Nitroxide Human Serum Albumin Incorporated Nanoflowers With Dual Enzyme-Like Activities

Can Cai

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NITROXIDE HUMAN SERUM ALBUMIN INCORPORATED NANOFLOWERS WITH DUAL ENZYME-LIKE ACTIVITIES

by

CAN CAI

(Under the Direction of Xiao-Jun Wang)

ABSTRACT

In this work, nitroxide 2,2,6,6-tetramethylpiperdine 1-oxyl (Tempo), human serum albumin (HSA), CuSO$_4$ and phosphate buffered saline (PBS) were used to synthesis hybrid nanoflowers. In addition, scanning electron microscopy (SEM), electron paramagnetic resonance (EPR), and Fourier-transform infrared spectroscopy (FTIR) were used for the characterization of nanoflowers. SEM spectra illustrated the morphologies of hybrid nanoflowers, whereas FTIR spectra corroborated the presence of PNA in hybrid nanoflowers. In a similar vein, EPR spectra confirmed the intermolecular interaction of nitroxides bound to human serum albumin that was incorporated into nanoflowers. Catalase mimetic activity and corresponding kinetic parameters of nanoflowers were determined using H$_2$O$_2$ depletion and O$_2$ evolution assays. The xanthine oxidase/cytochrome c method was used to determine superoxide dismutase mimetic activity of nanoflowers and corresponding kinetic parameters of nanoflowers. Finally, reusability of hybrid nanoflowers were assessed via H$_2$O$_2$ depletion assay and xanthine oxidase/cytochrome c method. Kinetic analysis revealed that reactions of incorporated nanoflowers with H$_2$O$_2$ conform to Michaelis–Menton kinetics and that the nanoflowers possess a higher affinity for H$_2$O$_2$ than nature catalase. The hybrid nanoflowers with superior stability and desired ligand grafting sites is expected to find widespread utilization in medical sciences.

INDEX WORDS: Catalase, Superoxide dismutase, Hybrid nanoflowers
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by

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MASTER OF SCIENCE
NITROXIDE HUMAN SERUM ALBUMIN INCORPORATED NANOFLOWERS WITH DUAL ENZYME-LIKE ACTIVITIES

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CHAPTER 1

INTRODUCTION

Purpose of this Work

Acting as potential therapeutic agents, combined synthetic superoxide dismutase (SOD)/catalase mimetics can prevent oxidative stress in living organisms by removing the reactive oxygen species (ROS) that are responsible for aging. [1,2] Many researchers in this field have focused on investigating salen–manganese (III) complexes, which possess superoxide and hydrogen peroxide scavenging properties that could possibly counter ROS-associated diseases. [3,4] Although salen–manganese (III) complexes exhibit better bioavailability and enzymatic behavior than antioxidant enzymes, their stability can be significantly reduced under oxidizing conditions owing to ligand degradation. [5]

