyme-like activity of these nanozymes, researchers aimed to utilize them as enzyme alternatives to overcome the difficulties of the natural enzymes. In this regard, nanozymes had been used for developing different analytical sensing and biosensing methods, and organic dye degradation. However, the focus of the nanozyme-based research is on developing sensing and biosensing-based methods. In contrast, studies on their potential application toward the enhancement of environmental safety are limited. Hence, developing high throughput nanozyme-based methods for the biodegradation of organic dyes in real water media can be considered as high-impact and applicable systems for the enhancement of environmental safety. Considering the above-mentioned facts, in this contribution, a high throughput nanozyme-based catalytic system was developed for eco-friendly nanozyme-mediated biodegradation of methylene blue in real water media. The protein-protected gold nanozymes were synthesized via a protein-assisted method. The above-mentioned nanozymes were synthesized and then characterized for their morphology and size by TEM and DLS analysis, in order. The nanozyme activity was evaluated by standard enzyme-activity assay. Besides, kinetics studies were carried out for the developed nanozymes by the standard Lineweaver-Burk method. Hence, the as-prepared nanozymes were utilized for the degradation of organic dye utilizing methylene blue as a model dye. The factors affecting the dye degradation including pH, degradation time, ionic strength, nanozyme amount, etc. were optimized. The developed method was finally employed for the nanozyme-mediated degradation of methylene blue from real water media such as pool water, mineral water, river water, and tap water samples.

2. Materials and methods

2.1 Materials

H$_2$O$_2$, acetic acid, NaCl, HAuCl$_4$, H$_2$O, Methylene Blue (MB), Bovine Serum Albumin (BSA), NaOH, and TMB (3,3',5,5' tetramethylbenzidine), were from Merck Company and the deionized water samples were obtained from an Iranian company named Zolal Teb Shimico.

2.2 Instrumentation

The enzyme-like activity of the as-prepared gold nanozymes and the organic dye biodegradation were probed by the UV-Visible measurements utilizing a UV-Vis spectrophotometer manufactured by Pharmacia Biotech Ltd (model: Ultrospec 4000). Besides, a Shimadzu SALD-301V particle size analyzer, a Metrohm 827 pH meter, and a transmission
electron microscope (Zeiss, model EL10C) were utilized for size estimation of the as-prepared nanozymes, pH measurements, and morphology evaluation, in order. The elemental mapping of the as-prepared gold nanozymes were provided using Talos F200S HR-TEM (USA) equipped with an energy dispersive spectroscopy (EDX).

2.3 Green synthesis of 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 HAuCl₄·4H₂O solution and 50 mg mL⁻¹ of BSA solution at 37.0 ℃ for 12 hours. The as-prepared nanozymes were then collected and stored at 4 ℃.

2.4 Dye degradation protocol

The dye degradation activity of the as-prepared nanozymes toward organic dye, methylene blue, was evaluated by quantification of the dye concentration during the nanozyme-mediated reaction. In a typical experiment, 120.0 µL of the as-prepared nanozymes and 100.0 µL H₂O₂ (30.0%) were added to 10.0 mL of 20.0 mg L⁻¹ methylene blue solution (pH 4.0-5.0), followed by 7.0 min incubation at ambient temperature, the dye bio-degradation process was then estimated utilizing UV-Visible measurements. The concentration of non-degraded dye was calculated by measuring its absorbance at 661.0 nm after the nanozymatic reaction and the nanozyme-mediated degradation efficiency was estimated as follows:

\[
\text{Degradation efficiency (\%)} = \frac{C_0 - C}{C_0} \times 100
\]

It is notable that \(C_0\) (in the unit of mg L⁻¹) and \(C\) (in the unit of mg L⁻¹) are the MB initial and final concentrations, respectively.

