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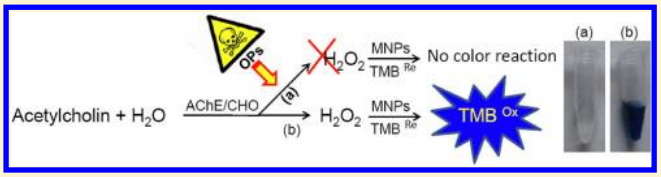
Fe_3O_4 Magnetic Nanoparticle Peroxidase Mimetic-Based Colorimetric Assay for the Rapid Detection of Organophosphorus Pesticide and Nerve Agent

Minmin Liang,[†] Kelong Fan,[†] Yong Pan,[‡] Hui Jiang,[‡] Fei Wang,[†] Dongling Yang,[†] Di Lu,[†] Jing Feng,[†] Jianjun Zhao,[‡] Liu Yang,[‡] and Xiyun Yan*[,†]

[†]Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

[‡]Research Institute of Chemical Defense, Beijing 102205, China

ABSTRACT: Rapid and sensitive detection methods are in urgent demand for the screening of extensively used organophosphorus pesticides and highly toxic nerve agents for their neurotoxicity. In this study, we developed a novel Fe_3O_4 magnetic nanoparticle (MNP) peroxidase mimetic-based colorimetric method for the rapid detection of organophosphorus pesticides and nerve agents. The detection assay is composed of MNPs, acetylcholinesterase (AChE), and choline oxidase (CHO). The enzymes AChE and CHO catalyze the formation of H_2O_2 in the presence of acetylcholine, which then activates MNPs to catalyze the oxidation of colorimetric substrates to produce a color reaction. After incubation with the organophosphorus neurotoxins, the enzymatic activity of AChE was inhibited and produced less H_2O_2, resulting in a decreased catalytic oxidation of colorimetric substrates over MNP peroxidase mimetics, accompanied by a drop in color intensity. Three organophosphorus compounds were tested on the assay: acephate and methyl-paraoxon as representative organophosphorus pesticides and the nerve agent Sarin. The novel assay displayed substantial color change after incubation in organophosphorus neurotoxins in a concentration-dependent manner. As low as 1 nM Sarin, 10 nM methyl-paraoxon, and 5 μM acephate are easily detected by the novel assay. In conclusion, by employing the peroxidase-mimicking activity of MNPs, the developed colorimetric assay has the potential of becoming a screening tool for the rapid and sensitive assessment of the neurotoxicity of an overwhelming number of organophosphate compounds.



Organophosphate (OP) neurotoxins are among the most toxic substances known. These compounds have been extensively used as pesticides and insecticides in modern agriculture and as chemical warfare agents in terrorist attacks or military activities.[1,2] According to statistics, about a billion pounds of OPs are released globally into the environment, our food, and water supplies each year, posing a great danger to human health, the ecosystem, and homeland security.[3] The acute toxicity of OPs is associated with their capacity to irreversibly inhibit the activity of acetylcholinesterase (AChE) in the central and peripheral nervous system, resulting in the accumulation of the neurotransmitter acetylcholine in the body, which can lead to organ failure and eventual death.[4] Therefore, there is an urgent demand for the rapid screening of OP compounds in the environment, public places, or workplaces to provide an early warning of sudden OP contamination or potential terrorist attack.