In recent years, a number of nanomaterials possessing SOD and catalase activities, such as nanoceria, Mn₃O₄, and platinum nanoparticles, have elicited much attention. [6-8] Affinity ligands must be grafted to the surface of SOD/catalase mimetics using nanostructures in order to be used as therapeutic agents for additional applications, such as for targeted drug delivery. [9] Given that the majority of the reactions of SOD/catalase mimetics with nanostructures occur on the surface of these nanomaterials, surface grafting causes a decline in the dual enzyme-like activities of mimetics. [10] Hence, this continues to pose a critical challenge in constructing combined synthetic SOD/catalase mimetics that possess perfect stability and sufficient grafting sites. It is generally accepted that amino acid side chains in proteins are
capable of providing sufficient enough grafting sites for affinity ligands. Hence, we propose that biomolecule-inspired synthesis would be a suitable approach for introducing protein into the nanostructure of mimetics with SOD and catalase activities, thereby overcoming the problems arising from surface grafting. In a previous report, protein-incorporated nanoflowers were fabricated using a molecular assembly of protein and copper phosphor. [11] The protein molecules serve as “glue” to bind the petals together during the growth of nanoflowers, which consequently spreads into mature Cu₃(PO₄)₂ nanoflowers. The grafting sites for the required ligands are believed to have been supplied by the amino acid side chains of the protein incorporated into the nanoflowers; these hybrid nanoflowers are regarded as mimetics possessing dual enzyme activities. Moreover, hybrid nanoflowers have been shown to exhibit excellent operational stability in the resolution of (R, S)₂-pentanol. [12] It is noteworthy that the skeletal stability of Cu₃(PO₄)₂ hybrid nanoflowers enables their use within medical and industrial fields. It has been confirmed that hybrid nanoflowers exert a peroxidase-like activity that relies on a Fenton-like reaction mechanism. [13] Could it be possible to endow them with a catalase-like activity using this ingenious approach? In a prior study, nitroxide radicals were used to stimulate the catalase mimetic activity of the heme protein. [14] The mechanism of stimulation was attributed to the replenishment of MbFe III and to the detoxification of MbFe IV, which, in turn was accelerated by nitroxide radicals that can “shuttle” among three oxidation states. Inspired by this work, we surmised that, if nitroxide radical molecules had been incorporated into the nanoflowers, then these radicals should accelerate the conversion of copper (II) ions to copper (I) ions in the nanoflowers by shuttling among their different oxidation states, thus facilitating H₂O₂ disproportionation. More specifically, the catalase
mimetic activity of the nanoflowers should be activated by the nitrooxide radicals incorporated into the nanoflowers. Furthermore, although nitrooxides have SOD-like activity, their utilization is limited owing to their short half-life in vivo. [15] Fortunately, it is possible to improve the half-life and SOD-like activity of nitrooxides by covalent attachment to the backbone of protein. [16,17] Based on the above-mentioned reports, we posited that human serum albumin decorated by nitrooxides is the perfect candidate to mediate the formation of nanoflowers with SOD and catalase mimetic activities. We also hypothesized that the nitrooxides would stimulate the catalase mimetic activity of copper ions in the nanoflowers so as to induce the corresponding activity of MbFe III in the heme protein. Furthermore, we predicted that the amino acid groups of the human serum albumin would provide sufficient grafting sites for the affinity ligands to avoid the capping of copper ions on the surface of the nanoflower, and that nitrooxide covalently attached to human serum albumin incorporated into the nanoflowers would still be able to maintain its initial SOD-like activity. In this study, polynitroxylated human serum albumin (PNA) were employed in order to direct the synthesis of the hybrid nanoflowers (See chapter 3). The material characteristics of these nanoflowers were assessed using scanning electronic microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and electron paramagnetic resonance (EPR). Notably, the catalase mimetic activity and corresponding kinetic parameters of nanoflowers were determined using the \( \text{H}_2\text{O}_2 \) depletion and \( \text{O}_2 \) evolution assays. The SOD-like activities of the nanoflowers were determined by utilizing the xanthine oxidase/cytochrome c method. Finally, the reusability of the hybrid nanoflowers was assessed via the \( \text{H}_2\text{O}_2 \) depletion assay as well as the xanthine oxidase/cytochrome c method.
Nanoflower

The general meaning of nanoflower is a microscopic flower shape substance by the formation of certain chemical compounds. There are several types of nanoflowers, such as gold based, titanium dioxide, and copper based etc. Copper based nanoflowers have been emphasized in this work; they were found by a group of scientists who had accidently mixed up the bovine serum albumin (BSA) with CuSO\(_4\) in phosphate buffered saline (PBS). In addition, the formation of copper based nanoflowers was interpreted in such a manner that suggested nitrogen atoms from amino acid residuals in the protein backbone form complexes with Cu (II) through covalent bonds and construct the protein-inorganic hybrid nanoflowers. [11] Thus, in the absence of protein, there will be no flower shape substance but amorphous Cu\(_3\)(PO\(_4\))\(_2\), as illustrated in Figure 1 (a). With the inclusion of any protein, figure 1 (b) was observed by the previous report. Meanwhile, figure 1 (c) was obtained by SEM in this work. Protein nitroxide human serum albumin was used in conjunction with copper ion. It can be seen that the nitroxide human serum albumin incorporated functional nanoflowers have catalase and superoxide dismutase mimic activities.

Figure 1.1 Illustration of Amorphous Cu\(_3\)(PO\(_4\))\(_2\) (a) and Nanoflowers (b) and (c) by SEM Images
Catalase

Nanoflowers exhibit catalase like activity. Catalase is a common enzyme existing in many kinds of organisms. Its function is to catalyze the decomposition of hydrogen peroxide into water and oxygen. [39] and plays an important role in shielding organisms from reactive oxygen species (ROS) associated diseases. Catalase was discovered by Louis Jacques Thénard (the founder of $\text{H}_2\text{O}_2$). In 1900, Oscar Loew named the enzyme with the function of catalyzing the decomposition of hydrogen peroxide “catalase”. [40] In 1937, James Batcheller Sumner crystallized catalase from beef liver [41] and found the molecular weight of the crystalline catalase a year later. [42] In the year 1967, a preliminary report of amino acid sequence of bovine liver catalase was published, [43] whereas the structure of beef liver catalase was determined in 1981. [44]

Superoxide Dismutase

Nanoflowers are also known to exhibit superoxide dismutase (SOD) like activity. As with catalase, superoxide dismutase is also a common enzyme existing in various kinds of organisms. Its function are to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen, regulate the superoxide ion, and prevent it from causing oxidative damage from organisms. [45] SODs were nothing but metalloproteins before Irwin Fraovich at the Duke University collected evidence to demonstrate their enzymic function. [46]
Reactive Oxygen Species

