Magnetic nanoparticles encapsulated laccase nanoflowers: evaluation of enzymatic activity and reusability for degradation of malachite green

Tingting Sun, Meihua Fu, Jinfeng Xing and Zhiqiang Ge

ABSTRACT

Magnetic laccase nanoflowers (MNFs-Lac) were successfully prepared through encapsulating Fe_3O_4 magnetic nanoparticles into the interior of laccase nanoflowers by grafting N-(phosphonomethyl)iminodiacetic acid (PMIDA) as an interconnecting bridge between the magnetic nanoparticles and copper ions. The characterizations by scanning electron microscopy and transmission electron microscopy showed that MNFs-Lac were spherical, porous and flower-like crystals with diameters of ~10 μm, and Fe_3O_4 nanoparticles were encapsulated in the interior of MNFs-Lac evenly. The enzymatic activity and reusability of MNFs-Lac were evaluated based on the degradation efficiency for malachite green (MG). The degradation parameters, concerning initial MG concentration, dosage of MNFs-Lac, reaction temperature, pH value and reaction time, were optimized through single-factor experiments. Under the optimal conditions, 25 mg·L^{-1} MG can be degraded almost completely by 1.5 g·L^{-1} MNFs-Lac within 15 min. When the MNFs-Lac were reused for 18 times, the degradation efficiency of MG was still as high as 90%. These results suggested that the modified preparation method improved greatly the reusability of MNFs-Lac, which made them more suitable to degrade MG in a water environment.

Key words | degradation, laccase, magnetic nanoflowers, malachite green, reusability

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INTRODUCTION

Malachite green (MG) is a highly water-soluble triarylmethane compound (Rao 1995). Besides occurring as a fungicide, disinfectant, ectoparasiticide and antibacterial agent in fish farming, it is also used extensively for dyeing cotton, wool, silk, paper and leather (Mall *et al.* 2005). MG is carcinogenic, mutagenic and teratogenic to human beings (Yang *et al.* 2017) because it could bind DNA to form complexes through intercalation and electrostatic interactions (Hu *et al.* 2006). As early as 1993, MG was already considered to be a priority chemical for carcinogenicity by the United States Food and Drug Administration (Shang *et al.* 2019). Nevertheless, MG is still widely used in many areas of the world due to its low cost, efficiency, and availability, which gives rise to a greater threat to the ecological environment and human health. Hence, elimination of MG from water environments has attracted growing attention. Many methods such as adsorption, photocatalytic degradation and biological degradation have been investigated. However, there are still some problems such as the methods being very time-consuming, and having poor efficiency and high cost. So, it is imperative to develop an economical and efficient method for MG removal from the water environment.

The adsorption and degradation are two main ways that have been proposed for eliminating pollutants from aqueous solution (Ge *et al.* 2019). Among them, biodegradation of pollutants is a cost-effective and environment-friendly process. Laccases are a class of multi-copper-containing oxidase that can effectively oxidize a wide range of organic pollutants with the simultaneous reduction of oxygen to water without harsh conditions. Because of their low substrate specificity and high availability, laccases have drawn wide attention in the treatment of wastewater contaminants (Yang *et al.* 2017). Many studies have shown that laccases can successfully degrade MG. (Siroosi *et al.* 2018; Zhang *et al.* 2018). However, the large-scale application of laccases was restricted due to its high production cost, easy inactivation, poor reusability and low storage stability (Li *et al.* 2013). In order to offset those shortcomings, immobilization of laccases is becoming more and more popular. A number of studies have proved that laccases can be immobilized

doi: 10.2166/wst.2020.068

successfully on the various supports by entrapment, cross-linking, physical adsorption and covalent binding. Although these traditional immobilization methods have improved the properties of the free enzyme to some extent, the exploration of better immobilization methods is still ongoing.

Use of hybrid organic–inorganic nanoflowers of protein–copper phosphate, comprised of an organic and an inorganic component with flower-like spherical architecture, is a simple, versatile protein immobilization method (Ge *et al.* 2012). When the enzyme is used as the organic component of hybrid nanoflowers, it exhibits higher enzyme activity and stability than the free enzyme due to the higher specific surface area and immobilization of the nanoflowers (Li *et al.* 2016). It has been reported that the enzyme activity of laccase nanoflowers was 6.5 times higher than free laccase, and the activity of horseradish peroxidase (HRP) nanoflowers was three times higher than free HRP (Ge *et al.* 2012; Somturk *et al.* 2015). Nevertheless, the separation and recovery of nanoflowers can only use a centrifugal method, which critically limits their recycling in practical applications. Magnetic nanoparticles have been widely used in the field of materials recycling because of the property of easy separation by an external magnetic field. In terms of this issue, laccase-loaded magnetic nanoflowers (MNFs) have been successfully prepared (Fu *et al.* 2019), and displayed excellent degradation efficiency of bisphenol A. However, the reusability of the MNFs was not quite satisfactory. The degradation efficiency was reduced by 30% after eight cycled runs. Besides the inactivation of laccase resulting from continuous operations, another important reason for the reduced degradation efficiency is that the magnetic nanoparticles adhering to the surface of MNFs could easily break away from the surface, eventually resulting in the decrease in recovery and reusability of MNFs. Consequently, it is urgently desired to develop a new facile strategy to bestow MNFs with desirable enzyme activity and superior reusability.

In order to overcome the defects of the present MNFs, the existing preparation method was modified by encapsulating magnetic nanoparticles into the interior of the laccase nanoflowers. The activity and reusability of obtained product were evaluated by degrading MG in aqueous solution.

METHODS

Chemicals and reagents

Laccase (from *Trametes versicolor*, 3,000 units·g^{-1}) and fluorescein isothiocyanate isomer I (FITC) were purchased from Beijing Solarbio Science & Technology Co., Ltd (China). Bovine serum albumin (BSA), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥99.0%), tetraethyl orthosilicate (TEOS), N-(phosphonomethyl) iminodiacetic acid (PMIDA, ≥99.0%) and copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) were obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd (China). MG was obtained from Sangon Biotech (Shanghai, China). 1-Hydroxybenzotriazole (Hobt, 99%) was purchased from Energy Chemical (Shanghai, China). Ultrapure water (Millipore, Schwalbach, Germany) was used as dispersion medium. All chemicals and reagents were of analytical grade and used without further purification unless otherwise specified.