3. Results and discussion

3.1 Characterization of as-prepared nanozymes

The as-prepared nanozymes were synthesized via a simple, high throughput and green method at physiological temperature and then characterized for their morphological properties, and size by TEM imaging method, and DLS analysis, in order. The morphological properties and the mean size of the as-prepared nanozymes were investigated by the TEM imaging method. To do this, the TEM image of the as-prepared nanozymes was recorded. The results are shown in Figure 1A, as shown in this figure, the as-mentioned nanozymes show a semi-spherical morphology with uniform particles. It is mentionable that, the uniformity of the particles of the as-prepared nanozymes is a significant advantage from an enzymatic point of view because the uniform particles showed higher enzyme-like activity than the particles with low uniformity. Besides, the results showed that the as-prepared nanozymes have a narrow size distribution of 7.7-18.3 nm with a mean size of about 13.2 nm which makes them suitable for enzyme-mimicking applications, considering the fact that the size of nanozymes can strongly affect their enzyme-like activity.²⁵ It is notable that the high-resolution TEM was also recorded for the as-prepared nanozymes (inset of Figure 1A), the results showed a spherical morphology with a 10.5 nm particle size. For exploring more precise size reporting of the as-prepared nanozymes, the DLS analysis was performed. The results are shown in Figure 1B, as shown in this Figure, the as-prepared nanozymes show a size distribution over 7.3-31.3 nm with a mean diameter of 13.16 nm. However, the maximum of particles has a size in the range of 10.5-17.2 nm. Besides, the mode of the particles has a size of about 13.0 nm. It is notable that the results of the DLS analysis (mean size of 13.16 nm) are close to those of the TEM imaging method (mean size of 13.2 nm). Moreover, the elemental mapping was also recorded for the characterization of the as-prepared nanozyme and proving the presence of the carbon skeleton of protein in its structure, the results are shown in Figure 1C. As can be seen in this figure, the presence of gold (Au), carbon (C), nitrogen (N), and oxygen (O) in the structure of the as-prepared nanozymes was proved by mapping results. It is mentionable that the appearance of
nitrogen, oxygen, and carbon elements is related to the presence of bovine serum albumin in the nanozyme structure.

3.2 Evaluation of nanozymatic properties

The nanozymatic properties of the as-mentioned nanozymes including their peroxidase-like activity and their kinetics parameters toward enzyme-mediated oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) were evaluated to prove the enzyme-like activity and power as well as to estimate the catalytic efficiency of the as-prepared nanozymes.

3.2.1 Peroxidase-like activity of the as-prepared nanozymes

The semi-enzyme activity or more precisely, the nanozymatic (peroxidase-like) activity of the as-synthesized gold nanozymes was probed toward the standard peroxidase substrate (i.e., 3,3',5,5'-tetramethylbenzidine (TMB)) oxidation as the standard enzyme/nanozyme activity assay. The activity was evaluated by probing the blue-colored TMB oxidation product (i.e., TMB-ox) via measurement of its visible region absorbance positioned at 655 nm based on the standard enzyme assay. It is notable that to prove the catalysis of the oxidation process by the as-mentioned nanozymes, the oxidation was carried out in the presence and the absence of the above-mentioned nanozymes. The results are represented in Figure 2A, based on the obtained results in this figure, the nanozyme-mediated oxidation of TMB to its corresponding blue-colored product cannot efficiently proceed in the absence of the as-mentioned nanozymes, revealing very slow kinetics of the oxidation process of TMB by hydrogen peroxide. In contrast by introducing the as-mentioned gold nanozymes into the mixture of TMB and hydrogen peroxide, the oxidation quickly proceeded and a significant absorbance was observed at 658 nm which can be assigned to the nanozyme-mediated oxidation product of TMB. Hence, it can be concluded that the as-mentioned nanozymes show characteristic peroxidase-like activity. In fact, the as-synthesized nanozymes can produce active hydroxyl radicals by acting on hydrogen peroxide, as reported, then, the produced hydroxyl radical reacts with the TMB molecules and oxidize them to their corresponding cation radicals through a 2-electron reversible oxidation mechanism.
3.2.2 Kinetics behavior of the as-prepared nanozymes