Reactive oxygen species (ROS) are reactive byproducts of oxygen and play an important role in cell signaling and homeostasis. However, excessive ROS tend to cause cell damage and are responsible for triggering many diseases. Among all kinds of reactive oxygen species, hydrogen peroxide is very common in organisms, and superoxide is the precursor of most other reactive oxygen species. For this reason, many researchers are dedicating themselves to finding a combined synthetic superoxide dismutase /catalase mimetic in order to eliminate ROS.
CHAPTER 2

PRINCIPLES OF SUPEROXIDE DISMUTASE AND CATALASE

Mechanism of Superoxide Dismutase and Catalase in Heme Proteins

Myoglobin and hemoglobin are two of the most common heme proteins found in mammals. Myoglobin is known for both transmitting and storing oxygen in muscle tissues. Due to oxidation from myoglobin, it mainly occurs in oxymyoglobin (O-MbFeII) form. In addition, oxymyoglobin can also undergo oxidation and generate metmyoglobin (MbFeIII) along with superoxide free radical (\(\text{O}_2^-\)). It has also been demonstrated that \(\text{H}_2\text{O}_2\), as the product of superoxide free radical spontaneous dismutation, must be eliminated. [49] Similar reactions also occur in hemoglobin. [50] Although the process of catalase in heme proteins is yet to be precisely solved, extensive efforts have been put in thus far in this regard. [14].

The mechanism of catalase in heme proteins (Hp) is as follows:

\[
\text{HpFe}^{\text{III}} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} + \text{H}_2\text{O}
\]

\[
\text{compound I} + \text{H}_2\text{O}_2 \rightarrow \text{HpFe}^{\text{III}} + \text{H}_2\text{O} + \text{O}_2
\]

The exact structure of compound I is uncertain - the iron is oxidized from Fe (III) to a nominal valency of Fe(V). Then, an Fe (IV) oxoporphyrin-cation radical, (haem \(^+\)Fe(IV)O is probably formed.

Due to the presence of nitroxides, the mechanism of superoxide dismutase in heme proteins can be explained as follows:

\[
\text{HpFe}^{\text{IV}} + \text{O}_2^- \rightarrow \text{HpFe}^{\text{III}} + \text{O}_2
\]
Stable Free Radical Nitroxide as Superoxide Dismutase Mimetic

Figure 2.1 Nitroxides Can Shuttle between Two States and Exhibit SOD Mimic Activity [14]

Nitroxide reacts with the first superoxide to form Oxoammonium and hydrogen peroxide. Thereafter, Oxoammonium reacts with the second superoxide to form oxygen and nitroxide again. Hydrogen peroxide and oxygen are the net reaction products.

Hydrogen Peroxide in Industrial Pollution

Hydrogen peroxide is a powerful oxidizer that is used extensively for industry purposes. Examples include bleach textiles and paper products, food processing, landfills, timber products, etc. Hydrogen peroxide can also be decomposed into hydrogen and water. While these two products are safe and green, this process is slow; thus, catalase mimetics can be used to treat the hydrogen peroxide associated with industrial pollution.

\[
\text{HpFe}^{\text{III}} + 2\text{H}^+ + \text{O}_2^- \rightarrow \text{HpFe}^{\text{IV}} + \text{H}_2\text{O}_2
\]
Mechanism of Superoxide Dismutase and Catalase in Nanoflowers

As mentioned before, nitroxides can shuttle between two states and exhibit SOD mimic activity. When combined with copper ions’ catalase-like activity in nanoflowers (similar with iron ions in heme proteins), they scavenge ROS species such as superoxide and hydrogen peroxide, with water and oxygen being the environmentally-friendly products. Notably, the superoxide generated by copper ions’ reaction will be taken care of by nitroxides, while the hydrogen peroxide generated by nitroxides’ reaction will be taken care of by copper ions.
CHAPTER 3

SYNTHESIS

Preparation of Reagent and Materials

The following reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA): nitroxide 2,2,6,6-tetramethylpiperdine 1-oxyl (Tempo), human serum albumin (HSA); catalase (from bovine liver; 2000–5000 units/mg protein; one unit decomposes hydrogen peroxide at a rate of 1.0 µmol/min at pH 7.0 at 25°C. In a span of 1 min, the hydrogen peroxide concentration reduced from 10.3 mM to 9.2 mM); bovine superoxide dismutase (≥2500 units/mg protein; one unit inhibits the rate of reduction of cytochrome c by 50% in a coupled system using xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3.0 mL reaction volume; the xanthine oxidase concentration used causes a change in absorbance of 0.025 min⁻¹ at 550 nm); xanthine oxidase (from bovine milk; ≥0.4 units/mg protein; one unit converts xanthine to uric acid at a rate of 1.0 µmol/min at pH 7.5 at 25°C); cytochrome c (≥95%, SDS-PAGE); and acetaldehyde (≥99.0%, GC). All chemicals and reagents were found to be of analytical grade. In addition, all aqueous solutions were prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).
Procedure