Preparation of magnetic nanoparticles encapsulated laccase nanoflowers

Although the interaction mechanism between proteins and metals is still poorly understood, most researchers deem that the formation process of protein–inorganic hybrid nanoflowers involves three major successive steps: nucleation, growth and development of nanoflowers (Somturk *et al.* 2015). In principle, to generate magnetic nanoparticles encapsulated laccase nanoflowers (MNFs-Lac), it is first necessary to compel the nucleation on the surface of magnetic nanoparticles, in which Cu^{2+} ions react with phosphate to form primary copper phosphate nanocrystals. The amine groups in laccases preferentially coordinate to copper phosphate to initiate the growth process of nanocrystals. As a consequence, the hierarchically flower-like MNFs-Lac can be fabricated at the end of the growth process with continuous supply of Cu^{2+} ions and protein molecule. Therefore, the initiation sites of the nanocrystals are very important for preparing magnetic nanoparticles encapsulated laccase nanoflowers. In order to ensure that the initiation sites of nucleation are located on the surface of magnetic nanoparticles, we used PMIDA as a bridge to connect magnetic nanoparticles and Cu^{2+} ions. These anchored Cu^{2+} ions would become nucleation sites for primary copper phosphate nanocrystals and act as initiator sites for anisotropic growth and formation of laccase nanoflowers. PMIDA possesses an organic moiety composed of two carboxylic acid groups and a central N-donor atom, having strong metal-chelating activity, and an inorganic phosphonate moiety that easily bonds to hydroxylated surface oxides resulting in P-O-Si bonds through a condensation reaction between its P-OH groups and Si-OH on the magnetic nanoparticles surface (Majewski *et al.* 2011).

The synthesis procedure of MNFs-Lac involves two steps. The first step is to graft PMIDA by condensation reaction onto the magnetic nanoparticles surface (Majewski *et al.* 2011). Silica-coated Fe_3O_4 nanoparticles (Fe_3O_4@SiO_2 NPs) were prepared by a previous method (Gao *et al.* 2018), which is specifically described in the Supporting Information. As-prepared Fe_3O_4@SiO_2 NPs (10 mg) were dispersed into 10 mL PMIDA aqueous solution at preselected particle/PMIDA mass ratio (1:2.5) under stirring; then the mixture pH was adjusted to about 10 with 2.5 M NH_4OH and shaken for 12 h at room temperature. The products were separated by an external magnet and washed three times with ultrapure water to remove unreacted components. The second step is to complex Cu^{2+} ions and to promote the formation of MNFs-Lac. PMIDA grafted magnetic nanoparticles (Fe_3O_4@SiO_2-PMIDA NPs, 10 mg) were transferred into $CuSO_4$ aqueous solution (0.5 M, 5 mL) and stirred continuously for 2 h at room temperature. Some of the Cu^{2+} ions were chelated by PMIDA on the magnetic nanoparticles to form Fe_3O_4@SiO_2-PMIDA-Cu^{2+} NPs and the rest of the Cu^{2+} ions were free in the mixture. Fifty millilitres of phosphate buffered saline (PBS, 0.01 M, pH 6.5) containing 0.4 mg·mL^{-1} laccases was added into the mixture and shaken for 5 min to blend well. After 24 h of incubation at 4 °C, MNFs-Lac were formed in the mixture and obtained by an external magnet. The resulting MNFs-Lac were washed three times with ultrapure water and stored at 4 °C for subsequent evaluation.

Characterizations

The morphology of samples was observed by scanning electron microscopy (SEM, S-4800, Hitachi) and transmission electron microscopy (TEM, JEM-2011F, JEOL). Crystal structure of samples was investigated by X-ray diffraction (XRD, D8-Focus, Bruker AXS) using CuKα radiation (40 kV, 40 mA) from 5° to 85° at 2θ with a scanning rate of 8°·min^{-1}. Magnetization curves were recorded on a vibration sample magnetometer (VSM, Lake Shore 7404, Lake Shore). Infrared spectra of the Fe_3O_4@SiO_2-PMIDA NPs and MNFs-Lac were inspected by Fourier transform infrared (FT-IR) spectroscopy in the range of 4,000–400 cm^{-1} (Excalibur FTS 300, Bio-Rad). The elemental compositions of Fe_3O_4@SiO_2-PMIDA-Cu^{2+} NPs were visible by energy-dispersive spectroscopy (EDS, Octane super, Edax). The presence and distribution of protein molecules in MNFs-Lac were investigated by fluorescence microscopy (Eclipse Ci-L, Nikon). Prior to observing, the MNFs-Lac were dyed with FITC, a specific protein-molecule fluorescent probe, for 15 min in the dark condition, and then washed by ultrapure water to remove the unbound dyes. The content of laccase loading on the MNFs-Lac was determined using the Bradford method (Bradford 1976), and calculated using the following Equation (1):

$$\text{Laccase loading } (\text{mg} \cdot \text{g}^{-1}) = \frac{m_0 - m_s}{m} \quad (1)$$

where m_0, m_s, and m are added protein amount in the mixture (mg), residual protein amount in supernatant (mg) and acquired amount of MNFs-Lac (g), respectively.

The expressed activity of MNFs-Lac can be estimated by the following Equation (2):

$$\text{Expressed activity } (\text{U} \cdot \text{g}^{-1}) = \frac{A}{m} \quad (2)$$

where A is activity of laccase (U), and m is amount of MNFs-Lac (g).

Determination of laccase activity and MG concentration

The activity of free laccase and concentration of MNFs-Lac were determined by detecting the absorbance of reaction mixtures at 417 nm according to a previous method (Fu *et al.* 2019). Briefly, 6 mL PBS (0.05 M, pH 4.5) containing 1 mM ABTS and 400 μL samples (free laccase or MNFs-Lac) were respectively preheated at 35 °C, and then mixed to react for 2 min at 35 °C. The suspension was separated and the supernatant was measured at 417 nm by a microplate reader with a 96 well plate. One unit of laccase activity (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per second under the assay conditions.

