Analyst

PAPER

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2014, **139**, 5466

A phosphorylation-sensitive tyrosine-tailored magnetic particle for electrochemically probing free organophosphates in blood

Yanmei Si, Ning Zhang, Zongzhao Sun, Shuai Li, Liyang Zhao, Rui Li and Hua Wang*

A simple, rapid, sensitive, selective, and field-deployable detection protocol has been initially proposed for the early warning and diagnosis of exposure to organophosphates (OPs) by electrochemically monitoring the direct biomarkers of free OPs in blood. Phosphorylation-sensitive tyrosine (Tyr), which was tested with unique electroactivity, was bound onto Fe_3O_4 particles mediated by the mussel-inspired dopamine to form Fe_3O_4@Tyr particles with well-defined shape and well-retained Tyr electroactivities, as characterized separately by electron microscopy and electrochemical measurements. A "lab-on-a-particle"-based detection procedure combined with a magnetic electrode was thus developed by employing Fe_3O_4@Tyr particles as capturing probes for detecting free OPs in blood, dimethyl-dichloro-vinyl phosphate (DDVP) as an example. A significant difference in electrochemical responses could be obtained for Fe_3O_4@Tyr particles before and after DDVP exposure, based on the phosphorylation-induced inhibition of electroactivities of loaded Tyr. Investigation results indicate that highly specific and sensitive phosphorylation for the inhibition of Tyr electroactivities by sensitive electrochemical outputs could endow the OP detection with high selectivity and sensitivity (*i.e.*, down to about 0.16 nM DDVP in blood). Moreover, strong and stable Tyr–OP bindings especially irreversible electrochemical oxidization of the Tyr probe could facilitate the OP evaluation with high reproducibility and stability over time. In particular, the simple "lab-on-a-particle"-based detection procedure equipped with a portable electrochemical transducer can be tailored for the field-deployable or on-site monitoring of the exposure to various nerve agents and pesticides.

Received 14th June 2014
Accepted 6th August 2014

DOI: 10.1039/c4an01074d

www.rsc.org/analyst

1. Introduction

Neurotoxic organophosphates (OPs) including pesticides and chemical nerve agents have been widely used in the agricultural industry and chemical warfare.[1,2] According to statistics, there are at least 13 types of OPs and hundreds of OP compounds in use, which are derivatives of phosphoric, phosphonic, or phosphinic acids.[3] As a result, there is potential of exposure for humans and animals. Moreover, after a number of terrorist attacks such as the Tokyo subway attack in 1995 and the tragic events of September 11, 2001, the fatal damage of nerve agents has been realized. The need for more effective methods for early warning of potential terrorist attacks and for the rapid screening of OPs in air, water, soil, and food has thus become increasingly urgent.[3–5]

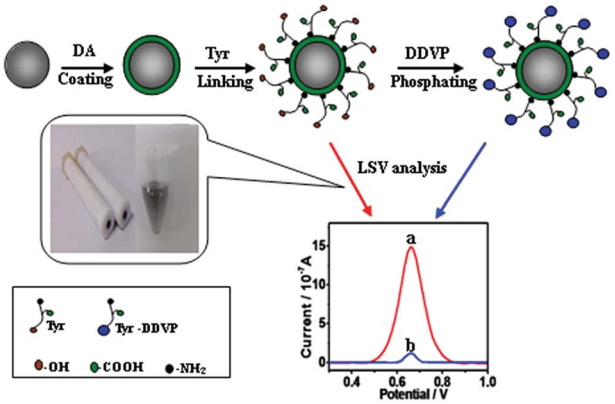
It is generally recognized that selecting suitable biomarkers of OP exposure is of central importance for developing an efficient strategy to prove the use of a chemical agent and find its application in diagnosis to ensure that appropriate medical countermeasures are administered. The exposure of OP agents with proteins and enzymes such as butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) in the biological matrix will produce four types of different kinds of biomarkers, including free OP, inhibited cholinesterase (ChE), phosphorylated adducts and their hydrolyzed metabolites.[6] Obviously, free OPs in blood are the most direct and accurate biomarkers for OP identification for warning of the exposure to pesticides and chemical warfare agents. To date, many methods, such as liquid chromatography (LC), gas chromatography (GC), and mass spectrometry (MS), have been employed for the determination of free OPs and multiple analogues with high sensitivity and specificity.[7–10] However, they may be limited by the need for expensive and complex analysis settings, well-trained personnel, and inconvenience for rapid field applications. Alternatively, recent years have witnessed the detection of phosphorylated adducts and enzyme (*i.e.*, BChE and AChE) activities as the indicators of OP exposure.[11] Nevertheless, the majority of current detection methods of OP exposure might encounter some formidable disadvantages. For example, phosphorylated adducts like enzymes were examined as the indicators of OP exposure, a challenge might lie in the interference of complicated sample backgrounds and unavailability