PNA was prepared in accordance with a previously published method, [15,18-20] using a nitrooxide/albumin molar ratio of 50:1. Synthesis of the hybrid nanoflowers was performed based on the classical method, with necessary modifications. [11] PNA (150 mg/mL) was diluted to 0.02, 0.05, 0.10, and 0.50 mg/mL concentrations in PBS buffer (0.05 M, pH 7.4). The synthesis was started by adding 20 mL CuSO$_4$ solution (120 mM) to 3 L of the PBS buffer that contained PNA. After incubation at 25° C for 3 d, the precipitate was separated from the mixture by centrifugation (10 min, 8000 g, 25° C). The precipitate was then washed with deionized water three times before being dried. The final product was termed as the “PNA-incorporated nanoflowers.” In order to calculate the production yield, the residual protein content in the supernatant was determined by the Bradford method [21] using bovine serum albumin as a standard. The encapsulation yield of fixed PNA was arrived at by calculating the difference between the amounts of PNA used and the amounts recovered in the supernatant. All samples were measured by means of EPR spectroscopy, and in each sample, the amount of nitroxides per gram of flowers was calculated by double integrating the EPR spectrum against a standard curve. The standard curve was constructed by spiking a test solution with multiple known amounts of Tempo. Notably, the encapsulation yields were 100%, 97%, 70%, and 18% for the samples prepared using 0.02, 0.05, 0.10, and 0.50 mg/mL PNA, respectively, which is consistent with the results derived from the EPR data. The hybrid nanoflowers prepared from 0.02, 0.05, 0.10, and 0.50 mg/mL PNA were called PNA-nano-A, PNA-nano-B, PNA-nano-C, and PNA-nano-D, respectively. With regard to the control group, HSA solutions of differing
concentrations in PBS in the range of 0.02–0.50 mg/mL were also used for synthesizing HSA-incorporated nanoflowers (which are referred to using this name hereafter).

Figure 3.1 The Formation of Nitrooxide Human Serum Albumin Incorporated Nanoflowers
CHAPTER 4

EXPERIMENTAL METHODS

Physical and Chemical Characterization Instruments

The morphologies of the samples were surveyed using a SEM (JEOL JSM-7600F, JEOL Ltd., Tokyo, Japan) that operated at 25kV. The samples’ FTIR absorption spectra were taken over a 400–4000 cm\(^{-1}\) range using a Thermo iS10 FTIR (Thermo Fisher Scientific, Waltham, MA, USA) (×2) with a resolution of 4 cm\(^{-1}\). Thereafter, EPR spectra were recorded by using an X-band EPR spectrometer (Bruker EMXplus, Bruker Instruments, Inc., Berlin, Germany). Parameters of spectroscopic data collection were as follows: center field, 3340 G; scan range, 200 G; field modulation, 0.79 G; microwave power, 0.5 mW; time constant, 0.1 s.

SEM shoots electrons onto samples and detects the scattered electrons in order to convert them into images, anode that are positively charged to direct and accelerate the electrons, thus providing us with a microscopic view of the tiny substance.

Unlike UV-visible spectrophotometer, FTIR utilizes infrared spectrum; it also absorbs particular chemical bonds energy to cause molecular vibrations instead of the transitions of atoms energy, thereby providing us with the molecule’s chemical structure information.

When matching the resonance frequency, EPR can be used to detect unpaired electrons, by applying a microwave and increasing magnetic field. Electrons then accept the microwave radiation, which, in turn, will cause the electron spin to flip over. In a practical example of standard nitrooxide EPR spectra (Figure 4.1), a nitrogen atom has five electrons whereas an
oxygen atom has six electrons. This implies that there will be one unpaired electron.

Importantly, 99.632% of nitrogen atoms are isotope $^{14}$N with nuclear spin 1, and 99.962% of oxygen atoms are nuclear spin 0. Approximately, a nitrogen atom’s nuclear spin is 1 and an oxygen atom’s is 0, which means that the nitrogen atom nuclear spin interacts with the electron spin and produces a hyperfine structure in EPR spectra. Due to the selection rule, three identical lines will appear when the nitrogen atom nuclear are +1, 0, and -1 respectively.