MG concentrations were determined by measuring the absorbance at the wavelength 617 nm by using a calibration plot achieved at the same conditions. All of the measurements were carried out in triplicate.

Storage stability experiment

The enzyme activities over time were used to assess the storage stability of MNFs-Lac and free laccase in PBS solution (0.05 M, pH 4.5) at 4 °C and room temperature (25 ± 2 °C) during the 60 days of storage period, respectively. The initial activity of MNFs-Lac and free laccase was defined as 100%, and the remaining activities were then determined at its optimum pH. Simultaneously, the leaching amount of protein from MNFs-Lac was also monitored by the Bradford

method using BSA as a standard. All of the measurements were carried out in triplicate.

Optimization of degradation conditions of MG

Hobt was chosen as a redox mediator in the degradation reaction of MG by MNFs-Lac. The concentration of Hobt was optimized in a PBS solution (0.05 M) of 25 mg·L^{-1} MG with various concentrations of Hobt ranging from 0.1 to 0.5 mM at pH 4.5 and 35 °C over a duration of 60 min.

A single-factor experiment design in batch style tests was carried out to explore the individual effects of the degradation conditions, including the initial MG concentrations, dosage of MNFs-Lac, pH and temperature of the reaction, on the degradation efficiency of MG. One factor was changed while the other factors were fixed. A PBS solution (0.05 M) of MG was employed throughout the experiments by adding 50 mL of MG solution of desired concentration (from 10 to 100 mg·L^{-1}) at pH 3.5–6.5 with a certain amount of MNFs-Lac (0.5–2.0 g·L^{-1}) to 150 mL flasks. Mixtures were then placed into an incubator shaker at 25–65 °C and stirred at 120 rpm for 60 min. The MNFs-Lac were separated from the mixture by an external magnet at specified time intervals, and the MG concentrations in supernatant were detected with a microplate reader at 617 nm. The degradation efficiency (D%) of MG in the mixture was calculated using Equation (3):

$$D\% = \frac{C_0 - C_e}{C_0} \times 100 \quad (3)$$

where C_0 is the initial MG concentration (mg·L^{-1}) and C_e is the final MG concentration in the supernatant (mg·L^{-1}). At least three repeated tests were conducted per reaction condition.

Reusability of MNFs-Lac

The reusability of MNFs-Lac was evaluated through a series of batch experiments using 0.3 mM Hobt as mediator to degrade MG in PBS solution (0.05 M). Based on the optimal results above, the reusability of MNFs-Lac was conducted in a constant temperature shaker at 120 rpm, reaction temperature of 35 °C, pH 4.5 and reaction time of 15 min. The reaction mixture contained 25 mg·L^{-1} MG and 1.5 g·L^{-1} MNFs-Lac. After each reaction cycle, the mixture was separated by an external magnet, and the MG concentration in supernatant was measured by a microplate reader at 617 nm. The recovered MNFs-Lac were washed three times with PBS solution (0.05 M, pH 4.5) and then transferred into the next fresh reaction solution to start a new operation cycle. Thirty consecutive reaction cycles were performed. The degradation efficiency of MG was calculated by using the concentrations of MG in the initial solution and the end of one cycle reaction, similar to as described in the 'Optimization of degradation conditions of MG' section.

RESULTS AND DISCUSSION

Characterizations

Figure 1(a) presents the SEM morphology of MNFs-Lac, showing the spherical, porous and flower-like structure with size diameter of ~10 μm. A smooth and flat surface of the petals is clearly visible in Figure 1(b), which indicates few magnetic nanoparticles on the surface of MNFs-Lac. Figure 1(c) shows the TEM image of Fe_3O_4@SiO_2. The nanoparticles were spherical with obvious core/shell structure. Figure 1(d) and 1(e) are TEM images of one of the petals consisting of laccase and magnetic nanoparticles. At high magnification (Figure 1(e)), it can be seen that scattered magnetic nanoparticles have been successfully incorporated into the interior of the petals, which could result in a tighter binding between magnetic nanoparticles and MNFs-Lac to form a more stable three-dimensional structure. Meanwhile, it was clearly observed that the strong signal of Fe_3O_4 and $Cu_3(PO_4)_2{\cdot}3H_2O$ still presented in the XRD spectrum of MNFs-Lac (Figure S1, Supporting Information), demonstrating that the formed MNFs-Lac did not cause changes in the phase property of Fe_3O_4. Moreover, the magnetic properties of MNFs-Lac were examined by a VSM and presented in Figure S2 (Supporting Information). Although the maximum saturation magnetization of MNFs-Lac was not as high as expected (about 20 emu·g^{-1}), the MNFs-LAC can still be separated rapidly from the MG solution and retrieved effectively, as shown in the insert in Figure S2.

The FT-IR spectra of PMIDA, Fe_3O_4@SiO_2-PMIDA, laccase and MNFs-Lac are shown in Figure 2. The adsorption peaks at 596 cm^{-1} and 1,091 cm^{-1} in curve 'b' came from the Fe-O vibration and Si-O-Si stretching vibrations of Fe_3O_4@SiO_2, respectively. And the stretching vibration of the P-O-Si band is shown at 1,123 cm^{-1} from Fe_3O_4@SiO_2-PMIDA. The band at 1,647 cm^{-1} in curve 'b' corresponds to the strong band at 1,743 cm^{-1} in curve 'a', which contributed to antisymmetric stretching vibrations of the C–O group (Demin *et al.* 2018), where the shift may result from the influence of the -COOH groups in the PMIDA molecule on the surface of the magnetic nanoparticles (Majewski *et al.* 2011).