To explore more precise 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 $[\text{TMB}]^{-1}$. It is notable that the velocity of the reaction was calculated based on the estimation of the concentration of the produced TMB-ox per one second of the reaction ($\mu$M sec$^{-1}$), considering the fact that the molar absorption efficient of TMB-ox at 655 nm was 39,000 $\mu$M$^{-1}$ cm$^{-1}$. The results are shown in Figure 2B, revealing a $V_{\text{max}}$ and a $K_m$ of 0.263 $\mu$M sec$^{-1}$ and 0.03 mM, in order. It is notable that the $V_{\text{max}}$ showed the catalytic efficiency of the nanozyme and the $K_m$ showed the affinity of an enzyme to its substrate, as reported. The higher $V_{\text{max}}$ and lower $K_m$ are assigned to higher efficiency and affinity, in order.$^{39}$ Hence, based on the above-mentioned considerations and facts, it is acceptable that the as-prepared gold nanozymes reveal very high enzyme (peroxidase)-like activity which makes them appropriate for potential practical application in the field of biocatalysis.

3.3 Nanozyme-mediated organic dye degradation

For developing a nanozyme-mediated dye degradation method utilizing the as-prepared nanozymes, 20 mg L$^{-1}$ methylene blue was utilized as a model dye. The efficiency percentage of the nanozyme-mediated dye degradation reaction was calculated by probing its UV-Visible absorbance at 661 nm and calculating its concentration before and after the reaction. The possible factors which may affect the nanozyme-mediated dye degradation for example, pH, degradation time, nanozyme amounts, hydrogen peroxide volume, and ionic strength were optimized to reach the best dye degradation performances, notably, at optimal experimental conditions the efficiency of the nanozyme-catalyzed degradation of MB was estimated as high as 99.0%.

3.3.1 Effect of degradation time

Since the nanozyme-mediated catalytic systems are time-dependent, hence, the effect of the degradation time on the nanozyme-mediated degradation of MB was evaluated over 0-10 min. The UV-Visible spectra of 20.0 mg L$^{-1}$ of MB as a function of degradation reaction time is represented in Figure 3A, revealing that the concentration of MB was decreased by increasing the degradation time and reached its minimum concentration after 7 min and after this time, the concentration of MB was not affected by the degradation time. Besides, the plot of degradation yield as a function of time was shown in Figure 3B, as seen, the degradation yield reached its maximum percentage at a degradation time as low as 7 min and then leveled off.
3.3.2 Effect of ionic strength and pH of media

The effect of the pH of the reaction medium on the yield of the nanozyme-mediated degradation of methylene blue as one of the most important and common parameters of the degradation process was investigated by testing a series of MB solutions with different pHs ranging from 2.0 to 8.0. The results shown in Figure 4A showed that the efficiency of the nanozyme-mediated degradation of the as-mentioned dye reached its maximum value (99.0%) at pH = 4.0-5.0, 7.0 and then decreased by increasing the pH of the solution. It is maybe related to the degradation of hydrogen peroxide at higher pH values and decreasing the nanozymes activity at pH higher than pH = 4.0-5.0. Hence, pH = 4.0-5.0 was selected as the optimal pH for nanozyme-mediated degradation of MB. Besides, the effect of ionic strength on the nanozyme-mediated dye degradation was also checked. To do this the ionic strength of the dye solutions was adjusted to the desired values using NaCl solutions with different concentrations ranging from 1.0 mM to 5.0 mM, after performing the tests, the results were shown in Figure 4B, showing that the efficiency of the nanozyme-mediated dye degradation was not significantly affected by the variation of ionic strength of the reaction media (Figure 4B).

3.3.3 Effect of hydrogen peroxide and nanozyme volume

As reported in the literature, the semi-peroxidase nanozymes or peroxidase enzymes act on the hydrogen peroxide and produce active hydroxyl radicals. The produced radicals react with the organic dyes and degraded them to carbon
Hence, the amount of hydrogen peroxide is a key factor that significantly affects the nanozyme-mediated degradation of methylene blue dye. Considering this fact, the amount of hydrogen peroxide was optimized by degrading 20.0 mg L\(^{-1}\) MB in the presence of different volumes of 30% hydrogen peroxides (Figure 5A). As seen in Figure 5A, the obtained results revealed that the degradation percentage reached its highest value when 100.0 µL of hydrogen peroxide (H\(_2\)O\(_2\) (30%)) was applied as the oxidant agent. Also, the amount of the as-prepared nanozymes was also optimized by probing the degradation of 20.0 mg L\(^{-1}\) MB in the presence of different volumes of the as-prepared nanozymes over 60-160 µL, the results exhibited that the nanozyme-mediated biodegradation yield was increased by increasing the nanozyme amount, reached its maximum yield at 120 µL and then leveled off (Figure 5B).