![EPR Spectra of Nitroxide](image)

**Figure 4.1 Standard EPR Spectra of Nitroxide**

Catalase Mimetic Activity by H$_2$O$_2$ Depletion Assay

The decomposition of H$_2$O$_2$ by hybrid nanoflowers was measured in accordance with the spectrophotometric method. [22] The hybrid nanoflowers (10 mg) were suspended in 100 mL of deionized water (0.1 mg/mL) and then allowed to stand for 5 min. During magnetic stirring, the decomposition reaction of H$_2$O$_2$ was triggered by the addition of 150 μL hybrid
nanoflower mixture to 9 mL H$_2$O$_2$ (12 mM). The reaction mixture was incubated at 25° C for 3 min. An aliquot of the reaction mixture (1 mL) was withdrawn every 30 s before being filtered by a membrane filter (0.22 µm pore size). The absorbance decreases of the filtrate at 240 nm ($\varepsilon = 43.6$ M$^{-1}$cm$^{-1}$) was monitored using an Agilent 8453 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in order to determine the residual amount of H$_2$O$_2$. The specific activity (µmol min$^{-1}$ mg$^{-1}$) was defined as the amount of H$_2$O$_2$ decomposed per minute per milligram of the hybrid nanoflowers.

![Figure 4.2 Illustration of UV-VIS Spectrophotometer](image1)

![Figure 4.4 Sample Curves of Spectrophotometer](image2)

**Catalase Mimetic Activity by O$_2$ Evolution Assay**

The conversion of hydrogen peroxide to oxygen was monitored using the Clark-type oxygen electrode method. [23] The hybrid nanoflower mixture (0.10 mg/mL) prepared above was further diluted by 10 times using deionized water. The reaction mixture consisted of three mL H$_2$O$_2$ (12 mM) and 50 µL that was a diluted hybrid nanoflower mixture (0.01 mg/mL). The reaction was initiated following the addition of diluted nanoflowers. The oxygen concentration
was monitored for 180 s at 25° C using a 5300A biological oxygen monitor (YSI Incorporated, Yellow Springs, OH, USA).

Catalase-Like Activity Assay by Steady-State Kinetic

The Michaelis–Menten constants $K_m$ and $V_{\text{max}}$ for the catalase-like activity of hybrid nanoflowers were determined by measuring the initial rates at varying $\text{H}_2\text{O}_2$ concentrations, ranging from 3 to 60 mM with different concentrations of nanoflowers. The values of $V_{\text{max}}$ and $K_m$ were derived using a Lineweaver–Burk plot after obtaining the initial rate versus $\text{H}_2\text{O}_2$ concentration data. [24]

Turnover numbers ($k_{\text{cat}}$) of the hybrid nanoflowers were calculated as per the following equation:

$$K_{\text{cat}} = \frac{V_{\text{max}}}{[E]}$$

where $[E]$ denotes the amount of PNA or HSA incorporated into the nanoflowers in the reaction mixture. Catalytic efficiencies ($k_{\text{cat}}/K_m$) of the hybrid nanoflowers were also calculated.

Superoxide Dismutation Mimetic Activity Assay

The method of inhibiting cytochrome $c$ reduction in the acetaldehyde/xanthine oxidase system was employed in order to evaluate the SOD-like activity of superoxide dismutase and the hybrid nanoflowers. [25] The reaction system of superoxide generation contained 300 μL cytochrome $c$ (0.5 mg/mL), 20 μL acetaldehyde (100 mM), and 0.3 μL xanthine oxidase (11.2
(0.1 mg/mL) or 30 μL PNA-incorporated nanoflowers (1.2 mg/mL). Meanwhile, the reaction system was incubated at 25° C for 15 mins. The absorbance increase of the reaction system at 550 nm was monitored by using an Agilent 8453 UV-VIS spectrophotometer. With regard to PNA-incorporated nanoflowers, the preparation of 330μL solutions identical to those that were used to produce the superoxide was undertaken. After adding the PNA-incorporated nanoflowers, each above-mentioned solution was centrifuged at an interval of 3 mins from each other, after which the absorbance of the supernatant was recorded. The reduction rate of cytochrome c was calculated by the slope of the curve within the initial 3 mins. One unit of SOD is defined as the amount of enzyme required to inhibit the reduction rate of cytochrome c by 50% in adherence to the above-mentioned conditions.

Practical Reusability Test by Removing Reactive Oxygen Species

The repeated batch decomposition of 12 mM H$_2$O$_2$ by the hybrid nanoflowers was performed at 25° C for 3 min. These hybrid nanoflowers were separated from the reaction mixture by centrifugation (8000 rpm, 3 min) at the end of each batch, before being washed using distilled water to remove any residual substrate or product. After each round of centrifugation, the nitroxide signal of the suspension was detected using an EPR spectrometer. Then, the recycled hybrid nanoflowers were incubated overnight in a vacuum oven for drying. The dried powder was employed again for the subsequent reaction cycle. The residual activity of the recycled catalase mimetic was then compared with the initial catalase-like activity of the
first cycle (100%). The SOD-like activities of the hybrid nanoflowers before and after 10 cycles were also determined in accordance with the above-mentioned assay.
CHAPTER 5