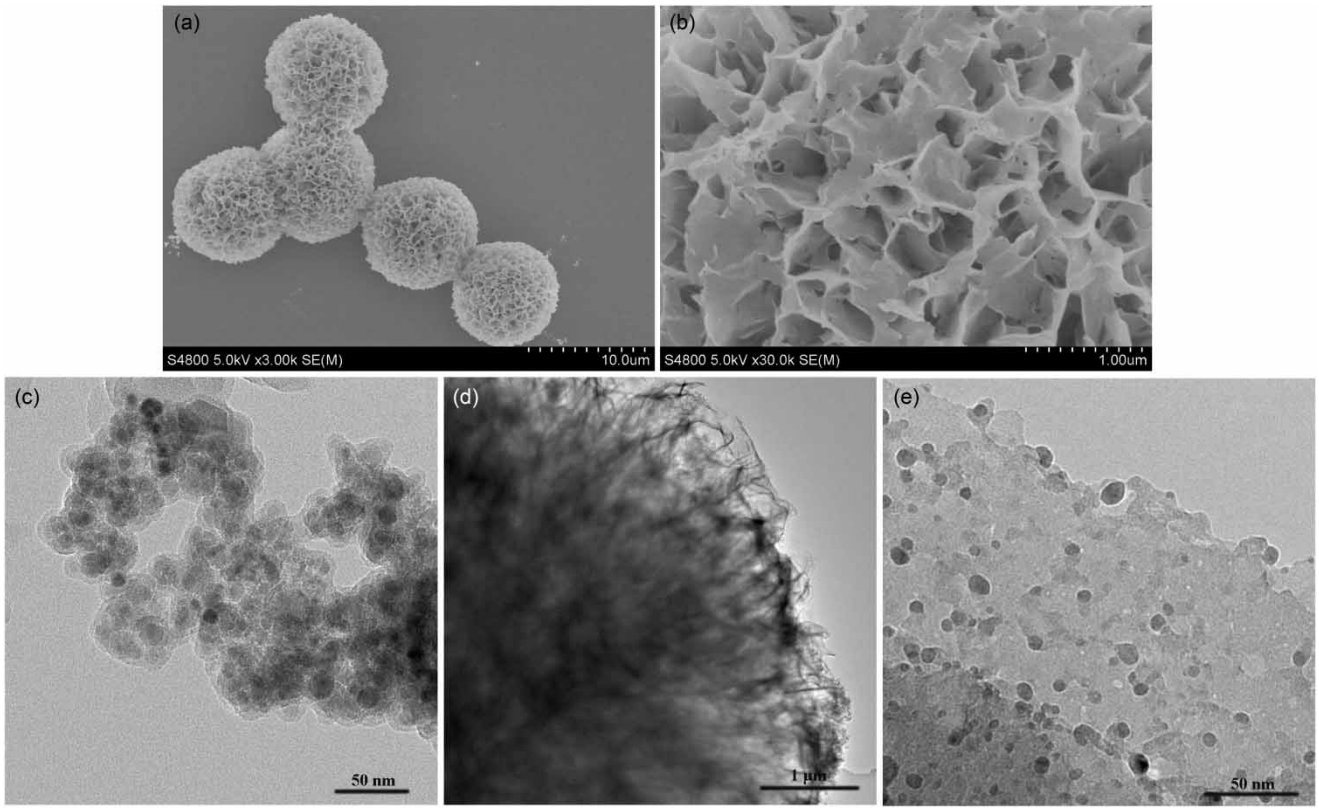

Figure 1 | SEM images of MNFs-Lac (a), (b); TEM images of Fe_3O_4@SiO_2 (c) and MNFs-Lac (d), (e).

The weak absorption peak appearing at 1,425 cm^{-1} in curve 'b' was the C-O-H in-plane bending vibration of PMIDA. The appearance of these peaks proved the successful grafting of PMIDA onto Fe_3O_4@SiO_2 NPs.

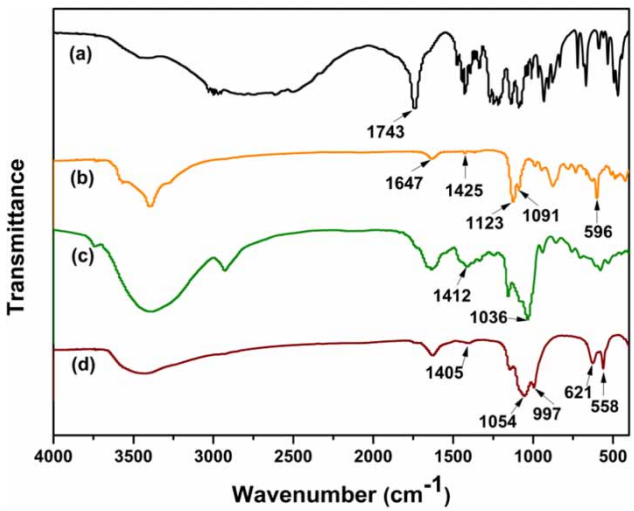

Figure 2 | FT-IR spectra of PMIDA (a), Fe_3O_4@SiO_2-PMIDA (b), laccase (c) and MNFs-Lac (d).

As presented, the adsorption peaks at 1,412 cm^{-1} and 1,405 cm^{-1} in curve 'c' and 'd' were assigned to the stretching vibrations of -CH_3 from laccase. And the stretching vibrations peaks of C-O at 1,036 cm^{-1} in curve 'd' and 1,054 cm^{-1} in curve 'd' from laccase were clearly observed. In addition, the characteristic peaks at 621 cm^{-1} and 558 cm^{-1} in curve 'd' were ascribed to the bending vibration of O-P-O, and the peak at 997 cm^{-1} was due to the antisymmetric stretching vibrations of PO_4^{3-}. The results indicated that laccase was successfully immobilized on the MNFs-Lac.

In addition, the compositions of Fe_3O_4@SiO_2-PMIDA-Cu^{2+} NPs were characterized by EDS (Figure 3). The existence of N, O, Si, P, Fe, and Cu elements in Fe_3O_4@SiO_2-PMIDA-Cu^{2+} NPs can be seen from the EDS spectra. Among them, O, Si and Fe were the main elements of Fe_3O_4@SiO_2 NPs. N and P elements could come from PMIDA. The existence of Cu element demonstrated that copper ions in the reaction solution were successfully chelated with PMIDA on the surface of magnetic nanoparticles.