Shandong Province Key Laboratory of Life-Organic Analysis, School of Chemistry and Chemical Engineering, Qufu Normal University, Qufu City, Shandong Province 273165, P. R. China. E-mail: huawangqfnu@126.com; Fax: +86 537 4456306; Tel: +86 537 4456306

of appropriate recognition elements or receptors (*i.e.*, antibodies) for targeting the phosphorylated adducts.[12] As a hot choice, much effort has been devoted to the biological monitoring approaches based on the OP inhibition of catalysis activities of enzymes, mostly known as blood cholinesterase of BChE and AChE.[13] Gracefully successive as these methods are, their detections of low-level OPs may still require individual baseline measurements before the meaningful changes of enzyme activities can be measured; especially some of the results are non-specific for the cause of cholinesterase inhibition in occupational health monitoring.[14] In addition, these OP exposure evaluation methods may suffer from a long analysis time and expensive sample preparation and multiple washing steps.[15,16] Hence, the development of simple, sensitive, selective, and field-deployable tools is highly desired for biomonitoring and diagnostic evaluation of OP exposure, especially to enhance our response to a sudden emergency and our ability to medically counteract the effects.

Moreover, the biochemical targets for organophosphorus nerve agents in humans or animals involve some special proteins (*i.e.*, serum albumin) and enzymes such as BChE, AChE, serine esterase, trypsin, and chymotrypsin.[17–21] The phosphorylated sites of them *in vivo* generally include serine (Ser), threonine (Thr) residues, and tyrosine (Tyr) residues.[22–24] For example, the abundant blood protein albumin can be easily phosphorylated at the Tyr residue.[14,22] These sets of evidence indicate that Tyr should conduct a strong interaction with the OPs, serving as an ideal receptor for free OPs in blood for early warning of OP exposure.

Over the past decades, electrochemical assays have developed rapidly with high analysis selectivity and sensitivity, especially in combination with various nanoscale materials.[25–29] More importantly, the merits of simple operation, fast detection, and miniaturized analysis instruments of these detection devices meet the need for rapid field detection applications. In this work, we seek to develop a novel electrochemical sensing method to probe the free OPs in human blood based on the special interactions of OPs with the meaningful amino acids (Ser, Thr and Tyr), aiming to circumvent the current drawbacks in the detection of phosphorylated targets for warning of OP exposure. Herein, the electrochemical activities of the amino acids above have been screened, and only Tyr showed the electro-activity. A difference in electrochemical responses can be expected for Tyr before and after the OP inhibition. Moreover, considering the interference of the complex components in blood samples, magnetic particles were employed as the carriers for loading Tyr, resulting in Fe_3O_4@Tyr particles. A "lab-on-a-particle"-based detection protocol has thereby been proposed with a magnetic electrode for probing OPs in blood, dimethyl-dichloro-vinyl phosphate (DDVP) as an example, based on the phosphorylation-induced inhibition of electroactivities of Tyr. The detailed electrochemical detection procedure is illustrated in Scheme 1. To the best of our knowledge, this is the first report of a "lab-on-a-particle"-based sensing method by the phosphorylation-induced inhibition of electrochemical activities of Tyr on magnetic particles for the early warning of OP exposure through monitoring the direct biomarkers of free phosphorus agents in blood.



Scheme 1 Schematic illustration of Fe_3O_4@Tyr particle-based electrochemical detection of free OPs using magnetic electrodes, based on the phosphorylation-induced inhibition of Tyr electro-activity on Fe_3O_4@Tyr particles.