RESULTS AND DISCUSSION

Catalase Mimetic Activity by Dismutation of H$_2$O$_2$ and Evolution of O$_2$ Assay

Figure 5.1 H$_2$O$_2$ Depletion Catalyzed by PNA-Incorporated Nanoflowers

Figure 5.2 O$_2$ Evolution Catalyzed by PNA-Incorporated Nanoflowers
In order to evaluate the effect of the amount of PNA on catalase mimetic activity of PNA-incorporated nanoflowers, they were prepared at different PNA concentrations, ranging from 0.02 to 0.50 mg/mL. As evidenced from Figure 5.1, the concentration of hydrogen peroxide in the reaction solution continued to decrease with the extension of the reaction time. Accordingly, as illustrated in Figure 5.2, the concentration of oxygen was found to continuously increase with the accumulating reaction time. It is pertinent to note that the rate of H$_2$O$_2$ decay was about two times higher than that of O$_2$ evolution for every sample, which provides a direct evidence of the genuine catalase-like activity of nanoflowers. In the H$_2$O$_2$ depletion and O$_2$ evolution assays, PNA-nano-B demonstrated the optimal decay rate of H$_2$O$_2$ and evolution rate of O$_2$ as compared to other samples. The specific activities of the hybrid nanoflowers calculated according to the rate of H$_2$O$_2$ decay during the initial 1 min of the reaction time are listed in Table 5.1
The results supported the inference that PNA-nano-B possesses the optimal specific activity (1782.03 ± 37.60 µmol min⁻¹ mg⁻¹). It is for this reason that PNA-nano-B was used in subsequent experiments. In comparison with PNA-nano-A, PNA-nano-B exhibited a 1.77-fold increase in the specific activity. The results demonstrate that increasing the concentration of PNA will encourage an enhanced amount of nitroxide to bind to the HSA incorporated into the nanoflowers. Therefore, the 1.77-fold enhancement in the specific activity of PNA-nano-B should be attributed to the increased amount of nitroxide bound to HSA incorporated into the nanoflowers. Moreover, PNA-nano-B showed 3.05-fold and 10.87-fold increases in comparison to PNA-nano-C and PNA-nano-D, respectively.

<table>
<thead>
<tr>
<th>Nanoflower</th>
<th>Specific activity (µmol/min/mg)</th>
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<tbody>
<tr>
<td>PNA-nano-A</td>
<td>1004.02 ± 34.32</td>
</tr>
<tr>
<td>PNA-nano-B</td>
<td>1782.03 ± 37.60</td>
</tr>
<tr>
<td>PNA-nano-C</td>
<td>584.01 ± 29.88</td>
</tr>
<tr>
<td>PNA-nano-D</td>
<td>164.00 ± 32.45</td>
</tr>
</tbody>
</table>
Morphologies Study by SEM Images

As shown in the SEM images, PNA-nano-A and PNA-nano-B possess excellent branched, flower-like morphologies with a size of 4–10 μM (Figure 5.4 A–H). In contrast, PNA-nano-C and PNA-nano-D nanoflowers were found to lose their hierarchical structure, which plays an essential role in the mass transfer diffusion of substrate as well as the product in the enzymatic reaction (Figure 5.4 I–P). With regard to PNA-nano-C and PNA-nano-D, the mass transfer limitation of the substrate and product caused by the loss of hierarchical structure leads to a poor specific activity. Accordingly, it can be surmised that the catalase mimetic activity of PNA incorporated nanoflowers is contingent on the amount of nitroxide bound to PNA being incorporated into the nanoflowers and their hierarchical structures. No nanoflowers could be obtained (see Chapter 1 Nanoflower) for the corresponding sample without PNA, and there was no measurable catalase-like activity.
Confirmation of Nanoflower Components by FTIR

Figure 5.5 FTIR Spectra of Amorphous Cu$_3$(PO$_4$)$_2$ without PNA (a), PNA-Incorporated Nanoflowers (b), and PNA (c)

As shown by the curve (a) in Figure 5.5, the presence of the Cu$_3$(PO$_4$)$_2$ component was confirmed by the characteristic PO$_4^{3-}$ bands at 1053 cm$^{-1}$ and 556 cm$^{-1}$. [27] The existence of PNA was verified by the amide I and II bands of the protein at 1651 cm$^{-1}$ and 1542 cm$^{-1}$ in curve (c) of Figure 5.5. [28] In curve (b) of Figure 5.5, the presence of the characteristic PO$_4^{3-}$ bands and the amide I, as well as II protein bands, proves that PNA is present in the Cu$_3$(PO$_4$)$_2$ nanoflowers.
Demonstration of Active Centers of Nitroxides by EPR

![Figure 5.6 EPR Spectra of PNA (a) and PNA-Incorporated Nanoflowers (b)](image)

From Figure 5.6, it is evident that the nanoflowers exhibit characteristic nitroxide peaks in a magnetic field range of 3320–3360 G. As opposed to soluble PNA, a widening of the peak-valley (I, II, III) was observed for PNA-incorporated nanoflowers, which can be ascribed to the intermolecular interaction of nitroxides bound to the HSA incorporated into the nanoflowers.