Fluorescence microscopy was used to investigate the existence and distribution of laccase in MNFs-Lac. Figure 4

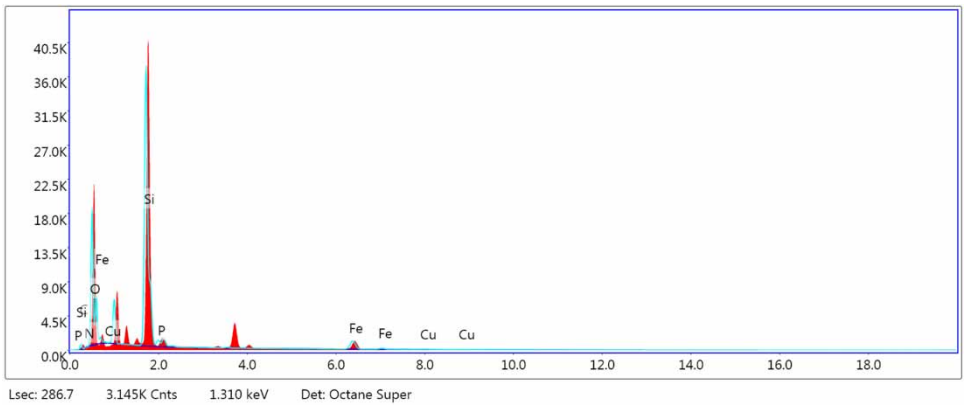

Figure 3 | EDS spectra of Fe_3O_4@SiO_2-PMIDA-Cu^{2+}.

shows fluorescence photomicrographs of MNFs-Lac. Figure 4(a) presents the status of MNFs-Lac stained with FITC under bright field as control. In Figure 4(b), the green fluorescence was attributed to the FITC combined with laccases of MNFs-Lac. The result visually demonstrated that laccases have been successfully loaded into MNFs-Lac.

The loading amount of protein was about 48.0 mg per gram of MNFs-Lac, and the expressed laccase activity was about 428.7 U/g MNFs-Lac.

Storage stability

The storage stability is one of the vital factors for evaluating the performance of immobilized enzymes because good stability means the enzymes can be useful for low-use practical applications (Vrsanska *et al.* 2018). The changes in activity of MNFs-Lac and free laccase with storage time are shown in Figure 5. After 30 days at 4 °C (Figure 5(a)), the retained activity of MNFs-Lac was still as high as 94%; when stored for 60 days, 90% of the initial activity still remained, but the activity of free laccase only retained 64% of its initial activity during an equal period of time. Simultaneously, it is obvious that the different storage temperatures can significantly affect the enzyme activity, whether that of the free laccase or the MNFs-Lac. After storage of 60 days at room temperature (Figure 5(b)), free laccase and MNFs-Lac lost 85% and 50% of their initial activity, respectively. The results revealed that the MNFs-Lac activity is more stable than the free form, which is consistent with the previously reported phenomenon of laccase immobilized on carriers with chelated Cu^{2+} (Bayramoglu *et al.* 2010; Fu *et al.* 2019).

Besides the conformational changes of laccase, the laccase leaching from MNFs-Lac could be another factor that affects the storage stability of MNFs-Lac. Hence, during the storage period of 60 days, the protein leaching amounts of MNFs-Lac stored at 4 °C and room temperature were measured respectively. Results showed that there was hardly any leaching of protein at the end of 60 days (data not shown), which indicates that the structure of MNFs-Lac was stable when stored in PBS (0.05 M, pH 4.5) for a period of 60 days.

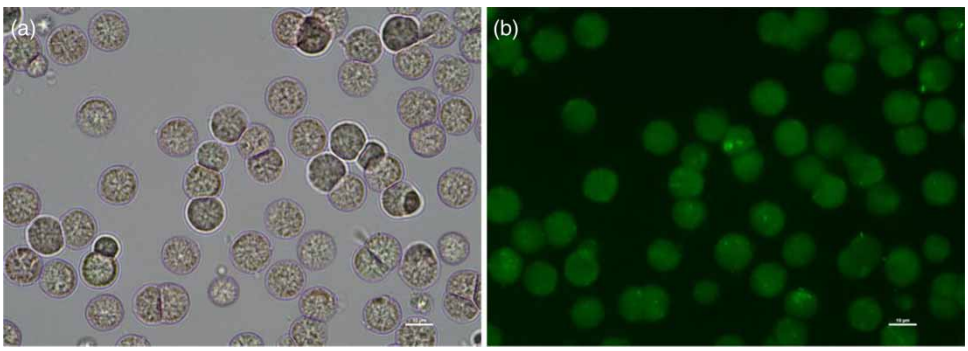

Figure 4 | Fluorescence photomicrographs of MNFs-Lac under bright field (a) and green exciting light (b), respectively (scale bar: 10 μm).