A number of analytical techniques are in use for OP determination in solution, such as liquid chromatography coupled with mass spectrometry (LC/MS),[5,6] gas chromatography (GC)/MS,[7] enzyme activity inhibition methods,[8] and several immunoassays.[9,10] Laboratory-based GC/LC/MS analytical techniques are commonly used and show detection limits in the nanomolar concentration range. However, these techniques are time-consuming, expensive, have to be performed by a highly trained technician, and not suitable for rapid analyses under field conditions, especially in emergency cases. For immunoassays, successful detection of low levels of OPs is challenged because of a lack of OP-specific antibodies. Enzyme activity inhibition methods have been developed as the most promising alternative to classical methods (GC/MS, LC/MS) for the faster and simpler detection of OPs. Acetylcholinesterase or butyrylcholinesterase are irreversibly inhibited by OP coumpounds, and a comparison of the enzyme activity before and after exposure to environmental samples can provide a detection of OPs. The enzyme activities are usually measured with the colorimetric Ellman assays,[11,12] radioactive assays,[13] fluorescence assays,[14] electrochemical assays,[15,16] or chemiluminescence.[17] However, these assays are not sensitive to the change of the enzyme activity after OP compound exposure and thus severely limit the detection sensitivity of these enzyme activity-based methods. In addition, optical detection assays such as fluorescence, chemilminescence, and electrochemiluminescence require complex and expensive optical imaging devices and sophisticated image-recognition software, which makes them unsuitable for a rapid on-site application. The Ellman's reagent usually results in a false-

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positive effect in a process called oximolysis and is subject to background perturbations.[11,12]

In previous studies, we first reported that Fe_3O_4 MNPs exhibit peroxidase activity that can catalyze the oxidation of peroxidase substrates in the presence of H_2O_2 to produce a color reaction.[18] Since reported in 2007, the novel catalytic property of MNPs has been widely used for laboratory tests, biomedical detection, and environmental analysis.[19−23] In this study, we make use of the novel property of Fe_3O_4 MNPs to detect OP pesticides and nerve agents in aqueous solution. The results show that our developed MNPs peroxidase mimetic-based method exhibited a sensitive response toward OPs exposure because of the highly effective catalytic activity of Fe_3O_4 MNPs.

EXPERIMENTAL SECTION

Reagents. All chemicals from commercial sources were of analytical grade. Acetylcholinesterase (AChE, from *Electrophorus electricus*), cholin oxidase (CHO, from *Alcaligenes* sp.), acetylcholine chloride (ACh), and 3,3,5,5-tetramethylbenzidine (TMB) were obtained from Sigma−Aldrich. Acephate and methy-paraoxon were from Huaerbo Chemical Reagent Co., (Beijing, China). $FeCl_3{\cdot}6H_2O$, ethylene glycol, acetic acid, and sodium acetate were purchased from Beijing Chemical Reagents (Beijing, China). Deionized water prepared by a Millipore system was used throughout the experiments.

Preparation of Fe_3O_4 MNPs. Fe_3O_4 MNPs were prepared as described previously.[23] Briefly, $FeCl_3{\cdot}6H_2O$ (0.6 g) was dissolved in 20 mL of ethylene glycol followed by addition of 1.5 g of sodium acetate. The mixture was stirred vigorously for 30 min, sealed in an autoclave, and heated at 200 °C for 30 h. The obtained Fe_3O_4 MNPs were collected by washing several times with ethanol and dried at 60 °C. Finally, the products were dispersed into water and stored at 4 °C for use.

The prepared MNPs were characterized with transmission electron microscopy (TEM, Tecnai F20, Philips), and the size was determined to be approximately 300 nm.

MNPs Peroxidase Mimetic-Based Colorimetric Assay. The colorimetric assay was constructed as follows: 50 μg of Fe_3O_4 MNPs were first mixed with 10 μM AChE, 1.2 mg/mL CHO, and 5 mM ACh in 500 μL acetate buffer (0.2 M, pH 5.0) using TMB as the colorimetric substrate. After incubation for 15 min at room temperature in the dark to allow color development, 2 M H_2SO_4 was added to stop the reaction. The Fe_3O_4 MNPs were then removed from the reaction solution by an external magnetic field. The absorbance of the resulting assay solution at 450 nm was determined on a Benhmark Plus microplate spectrophotometer (Bio-Rad Laboratories).