Kinetic Assays of Catalase-like Activities

The kinetics of the catalase-like activity of PNA-incorporated nanoflowers was further analyzed based on apparent steady-state kinetics. The initial reaction velocities were calculated from the slopes of the \( \text{H}_2\text{O}_2 \) depletion and \( \text{O}_2 \) evolution assays. As shown in Figures 5.7 and 5.8, the velocities of \( \text{H}_2\text{O}_2 \) depletion and \( \text{O}_2 \) evolution gradually approach plateau when the concentration of PNA incorporated into nanoflowers remains constant, which accompanies the rising \( \text{H}_2\text{O}_2 \) concentration. This is consistent with the enzymatic characteristics of native catalase. [29]
Figure 5.7 Steady-State Kinetic Assay of PNA-Incorporated Nanoflowers. Velocity Indicates the Rate of H$_2$O$_2$ Depletion

Figure 5.8 Steady-State Kinetic Assay of PNA-Incorporated Nanoflowers. Velocity Indicates the Rate of O$_2$ Evolution
Figure 5.9 Lineweaver–Burk Plots of Reaction Velocity of PNA-Incorporated Nanoflowers as a Function of H$_2$O$_2$ Concentration Ranging from 3 to 60 mM. V Indicates the Rate of H$_2$O$_2$ Depletion

Figure 5.10 Lineweaver–Burk Plots of Reaction Velocity of PNA-Incorporated Nanoflowers as a Function of H$_2$O$_2$ Concentration Ranging from 3 to 60 mM. V Indicates the Rate of O$_2$ Evolution

On the Lineweaver–Burk double reciprocal plot, a good linear relationship can be observed between $V^{-1}$ and $[S]^{-1}$ (Figure 5.9 and Figure 5.10). The $K_m$ and $V_{max}$ values of PNA-incorporated nanoflowers were calculated using the slope and intercept of the line in Figure 5.9 and are depicted in Table 5.2. From Table 5.2, the $K_m$ value of the native catalase is found to be 1.64 times higher than that of PNA-incorporated nanoflowers. This, in turn, suggests that PNA-
incorporated nanoflowers have a 1.64-fold increase in affinity for H₂O₂ in comparison to the native catalase. The increased affinity can be ascribed to the presence of more active sites on the surface of PNA-incorporated nanoflowers when compared with the native catalase, which has four active sites per enzyme molecule. However, the Vₘₐₓ, Kₗ (S⁻¹), and Kₗ/Kₘ (M⁻¹S⁻¹) of the native catalase are observed to be 32.50, 15.89, and 10.00 times higher than those of PNA-incorporated nanoflowers, respectively. The lower Vₘₐₓ, Kₗ (S⁻¹), and Kₗ/Kₘ (M⁻¹S⁻¹) of these nanoflowers could be attributed to two reasons: (i) PNA-incorporated nanoflowers lack the shape and properties of the heme distal pocket of the native catalase; and (ii) the ratio of Cu ions in the nanoflowers to nitroxide bound to HSA that is incorporated into the nanoflowers is relatively low. This means that some Cu ions in the nanoflowers cannot be stimulated by nitroxide. Given that the absorbance of catalase at 280 nm would be expected to interfere with H₂O₂ detection, the O₂ evolution assay was performed in order to make a comparison between the activity of native catalase, PNA-incorporated nanoflowers, and HSA-incorporated nanoflowers. According to the findings, PNA-incorporated nanoflowers exhibit 5.21% catalytic activity relative to the native catalase, as well as a 32.3-fold increase in catalase-like activity compared with that of HSA-incorporated nanoflowers (Figure 5.11). It has been reported in the presence of heme iron, the nitroxide catalase-like activity relies on the cyclical conversion of ferryl hemoglobin/methemoglobin. In the existing experiment, a distinct difference in the catalase-like activity of the hybrid nanoflowers could be observed in the presence or absence of nitroxides. For this reason, we propounded that the close proximity of nitroxide to the Cu ion in the nanoflowers potentially accelerates the oxidative/reductive cycle occurring between Cu II and Cu I (Figure 5.12), which creates genuine catalase-like
activity in the nanoflowers. Moreover, the experimental data show that the activity of native superoxide dismutase is 3.37 times higher than that of soluble nitroxide (2,2,6,6-tetramethylpiperidine 1-oxyl) and that the SOD-like activity of the nitroxide encapsulated into the hybrid nanoflowers is the same as that of the soluble nitroxide.