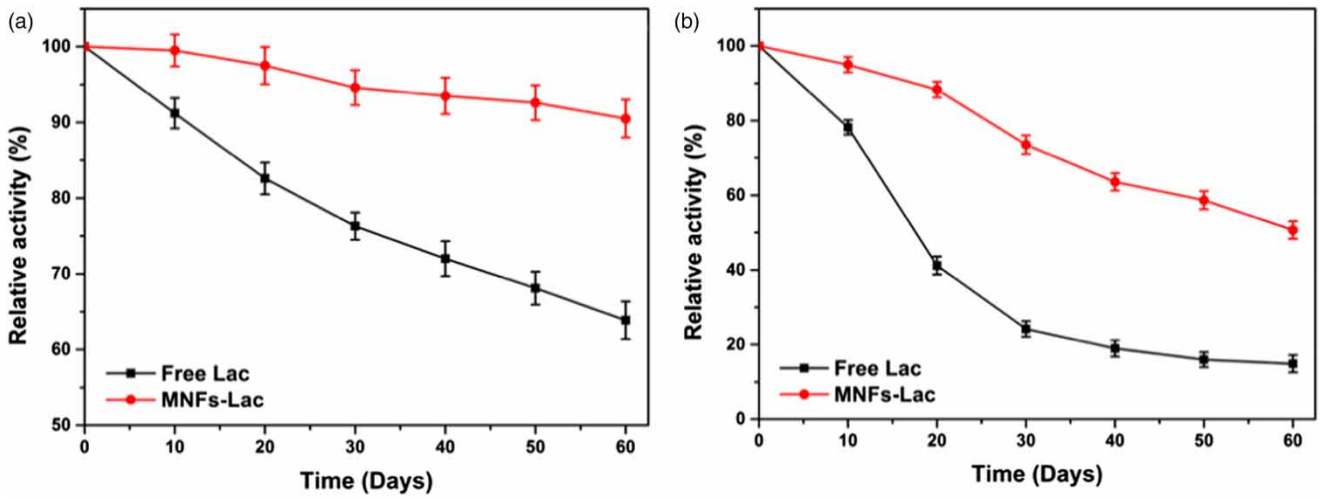

Figure 5 | Effect of storage time on the relative activity of free laccase and MNFs-Lac at 4 °C (a) and room temperature (25 ± 2 °C) (b) in PBS solution (0.05 M, pH 4.5), respectively.

Optimum conditions of MG degradation

Prior to detailed investigation on degradation of MG by MNFs-Lac, a mediator for the reaction was primarily selected and optimized, since as an electron vehicle between laccases and target compounds, it can greatly enhance the catalytic capacity and applicability of laccase. Mediators for laccases involve natural and synthetic mediators. Among them, ABTS is commonly used as a substrate and mediator to determine the activity unit of laccases (Fu *et al.* 2019; Guardado *et al.* 2019). However, it is not a suitable redox mediator for laccases degrading MG because the ABTS oxidized by laccases turns the reaction blue and disturbs the determination of MG concentration via spectrophotometry. Hobt is a colorless and efficient redox mediator that has been widely and successfully used in the decolorization and biodegradation of dye wastewaters and pharmaceuticals by laccase (Khlifi *et al.* 2010; Zeng *et al.* 2017). The redox potential of Hobt is approximately 1,100 mV at pH 4 and not affected by solution pHs in the range of 4–9 (Xu *et al.* 2000). In a pre-experiment, the reaction mixture containing laccase and Hobt was measured at absorbance wavelength of MG (617 nm) via spectrophotometry, and no absorbance was found, which means that Hobt does not interfere with the determination of the absorbance of MG. Therefore, Hobt was chosen as a redox mediator in the degradation reaction of MG by MNFs-Lac. The results showed that MG degradation efficiency using laccases was enhanced in the presence of Hobt at various concentrations. The maximum MG degradation efficiency was observed to be 99, 99, 94, and 83% for the Hobt concentrations of 0.5, 0.3, 0.2 and 0.1 mM, respectively. Considering the economic cost, 0.3 mM Hobt was selected as the optimum concentration for all subsequent MG degradation experiments in this study.

To investigate the degradation efficiency of MG by MNFs-Lac, the main influential factors including initial MG concentration, dosage of MNFs-Lac, temperature and pH value were explored. As shown in Figure 6(a), initial MG concentration is one of the key parameters in the degradation experiment, influencing the degradation rate and efficiency significantly. When a specified amount of MNFs-Lac (1.5 $g \cdot L^{-1}$) was added into a series of MG concentrations (10, 25, 50, 100 $mg \cdot L^{-1}$) in 0.05 M PBS buffer (pH 4.5), the degradation efficiency and rate of MG were markedly different with initial MG concentration. The degradation efficiency could reach up to 99% within 5 min and 15 min for 10 $mg \cdot L^{-1}$ and 25 $mg \cdot L^{-1}$ of MG, respectively. Although the degradation efficiency and rate were markedly decreased when the concentration of MG was raised to 50 $mg \cdot L^{-1}$ or 100 $mg \cdot L^{-1}$, the degradation efficiency and rate of MG by MNFs-Lac were still much higher than that of laccases immobilized on other carriers. For instance, to attain 95% degradation efficiency for 50 $mg \cdot L^{-1}$ MG concentration, the reaction between the laccases immobilized on magnetic grapheme oxide and MG would last at least 3 hours (Chen *et al.* 2017). Even if the MG concentration was only 20 $mg \cdot L^{-1}$, 90% degradation efficiency of MG by laccase–biotitania biocatalysts also needed 6 hours (Zhang *et al.* 2018). Therefore, compared with the reported literature, MNFs-Lac is an ideal carrier for laccase immobilization.