Measurement of OP Pesticides and Nerve Agents. OP compounds inhibit the activity of the AChE enzyme and induce a decreased color reaction over the MNP peroxidase mimetic-based assay. In the present study, OP pesticide acephate, methy-paraoxon, and nerve agent Sarin were determined by measuring the decrease in the color intensity catalyzed by the MNP peroxidase mimetics in the presence of the enzymes AChE and CHO. AChE at 10 μM was first incubated with various concentrations of acephate, methy-paraoxon, or Sarin in 50 μL of acetate buffer (0.2 M, pH 5.0) for 15 min at room temperature. The reaction solution was then added to the assay solution of MNPs (0.1 mg/mL), CHO (1.2 mg/mL), ACh (5 mM), and TMB (0.1 mg/mL) dissolved in 450 μL of acetate buffer (0.2 M, pH 5.0). The mixed solution was incubated for another 15 min in the dark at room temperature to allow color development. The reaction was then stopped by addition of 2 M of H_2SO_4. The MNPs were removed from the assay solution by application of an external magnetic field. The absorbance of the final reaction solution at 450 nm was determined.

Safety Considerations. The Sarin study was performed in the laboratory of the Research Institute of Chemical Defense (RICD). Storage with Sarin was carried out under special technical conditions. Tubes with stock Sarin was kept in boxes filled with active carbon. Manipulation and experiments were carried out in fume chambers.

RESULTS AND DISCUSSION

Detection Method. We present herein a MNP peroxidase mimetic-based colorimetric assay to detect OP compounds. The assay is composed of Fe_3O_4 MNPs and the enzymes AChE and CHO. The enzymes AChE and CHO catalyze the formation of H_2O_2 in the presence of acetylcholine (see eqs 1 and 2). The formed H_2O_2 then activates MNPs to catalyze the oxidation of colorimetric substrates to produce a color reaction (see eq 3), which is visible and can be quantitatively monitored by spectrophotometric absorption. After incubation with the OP compounds, the enzymatic activity of AChE is inhibited and produces less H_2O_2, which results in a decreased catalytic oxidation of colorimetric substrates over MNP peroxidase mimetics, accompanied by a drop in color intensity. Therefore, the color reaction catalyzed by the MNP peroxidase mimetics coupled with the enzymatic activity of AChE is expected to be a feasible measure of OP compounds.

$$\text{acetylcholin} + H_2O \xrightarrow{\text{AChE}} \text{choline} \quad (1)$$

$$\text{choline} + O_2 \xrightarrow{\text{CHO}} H_2O_2 \quad (2)$$

$$H_2O_2 + \text{TMB} \xrightarrow{\text{MNPs}} \text{oxidized TMB} \quad (3)$$

To establish the validity of the MNP peroxidase mimetic-based colorimetric assay, the following experiments were carried out. MNPs at 0.1 mg/mL were reacted with 10 μM AChE, 5 mM ACh, 1.2 mg/mL CHO, and 0.1 mg/mL TMB in 0.2 M pH 5.0 acetate buffer. After the reaction, MNPs were removed from the reaction solution by an external magnet, and the color absorbance at 652 nm was measured. As shown in Figure 1, MNPs catalyzed the oxidation of the colorimetric substrate TMB to give an intense color reaction in the presence of enzymes AChE and CHO after incubation in acetylcholine chloride solution, indicating the highly catalytic oxidation

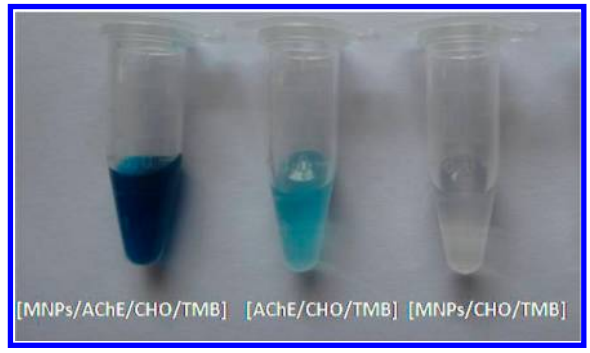

Figure 1. MNPs catalyzed the oxidation of colorimetric substrate TMB to produce a color reaction in the presence of AChE and CHO after incubation in acetylcholine chloride solution.