2. Experimental

2.1 Reagents and apparatus

Tyrosine (Tyr), serine (Ser), threonine (Thr), dopamine (DA), vitamin C (Vc), alanine (Ala), glycine (Gly), arginine (Arg), phenylalanine (Phe), aspartic acid (Asa), and human serum albumin (HSA) were purchased from Sigma-Aldrich (Beijing, China). Tri-hydroxymethyl aminomethane (Tris) was obtained from Sinopharm Chemical Reagent Co. (China). Organophosphorus (OP) agents of dimethyl-dichloro-vinyl phosphate (DDVP), methidathion (Met), and paraoxon (Par) were provided by Dibai Reagents (Shandong, China). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), glycol, sodium acetate and all other reagents were of analytical grade. Deionized water (>18 Mohm) used was obtained from an Ultra-pure water system (Pall, USA). The OP stock solutions, DDVP as a representative example, were simply dissolved in acetone and diluted to different concentrations.

Electrochemical measurements were conducted with an electrochemical workstation CHI760D (CH Instrument, Shanghai, China) connected to a personal computer. A three-electrode system was applied consisting of a glassy carbon working electrode with a magnetic core (Incole Union Technology, Tianjin, China), a Pt wire counter electrode, and an Ag/AgCl reference electrode. Characterization processes of the as-prepared materials were performed by using scanning electron microscopy (SEM, Hitachi E-1010, Japan) and transmission electron microscopy (TEM, FEI Tecnai G20, USA).

2.2 Synthesis and characterization of magnetic Fe_3O_4@Tyr particles

Magnetic Fe_3O_4 particles were prepared according to a modified synthesis procedure reported previously.[30] Briefly, 5.40 g of $FeCl_3 \cdot 6H_2O$ was dissolved in 40 mL of glycol to form a clear

solution. Following that, 3.28 g of anhydrous sodium acetate was added and vigorously mixed by ultra-sonication to give a homogeneous solution. Furthermore, the mixture was transferred into a Teflon-lined stainless steel autoclave for hydrothermal treatment at 200 °C for 12 h. Subsequently, the autoclave was cooled down to room temperature and the precipitate was magnetically collected and washed several times with water and ethanol by sonication. The so obtained Fe_3O_4 particles were then dried under vacuum and stored.

An aliquot of 5.0 mg Fe_3O_4 particles was added to 5.0 mL Tris-HCl buffer (pH 7.4) containing 20 mg DA and mixed by sonication for 5.0 min and then incubated overnight. The so prepared DA-modified Fe_3O_4 particles were magnetically washed three times and then diluted to 4.5 mL with Tris-HCl buffer. After that, 0.5 mL 25% glutaraldehyde and 18.0 mg Tyr (dissolved in NaOH) were added to the DA-modified Fe_3O_4 suspension and further sonicated for 5.0 min. The resulting mixture was finally incubated for 2 h, and then rinsed three times with Tris-HCl buffer to form Fe_3O_4@Tyr particles, stored at 4 °C for future use.

The so yielded Fe_3O_4@Tyr particles were sonicated for 30 min, and then characterized by SEM and TEM imaging. In addition, the electrochemical voltammetric characterization of the stepping process for the Fe_3O_4@Tyr setup was conducted using magnetic electrodes, which was performed following the electrochemical analysis procedure below.

2.3 Fe_3O_4@Tyr particle-based electrochemical measurements

An aliquot of Fe_3O_4@Tyr particles was added to the plastic tubes (0.5 mL), and OP samples with different concentrations were separately introduced and reacted for 20 min. Then, each of the reaction suspensions were magnetically separated and washed three times. 5.0 μL of the resulting magnetic mixture was added to the surface of magnetic working electrodes, which were regenerated after use by polishing procedures. Electrochemical measurements were performed in Tris-HCl buffer for each of the as-modified electrodes by linear sweep voltammetry (LSV), scanning at a potential range of 0.2–1.0 V at a scanning speed of 100 mV s^{-1}. A baseline correction of the resulting voltammograms was performed using CHI software.

Besides, the control tests for the selectivity investigation of Fe_3O_4@Tyr particle-based electrochemical responses to DDVP were conducted in the same way, comparing to Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Vc, Ala, Gly, Arg, Phe, Asa, HSA, Met, and Par.