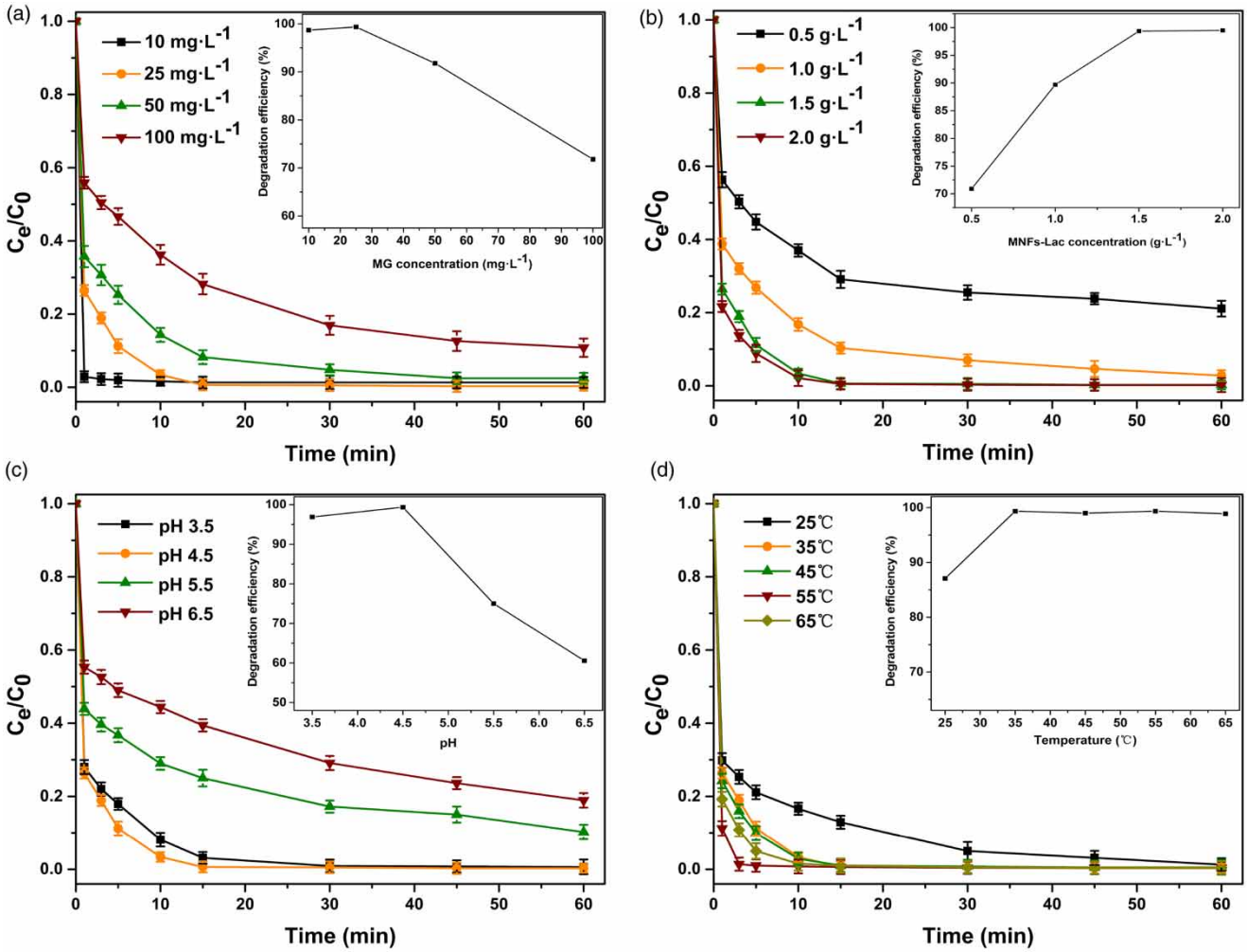

Figure 6 | Effects of different influencing factors on MG degradation with 0.3 mM Hobt. (a) Effect of initial MG concentrations on MG degradation (1.5 g·L^{-1} MNFs-Lac, pH 4.5, 35 °C); (b) effect of MNFs-Lac dosage on MG degradation (25 mg·L^{-1} initial MG concentration, pH 4.5, 35 °C); (c) effect of pH on MG degradation (25 mg·L^{-1} initial MG concentration, 1.5 g·L^{-1} MNFs-Lac, 35 °C); (d) effect of reaction temperature on MG degradation (25 mg·L^{-1} initial MG concentration, 1.5 g·L^{-1} MNFs-Lac, pH 4.5). Inset is the curve of degradation efficiency of MG at reaction time of 15 min.

The effect of MNFs-Lac dosage was tested at different levels of amount, 0.5, 1.0, 1.5, and 2.0 g·L^{-1} with an MG concentration of 25 mg·L^{-1} at pH 4.5. Figure 6(b) shows the results of the effect of the MNFs-Lac dosage on the MG degradation efficiency. It can be seen that there was an obvious increasing trend in the degradation efficiency and rate with MNFs-Lac dosage from 0.5 g·L^{-1} to 1.5 g·L^{-1}. However, there was no longer an apparent increase in the degradation efficiency when the MNFs-Lac dosage was increased from 1.5 g·L^{-1} to 2.0 g·L^{-1}. An explanation for this is that the higher the MNFs-Lac dosage, the more laccase molecules could possibly be introduced into the reaction system. This suggested that the amount of 1.5 g·L^{-1} MNFs-Lac was sufficient relative to 25 mg·L^{-1} MG. Under this circumstance, continually increasing the amount of MNFs-Lac could not effectively improve the catalysis efficiency of laccases. Hence, in terms of MG degradation and the cost-effectiveness of the process, 1.5 g·L^{-1} MNFs-Lac dosage would be seen as an optimum value of MNFs-Lac for 25 mg·L^{-1} MG concentration.

The effect of pH on MG degradation was evaluated at 35 °C in PBS solution (0.05 M) containing MG of 25 mg·L^{-1}, MNFs-Lac of 1.5 g·L^{-1} and 0.3 mM Hobt at different pH values ranging from 3.5 to 6.5. The pH dependence of the MG degradation efficiency induced by MNFs-Lac is presented in Figure 6(c). It can be seen that the MG degradation was more efficient at pH 4.5–3.5 than at pH 5.5–6.5, which suggested the pH value had vital impact on the degradation efficiency of MG. The pH influence on the enzymatic reaction can be described as two

mechanisms: one is the electrostatic interaction between laccases, redox mediator Hobt and substrate MG; another is the impact of pH on the conformation of laccases. The electrostatic interaction can be considered to affect the encounter probability of the three: laccases, Hobt, and MG. The enzymatic reaction would be enhanced or hindered depending on whether attractive or repulsive forces prevail, respectively. The isoelectric point (IP) of laccases is around 4.2 (Gascon *et al.* 2014). Laccase molecules will be positively charged when solution pH < 4.2, negatively when pH > 4.2 and neutrally when pH ≈ 4.2. The pKa for MG is 6.9 (Goldacre & Phillips 1949). MG molecules bear a positive net charge at 6.9 > pH > 4.2, whereas the laccases are charged negatively at this pH range. The optimal conditions may occur in a pH range between the IP (laccases) and pKa (MG), at which the positively charged MG and negatively charged laccases can readily attract each other. Both of them combine by hydrogen bonding easily, increase the adsorption amount and improve the degradation efficiency. Also, it is well known that the enzymatic activity is determined by the protein conformation of enzyme. The study by Saoudi *et al.* (2017) revealed that the conformational stability of the laccase decreased with increasing pH from 3 to 7. At pH 3–5, the laccase conformation was stable, while at pH > 6, the changes in the laccase conformation were initiated. This phenomenon, induced by varying the concentration of H^+ ions in the solution, was attributed to the increases in the values of free enthalpy and entropy of protein unfolding which are responsible for the folded state. Thus, the optimum pH of MG degradation by MNFs-Lac was selected as pH 4.5.