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activity of MNPs toward peroxidase substrates after activated by H_2O_2 generated by the AChE and CHO enzyme reactions. The produced color intensity of the reaction solution was dependent on the enzymatic activity of AChE (Figure 2),

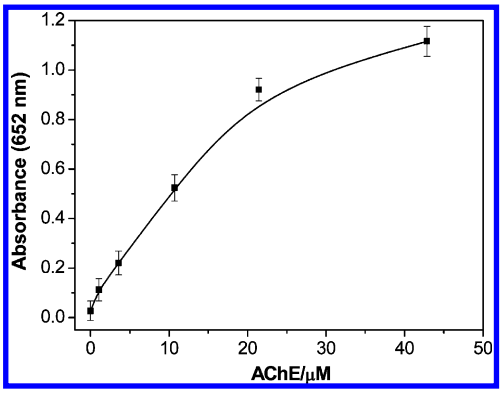

Figure 2. Color reaction of the MNP peroxidase mimetic-based colorimetric assay is AChE dose dependent. The error bars represent the standard deviation of three measurements.

demonstrating that the developed assay can be used to detect OP neurotoxins that inhibit AChE activity in a dose-dependent manner. The assay solution without MNPs demonstrated little color reaction because of the low oxidative capacity of H_2O_2 (Figure 1, middle). No color reaction was observed in the absence of enzyme AChE (Figure 1, right).

In the experiment, a 0.2 M pH 5.0 acetate solution was used as reaction buffer for the developed assay considering Fe_3O_4 MNPs has high catalytic activity in the pH range 3.0−5.0.[18] Although this acidic solution is not normally used for the AChE and CHO catalyzed H_2O_2 generation reaction, the pH 5.0 acetate buffered assay solution permitted the achievement of enough color intensity from the MNP-catalyzed color reaction to carry on the OP neurotoxin inhibition reaction (Figure 1 left). The results suggest that, even at the acidic pH, the activity of enzymes AChE and CHO is high enough to generate sufficient H_2O_2 for the MNP-catalyzed color reaction. Therefore, 0.2 M pH 5.0 acetate buffer was chosen for the assay reaction.

OP Pesticides and Nerve Agents Detection. To explore the feasibility of the MNP peroxidase mimetic-based colorimetric assay, two of the most commonly used OP pesticides methy-paraoxon and acephate and OP nerve agent representative Sarin were investigated. Given that OP compounds are fairly unstable at high pH (for example, the half-life of Sarin is 2 min at pH 10.0 and dramatically increases to approximately 8 days at pH 5.0),[24,25] pH 5.0 acetate buffer was used for OP compound incubation reaction. In the experiment, the enzyme AChE was incubated with various concentration of methyl-paraoxon, acephate, or Sarin in acetate buffer (0.2 M, pH 5.0) for 15 min at room temperature and then mixed with the developed MNP peroxidase assay solution as described above. After the reaction, the color intensity of the resulting solution was measured after removing the MNPs by a magnetic field.

Figures 3A and 4A show the dose-dependent responses of methyl-paraoxon and acephate, and Figures 3B and 4B show the corresponding semilogarithmic plots. As can be seen, the color intensity decreased as the concentration of OP pesticides increased, revealing a dependence of the color reaction on the OP pesticide concentration. Presumably, H_2O_2 is generated in the presence of both AChE and CHO in the acetylcholin solution, inducing colorimetric substrate TMB oxidation over MNP peroxidase mimetrics. After OP exposure, AChE activity is inhibited and produces much less H_2O_2 than the intact AChE enzyme, resulting in a decreased catalytic oxidation of colorimetric substrates over MNP peroxidase mimetics and a drop in the color intensity. On the basis of the results, the detection limit for the acephate and methyl-paraoxon are 5 μM and 10 nM (3 times the standard deviation of the response obtained for a blank), respectively, which is environmentally more relevant than the previously reported AChE-based Ellman colorimetric assays.[26] The final color absorbance of the assay solution after methyl-paraoxon exposure is much lower than that after acephate exposure, suggesting that methyl-paraoxon induced much more severe damage than acephate, which is consistent with the known neurotoxicity of these two OP pesticides.[27]