Table 5.3 Comparison of Kinetic Parameters between PNA-Incorporated Nanoflowers and Native Catalase

<table>
<thead>
<tr>
<th>Catalysts</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmol/min/mg)</th>
<th>$K_{cat}$ (S$^{-1}$)</th>
<th>$K_{cat}/K_m$ (M$^{-1}$S$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA incorporated nanoflowers</td>
<td>17.41</td>
<td>$0.44 \times 10^4$</td>
<td>$3.78 \times 10^4$</td>
<td>$0.21 \times 10^7$</td>
</tr>
<tr>
<td>Catalase from bovine liver [30]</td>
<td>28.60</td>
<td>$1.43 \times 10^5$</td>
<td>$60.06 \times 10^4$</td>
<td>$2.10 \times 10^7$</td>
</tr>
</tbody>
</table>

Figure 5.11 O$_2$ Evolution Catalyzed by Native Catalase (1), PNA-Incorporated Nanoflowers (2), and HSA Incorporated Nanoflowers (3). For Each Reaction Condition, the Amounts of Sample Used for the Assay Were: 10 μL Native Catalase (0.001mg/mL), 50μL PNA-Incorporated Nanoflowers (0.01 mg/mL), and 50 μL HSA-Incorporated Nanoflowers (0.01 mg/mL). One Unit of Activity is Defined as the Evolution of 1 μmol/min of Oxygen at 25° C in Deionized Water.
Reusability

Figure 5.12 Reuse of PNA-Incorporated Nanoflowers

Figure 5.13 The Relative EPR Intensities of Nanoflowers Before (A) and After (B) Ten Continuous Cycles
The reusability of PNA-incorporated nanoflowers was studied across 10 continuous runs in order to examine their utility and value in practical applications. The results indicate that after 10 cycles, the PNA incorporated nanoflowers maintain 83.3% of their initial catalase mimetic activity (Figure 5.12), which is consistent with the change in the intensity of nitroxide bound to the HSA being incorporated into the nanoflowers on an EPR spectrum (Figure 5.13). In addition, no absorption related to protein or nitroxide could be detected in the supernatant that was collected from the reaction system at the end of each run. This implies that, during the reaction, PNA does not escape from the nanoflower matrix, and that there is no decomposition of the nitroxide from the HSA. Hence, it can be surmised that the 16.7% decrease in catalase mimetic activity during the continuous runs is attributed to the loss of PNA-incorporated nanoflowers caused by repeated washing. Moreover, after 10 cycles, the nanoflowers were still found to retain 83.5% of their initial SOD-like activity (Figure 5.14). The decline in SOD-like activity...
activity should therefore be attributed to the loss of nanoflowers during continuous operational use. The excellent reusability of PNA-incorporated nanoflowers implies that they can potentially be industrially amplified for use in removing ROS and in preventing oxidative stress in clinical applications. The excellent performance of hybrid nanoflowers in mimicking catalase and SOD sets the stage for a plethora of radical scavenging reactions. It can be further speculated that the hybrid nanoflowers could be leveraged as a prospectively useful tool in controlling oxidative stress; this is similar to the antioxidant properties of other nanomaterials, which have been already been investigated in existing studies. [32-37]

Statistical Analysis

The data obtained from the various experiments were plotted using Origin 8.5 and expressed as the mean ± standard error. Each value denotes the mean of data from three independent experiments that were performed in duplicate, with an average standard of deviation <5%. 
CHAPTER 6

CONCLUSIONS

In this study, PNA-incorporated nanoflowers with catalase and superoxide dismutase-like activities were fabricated based on the inspiration from nitrooxide-modified HSA. Cu$^{2+}$ with PNA, as the skeleton components of hybrid nanoflowers, is used to mimic catalase-like and SOD-like activities instead of Fe$^{3+}$. These PNA-incorporated nanoflowers not only showed a higher affinity for H$_2$O$_2$ than that of the native catalase, but also exhibited perfect reusability across 10 continuous cycles in catalase-like and superoxide dismutase-like reactions. Resultantly, this endows the nanoflowers with the potential to treat diseases associated with ROS. As long as HSA in the hybrid nanoflowers can be replaced by or covalently linked to other enzymes that are commonly used in medical diagnostics, the applications to which PNA-incorporated nanoflowers with dual enzyme-like activities can be applied and be greatly expanded. Furthermore, the reactive functional groups (thiol, amino, and carboxyl) offered by the protein encapsulated into these nanoflowers can be employed to bind several drugs and endogenous molecules. This phenomenon implies that nanoflowers can potentially be used as a viable promising carrier system for drug delivery. These promising features of PNA-incorporated nanoflowers should encourage us to further exploit their applications in medical sciences and H$_2$O$_2$ industrial pollution.
REFERENCES