The effect of temperature on the degradation efficiency was carried out in 25 mg·L^{-1} MG solution containing 1.5 g·L^{-1} MNFs-Lac and 0.3 mM Hobt at temperature range of 25 °C–65 °C and pH 4.5. The results are shown in Figure 6(d). It can be seen that the temperature had more influence on the degradation rate than degradation efficiency. When the reaction temperature was 25 °C, only about 80% of MG was degraded within the first 5 min, and the 99% of degradation efficiency needed took about 60 min to attain. Correspondingly, as the reaction temperature was increased to 55 °C, 99% of MG was degraded within the first 5 min, which indicated that appropriate increasing in reaction temperature was conducive to enhance the enzymatic activity of laccase. However, as the reaction temperature was increased to 65 °C, the degradation efficiency and rate of MG decreased slightly. This could be attributed to the fact that the unfolded energy of laccase decreases with increasing temperature. The decrease in the unfolded energy of laccase signifies the existence of a protein denaturation process with increasing temperature (Saoudi *et al.* 2017). Therefore, the higher temperature was unfavorable for the enzymatic activity of laccase, even completely destroying its activity at 85 °C (Fu *et al.* 2019). When the reaction temperature was set at 35 °C–45 °C, the degradation efficiency reached 99% within 15 min. Consequently, considering the practical operating cost for the MG degradation system, 35 °C was considered as the optimum degradation temperature in all subsequent experiments.

Based on the results from the single-factor optimization experiments above, the optimum conditions for degrading MG of 25 mg·L^{-1} were as follows: 0.3 mM Hobt as the redox mediator of laccase, reaction temperature of 35 °C, MNFs-Lac dosage of 1.5 g·L^{-1}, reaction solution of pH 4.5 and reaction time of 15 min. Under the optimum conditions, MNFs-Lac exhibited higher degradation efficiency toward MG than did free laccase (as evidenced in Figure S3, Supporting Information).

Reusability of MNFs-Lac

Under the optimal conditions, the reusability of MNFs-Lac was evaluated with a 30-cycles degradation test. As shown in Figure 7, MNFs-Lac exhibited remarkable reusability. Even after 13 cycles, the degradation efficiency of MG still remained 99%. Only from cycle 14 was a progressive decrease of the degradation efficiency observed. However, the decrease of degradation efficiency was slow. The

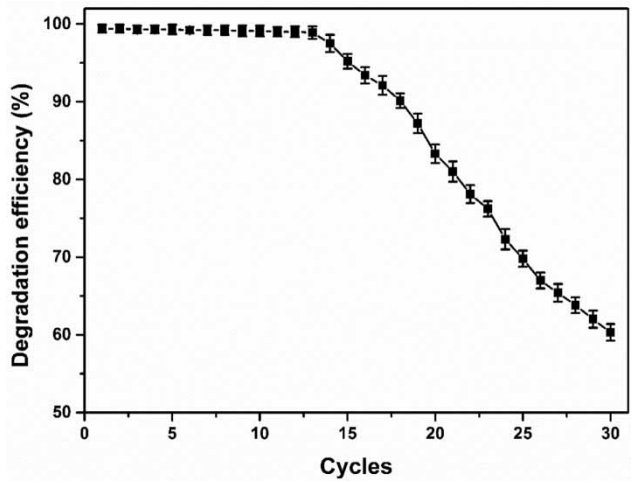

Figure 7 | Effect of cycles on degradation efficiency (25 mg·L^{-1} initial MG concentration, 1.5 g·L^{-1} MNFs-Lac, 0.3 mM Hobt, pH 4.5, 35 °C).

degradation efficiency of MG was still as high as 90% when the MNFs-Lac were reused for 18 times. Compared to previous results of Fu *et al.* (2019), the perfect reusability and enzymatic activity of MNFs-Lac could be attributed to the modified preparation method, by which magnetic nanoparticles were encapsulated into the interior of nanoflowers, reducing the coverage of the magnetic nanoparticles on the surface active sites of MNFs-Lac, which makes MG have more contact with laccase. In addition, the MNFs-Lac produced by the new method prevented the magnetic nanoparticles moving away from the surface of MNFs-Lac, which would greatly improve the recovery of MNFs-Lac during the recycled process. Thus, magnetic nanoparticles encapsulated laccase nanoflowers have a greater advantage because of its recyclability.

CONCLUSIONS

In order to improve the reusability of MNFs-Lac, magnetic nanoparticles were successfully encapsulated into the interior of laccase nanoflowers by grafting PMIDA as an interconnected bridge between the magnetic nanoparticles and copper ions. The morphology and compositions of as-prepared MNFs-Lac were characterized by SEM, TEM, FT-IR, EDS and fluorescence microscopy. The enzymatic activity of MNFs-Lac as a function of degradation efficiency of MG was optimized using a series of batch tests with single-factor experiment design. Under the optimum conditions, the degradation efficiency of MG has stayed at around 99% after 13 times cycles. Even if after 18 times cycles, the degradation efficiency still remained 90%. These results indicated that the modified preparation method improved greatly the reusability of MNFs-Lac. Improvements in the reusability will be beneficial for MNFs-Lac as a more promising material for degrading MG in a water environment.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors are responsible for the content and writing of the paper.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of Chinaunder Grant (number 31771094).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available online at https://dx.doi.org/10.2166/wst.2020.068.

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First received 29 October 2019; accepted in revised form 6 February 2020. Available online 18 February 2020