3. Results and discussion

3.1 Comparison of electrochemical activities of three OP-sensitive amino acids

It is well established that the attacking sites of organic phosphorus for proteins (*i.e.*, serum albumin) and enzymes (*i.e.*, AChE and BChE) mainly include Ser, Thr and Tyr. In the present work, the electrochemical activities of free three amino acids in solutions are investigated (Fig. 1). Fig. 1A shows that Tyr has a significant peak of oxidation current at about 0.67 V, in contrast to Ser and Thr with no obvious redox peaks. It is thought that the electro-oxidation of Tyr residues involves two-electron and two-proton transfer, the electrode process of which is similar to that of *p*-substituted phenols.[31–34] Ser and Thr, however, do not have a relevant structure to obtain obvious electrochemical signals in the experiment. Further investigations indicate that the peak currents of Tyr could be reduced depending on different concentrations of exposed organophosphorus (Fig. 1B). Herein, organic phosphorus can react with phenol hydroxyl of Tyr,[14] that is, after OP exposure, the phosphorylated phenol group on Tyr would not be electrochemically oxidized to give the corresponding signals. Such a finding suggests that Tyr may be used as the phosphorylation receptor for probing free OPs in blood by the electrochemical analysis.

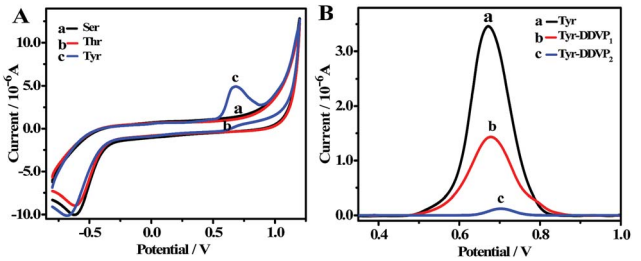


Fig. 1 Initial investigation of electrochemical activities of three OP-sensitive amine acids. (A) LSV responses of Ser, Thr, and Tyr of 1.0 mM in Tris-HCl buffer; (B) LSV responses of 80 μM Tyr (control) in the presence of 695 nM and 1135 nM DDVP in Tris-HCl buffer.

3.2 Characterization of Fe_3O_4@Tyr particles

The as-prepared Fe_3O_4@Tyr particles were systemically characterized (Fig. 2). The scanning electron microscopy (SEM)

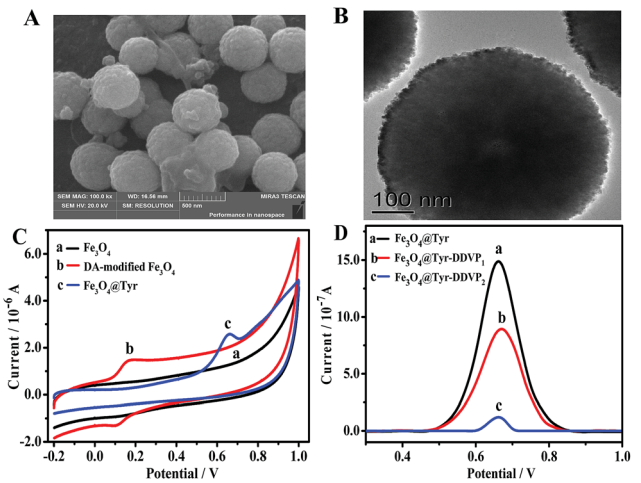


Fig. 2 Characterization of Fe_3O_4@Tyr particles by (A) SEM image, (B) TEM image, (C) electrochemical voltammetric responses of Fe_3O_4 particles, DA-modified Fe_3O_4 particles, and Fe_3O_4@Tyr particles of 1.67 mg mL^{-1}, and (D) LSV responses of Fe_3O_4@Tyr particles before and after treatment with 2.5 nM and 500 nM DDVP for 20 min.

image shows that Fe_3O_4@Tyr particles displayed a defined spherical shape but a scabrous surface with an average size of about 500 nm (Fig. 2A), which is also manifested in the transmission electron microscopy (TEM) image (Fig. 2B).