**Figure 5.** The effect of (A) hydrogen peroxide and (B) nanozyme amount on the nanozyme-mediated methylene blue degradation. Error bars are represented in 3 replicate experiments

<table>
<thead>
<tr>
<th>Real sample</th>
<th>Spiked MB conc. (mg L(^{-1}))</th>
<th>Degradation yield (%)</th>
<th>RSD (%; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>10.0</td>
<td>99.0</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>98.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Pool water</td>
<td>10.0</td>
<td>97.5</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>96.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Mineral water</td>
<td>10.0</td>
<td>99.0</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>98.5</td>
<td>3.5</td>
</tr>
<tr>
<td>River water</td>
<td>10.0</td>
<td>96.1</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>94.3</td>
<td>4.6</td>
</tr>
</tbody>
</table>

### 3.4 Practical application

The feasibility of the developed nanozyme-mediated methylene blue degradation for the degradation of this dye in the real aqueous samples was examined by probing its nanozyme-mediated biodegradation in different real aqueous media for instance, tap water, mineral water, pool water, and river water. To do the degradation experiments, 10.0 mg L\(^{-1}\) or 20.0 mg L\(^{-1}\) MB was initially spiked to each water sample, and the nanozyme-mediated dye degradation system was applied for the dye degradation, after these investigations, the obtained results of the study were summarized in Table 1, revealing an excellent biodegradation yield over a minimum value of 94.3% (river water) and a maximum value of 99.0% (tap water) for the different real samples, proving the suitability of the developed nanozyme-mediated method.
for the dye degradation in the real aqueous media. Despite the significant advantages of the developed nanozymatic system such as eco-friendly nanozyme, very short degradation time, high degradation yield, needing small quantities of nanozyme for degradation, and applicability for dye degradation in different real water media, the reusability/recoverability of the proposed nanozymes is difficult due to its miscibility with water.

4. Conclusions

In this work, a high throughput nanozyme-based catalytic system was developed for nanozyme-mediated biodegradation of methylene blue in real water media. The above-mentioned nanozymes were synthesized and then characterized for their morphology, composition, and size by TEM, elemental mapping, and DLS analysis, in order. The nanozymatic properties of the as-prepared nanomaterials were evaluated by standard enzyme activity assay, revealing high peroxidase-like performances for the as-prepared nanozymes. Therefore, for more precise reporting of the peroxidase-like performances of the nanozymes, the kinetics behavior of the as-prepared nanozymes was evaluated by the standard Lineweaver-Burk method, revealing a $V_{\text{max}}$ as high as 0.263 $\mu$M sec$^{-1}$ and a $K_m$ as low as 0.03 mM, revealing high catalytic efficiency and affinity of the as-prepared nanozymes. Hence, the as-prepared nanozymes were utilized for the degradation of organic dye in real water utilizing methylene blue as a model dye. The factors affecting the dye degradation including pH, degradation time, ionic strength, nanozyme amount, etc. were optimized utilizing the one-factor-at-a-time optimization method. At optimal experimental conditions, the as-prepared nanozymes can degrade about 99.0% of the organic dye within a degradation time as low as 7 min. The developed method was finally employed for the nanozyme-mediated degradation of methylene blue from real water media such as pool water, mineral water, river water, and tap water samples. The results of this study revealed an excellent biodegradation yield of over 94.3%-99.0% for the different real samples, proving the suitability of the developed method for the degradation of dyes in water media. Notably, due to the approximately complete degradation (99%) of the organic dye in real media using small quantities of the developed nanozymes (120 $\mu$L) at a very short time (7.0 min), the developed system can be considered a high throughput nanozymatic method toward dye degradation. Besides, since the gold nanozymes were synthesized via a green protein-assisted method in physiological temperature, the nanozymes are considered green and the reaction can be called an “eco-friendlnanozyme-mediated degradation reaction”.

Acknowledgments

The authors gratefully thank the Hormozi Laboratory of Chemistry and Biochemistry for the support of this work.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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