OP nerve agents (Sarin, Soman, Tabun, and VX) are extremely toxic and have been effectively used in warfare and terrorist attacks because they are fairly easy to manufacture and only low cost and simple technology is required for their synthesis.[28] Therefore, there are urgent needs for rapid and reliable methods for the determination of OP nerve agents. However, in literature, there are only a few papers that report the detection of the OP nerve agents because it is difficult to work with them in a nonspecialized laboratory. Nerve agent mimics such as dimethyl methyl phosphonate (DMMP), diethylcyanophosphonate (DCNP), or diisopropylfluorophos-

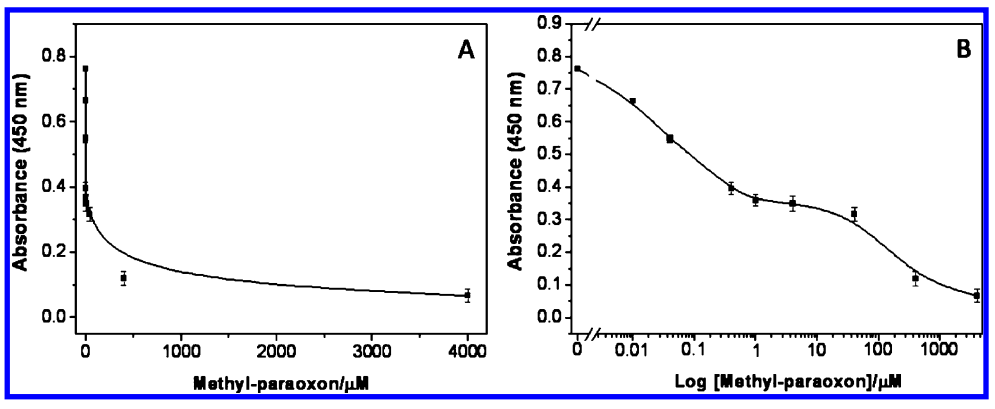

Figure 3. (A) Dose−response curve for methyl-paraoxon detection using the MNP peroxidase mimetic-based colorimetric assay. (B) Semilogarithmic plot of the data in panel A. The error bars represent the standard deviation of three measurements.

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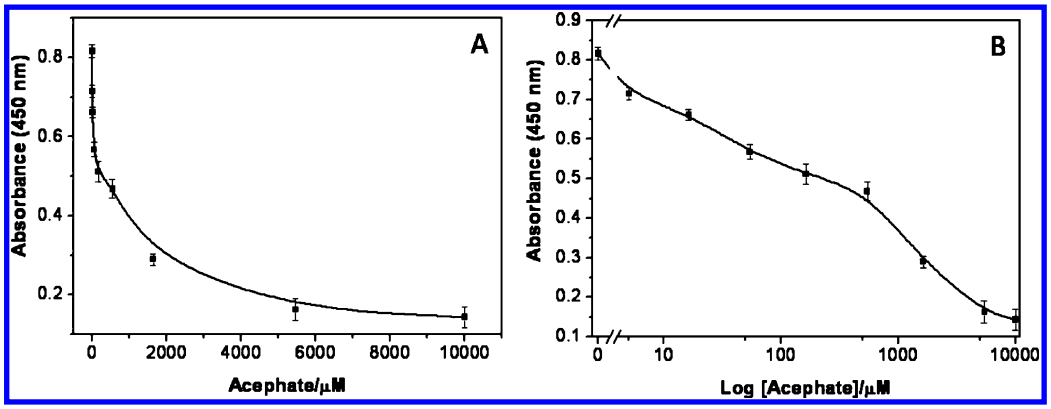

Figure 4. (A) Dose−response curve for acephate detection using the MNP peroxidase mimetic-based colorimetric assay. (B) Semilogarithmic plot of the data in panel A. The error bars represent the standard deviation of three measurements.