Moreover, the electrochemical characterization of the stepping setup process for Fe_3O_4@Tyr particles was conducted comparably using magnetic electrodes, with results shown in Fig. 2C. One can find that the electrode with Fe_3O_4 particles presents no electrochemical properties. A couple of redox peaks at 0.10–0.20 V were witnessed for the one with DA-modified Fe_3O_4 particles, indicating the stable coatings of DA onto magnetic particles. Importantly, the electrode with Fe_3O_4@Tyr particles could exhibit a well-defined irreversible oxidation peak at 0.67 V of Tyr. It thus verifies that Tyr was successfully attached onto DA-modified Fe_3O_4 particles with well-retained electrochemical activity, presumably resulting from the mussel-inspired biological compatibility of DA coating particles. However, the redox peaks of DA might disappear after being activated by glutaraldehyde that could cause the chemical change of its electroactive groups. Furthermore, Fe_3O_4@Tyr particles were added into the DDVP solutions with different concentrations for phosphorylation, and further magnetically separated and attached onto the magnetic electrodes for electrochemical LSV measurements (Fig. 2D). It is noted that the peak current of Tyr on Fe_3O_4@Tyr particles decreased significantly with increasing OP content. Of note, the phosphorylation of the Tyr residue is considerably stable with no aging,[14,22] and Tyr on Fe_3O_4@Tyr particles can be electrochemically oxidized with irreversible characteristics, as confirmed elsewhere.[31] Therefore, based on the OP inhibition of Tyr electroactivity of Fe_3O_4@Tyr particles, a "lab-on-a-particle" protocol can be established to facilitate the monitoring of OP exposure with high sensitivity, selectivity, and stability afterwards.

3.3 Electrochemical sensing procedure for OPs with Fe_3O_4@Tyr particles

The OP inhibition of Tyr electroactivities was employed in combination with electrochemical outputs to determine the content of free organic phosphorus in blood. The main detection procedure is illustrated in Scheme 1. Herein, considering the interference from the complex components in blood, magnetic separation has also been introduced by using magnetic particles as carriers to load the OP-sensitive Tyr. DA was modified onto Fe_3O_4 particles, followed by the linking of Tyr through glutaraldehyde cross-binding chemistry. The so formed Fe_3O_4@Tyr particles were used to capture free OP, DDVP as a model, from the blood. The DDVP-induced phosphorylation of Tyr on Fe_3O_4@Tyr particles (Fe_3O_4@Tyr-DDVP) was then electrochemically measured compared to original Fe_3O_4@Tyr particles with magnetic electrodes. An obvious difference in signals of linear sweep voltammetry (LSV) was obtained for the particles before and after DDVP treatment, thus profiling the "lab-on-a-particle" detection procedure for electrochemically monitoring OP exposure.

3.4 Optimization of detection conditions of Fe_3O_4@Tyr particle-based electrochemical assays for OP exposure

The detection conditions of Fe_3O_4@Tyr particle-based electrochemical assays for OP exposure are optimized (Fig. 3). The amounts of Fe_3O_4@Tyr particles were first investigated for the electrochemical Tyr responses to DDVP (Fig. 3A). As shown in Fig. 3A, Tyr responses could increase with the increasing amounts of Fe_3O_4@Tyr particles. Interestingly, too high concentrations of Fe_3O_4@Tyr particles might lead to greatly decreased signals presumably due to too high density of nonconductive particles on the electrode surfaces. Accordingly, 1.67 mg mL^{-1} of Fe_3O_4@Tyr particles was chosen in the experiments. Moreover, the pH values can be another important parameter for OP detection (Fig. 3B). One can see that the highest response was observed at pH 7.4. Herein, harsh acid or base solutions might either induce the instability of Fe_3O_4 particles (including their loadings) or cause the rapid decomposition of DDVP. Also, the temperature for phosphorylation reactions could exert an influence on OP monitoring (Fig. 3C), and 25 °C is selected as the optimum reaction temperature. In addition, the interactions between DDVP with Tyr on Fe_3O_4@Tyr particles could depend on the reaction time (Fig. 3D). Apparently, the current changes increased with the increasing reaction time and tended to be steady after 20 min, upon which the reaction was stopped by magnetic separation. Such a reaction time is thus chosen to achieve the sufficient DDVP phosphorylation of Fe_3O_4@Tyr particles.

3.5 Detection selectivity and stability for electrochemical responses to DDVP with Fe_3O_4@Tyr particles

To determine the levels of OPs in complicated samples like blood, the potential interfering substances including common ions, vitamins, and amino acids with similar levels in blood were electrochemically tested (Fig. 4A). Compared with the

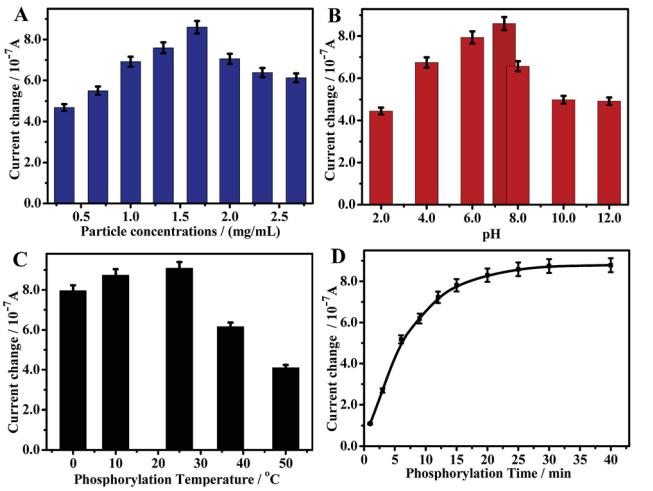


Fig. 3 Effects of experimental conditions on electrochemical responses of Fe_3O_4@Tyr particles to DDVP (15 nM) by using (A) amounts of Fe_3O_4@Tyr particles, (B) pH values, (C) phosphorylation temperature, and (D) phosphorylation time.

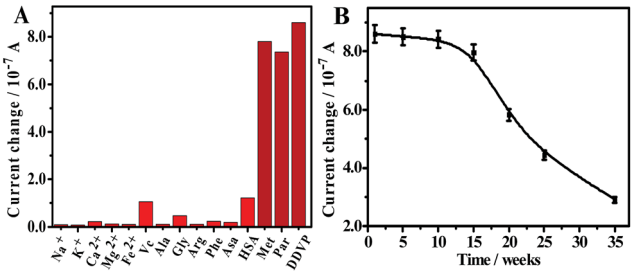


Fig. 4 (A) The detection selectivity of Fe_3O_4@Tyr particle-based electrochemical responses toward OPs (including Met, Par, and DDVP of 15 nM), Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Vc, Ala, Gly, Arg, Phe, Asa, and HSA, each with the concentrations similar to the levels in blood; (B) the detection stability of electrochemical responses to DDVP for the electrodes modified with Fe_3O_4@Tyr particles stored over time.

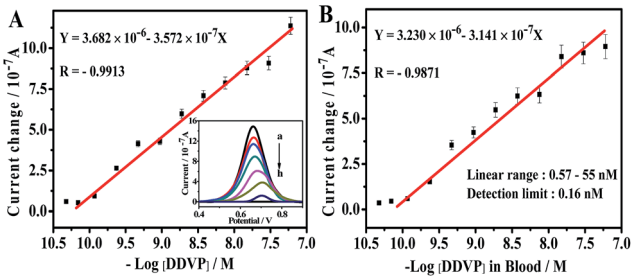


Fig. 5 Calibration curves of the relationships between the Fe_3O_4@Tyr particle-based electrochemical responses and DDVP of different concentrations in (A) water (inset: real-time LSV response profiles), and (B) blood, where the inserted a–g curves correspond to 0.0, 0.235, 0.470, 1.88, 15.00, 60.00, and 500 nM DDVP, and the h curve refers to the electrode baseline.

current changes for the OP solutions, no apparent current changes were observed for Na^+, K^+, Ca^+, Mg^{2+}, Fe^{2+}, Vc, HSA, and several kinds of amino acids indicated. Importantly, they could have no influence on the electrochemical responses to OP, even though they co-exist with high concentrations (data not shown). Accordingly, these common components in blood might have negligible impact on the detection of OPs. The high detection selectivity for OP exposure can thereby be expected, presumably resulting from the specific and strong phosphorylation between Tyr and OPs,[14,22] which in turn could induce the unique inhibition of Tyr electro-activities. Moreover, as shown in Fig. 4A, three kinds of OPs (Met, Par, and DDVP) could display approximately high electrochemical responses, suggesting that the proposed method can determine the total amounts of OPs in various media.