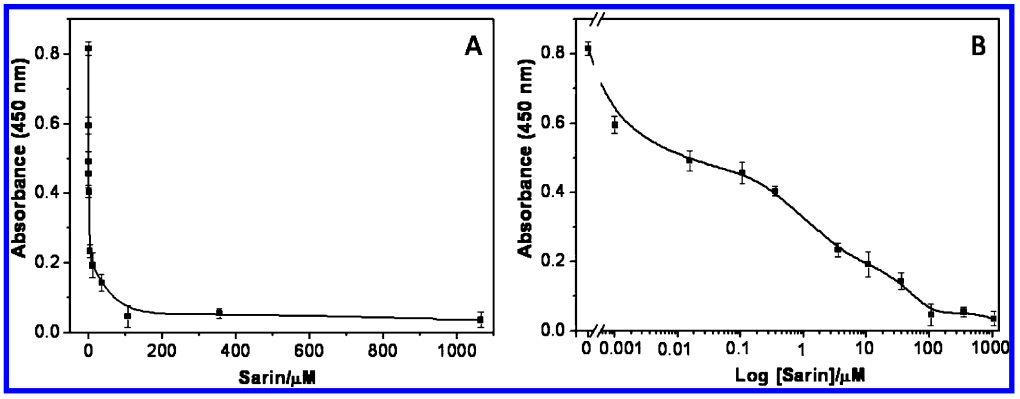

Figure 5. (A) Dose−response curve for Sarin detection using the MNP peroxidase mimetic-based colorimetric assay. (B) Semilogarithmic plot of the data in panel A. The error bars represent the standard deviation of three measurements.

phate (DFP) are generally tested.[29,30] In this work, Sarin was studied as a representative of OP nerve agents. The stock solutions of Sarin were prepared using appropriate safety conditions created in the laboratory of RICD. Storage and manipulation with Sarin were carried out under special technical conditions.

Considering the problem of hydrolysis, Sarin solutions were prepared fresh before each measurement. Thus, under our experimental conditions (pH 5.0) and time scale (15 min), the hydrolysis of Sarin can be neglected. Figure 5A shows the Sarin concentration−response curve, and Figure 5B shows their semilogithmic plots where as low as 1 nM of Sarin could be easily detected. This concentration is well under the permissible value for a short-term ingestion of military field drinking water by soldiers (10 μg Sarin/L water).[31] Thus, OP-containing samples can be analyzed at concentrations well below the hazardous level. Furthermore, the detection limit is better than that achieved with the AChE enzyme activity-based methods[32,33] or the immunoassays[34] and the chromatographic methods.[35] The obviously better detection sensitivity can be explained by the highly effective catalytic activity of Fe_3O_4 MNPs, which respond sensitively to the change of the enzymatic activity of AChE after OP exposure. In addition, the developed assay is lower in cost and more convenient to implement as the MNPs can be mass produced and easily separated from assay solution when compared with other colormetric assays that usually use expensive and unstable organic dyes as indicators.[11,12] These results suggest that the MNP peroxidase mimetic-based colorimetric method could be a potential organophosphorus assay with high detection sensitivity and practicability.

■ CONCLUSIONS

In conclusion, we have demonstrated the feasibility of using the MNP peroxidase mimetic-based colorimetric assay in the detection of OP neurotoxins in aqueous solution. As shown above, OPs were monitored rapidly and sensitively with the detection limit of 10 nM for methy-paraoxon, 5 μM for acephate, and 1 nM for Sarin. The developed MNPs peroxidase assay is more sensitive when compared with the traditional enzyme activity-based methods because of the highly effective catalytic activity of MNPs. More importantly, the developed MNP peroxidase assay is convenient to implement and requires only a simple, inexpensive detection apparatus, and thus, it is practical for rapid field detection. With further development, the assay can be configured into an array format for the rapid, low-cost, and large-scale field screening of OP neurotoxins.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86 10 6488 8583; fax: +86 10 6488 8584; e-mail: yanxy@ibp.ac.cn.

Notes

The authors declare no competing financial interest.

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