Moreover, the detection stability of electrochemical responses to DDVP was evaluated for the electrodes with Fe_3O_4@Tyr particles (Fig. 4B). Here, Fe_3O_4@Tyr particles were stored over time in a refrigerator at 4 °C and taken out at different time intervals for DDVP treatment and further electrochemical tests. As can be seen from Fig. 4B, no significant change of electrochemical signals was monitored up to ten weeks. Such high stability for the electrochemical DDVP detection might be related to the considerably strong interaction between DDVP and Tyr on Fe_3O_4@Tyr particles, as well as its unique merit of irreversible electrochemical oxidization. The above results indicate that the prepared Fe_3O_4@Tyr electrodes could present high detection stability for free OPs.

3.6 Electrochemical detection of DDVP samples with magnetic Fe_3O_4@Tyr electrodes

Under the optimized conditions, DDVP samples with different concentrations in Tris-HCl buffer were examined by the "lab-on-a-particle"-based detection method (Fig. 5A). It can be observed that the current responses could decrease with the increase of DDVP concentrations, as shown in the inset in Fig. 5A. A linear relationship was obtained for the electrochemical responses over −log[DDVP] of DDVP concentrations ranging from 0.12–60 nM (R = 0.9913), with the detection limit of about 0.056 nM, estimated according to the 3σ rule. The detection linearity range and limit of the developed method were compared with those of the electrochemical assays for DDVP reported elsewhere (Table 1), showing the better or comparable detection sensitivity. Herein, the high sensitivity and reproducibility of DDVP detection might presumably result from the highly sensitive and stable phosphorylation of Tyr on Fe_3O_4@Tyr particles, in addition to highly sensitive electrochemical LSV outputs as aforementioned.

Table 1 Comparison of detection performances among different electrochemical assays for DDVP detection

Detection methods	Linear ranges (M)	Detection limits (M)	Ref.
Amperometry	ND	1.0×10^{-10}	35
Amperometry	ND	7.0×10^{-12}	36
Amperometry	4.52×10^{-11}–4.52×10^{-8}	1.13×10^{-11}	37
Amperometry	Up to 8×10^{-6}	6.0×10^{-8}	38
LSV	1.2×10^{-10}–6.0×10^{-8}	5.6×10^{-11}	This study

Moreover, the Fe_3O_4@Tyr particle-based method was used to probe the levels of DDVP samples spiked in blood (Fig. 5B). Herein, Fe_3O_4@Tyr particles were added to the diluted blood samples containing different concentrations of DDVP, and then magnetically immobilized on the magnetic electrodes for electrochemical measurements under the optimal conditions. The linear detection relationship between the electrochemical responses and −log[DDVP] in blood was obtained over the DDVP concentration range of 0.57–55 nM (R = 0.9871), with the detection limit of about 0.16 nM. Therefore, the application feasibility of the developed "lab-on-a-particle"-based detection strategy for the early warning and diagnosis of OP exposure was demonstrated by probing the direct biomarkers of free OPs in blood with high sensitivity and reproducibility.

4. Conclusions

Electrochemically active Tyr was successfully bound onto Fe_3O_4 particles to form Fe_3O_4@Tyr particles as capturing probes for

detecting free DDVP in blood by electrochemical outputs. The sensitive and specific phosphorylation-induced inhibition of the electro-activities of Tyr on Fe_3O_4@Tyr particles could allow for the electrochemical detection of free OP with high sensitivity and selectivity. Also, the strong interactions between Tyr and OPs especially irreversible electrochemical oxidization of the Tyr probe could facilitate the OP evaluation with high stability over time. Moreover, the magnetic separation-based detection of the direct phosphorylation biomarkers of free OPs in complicated media could be achieved without any sample purification. Particularly, this simple and rapid detection procedure with a portable electrochemical transducer device can allow for the field-deployable or on-site OP monitoring. Therefore, such a "lab-on-a-particle" detection strategy may find wide applications for the early warning or diagnosis of the exposure to OPs in the environment (*i.e.* pesticides), warfield (*i.e.*, nerve agents), and clinical laboratories.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (nos 21375075, 21302109, and 21302110), the Taishan Scholar Foundation of Shandong Province, and the Natural Science Foundation of Shandong Province (ZR2013BQ017 and ZR2013BM007), P. R. China.

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