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A ratiometric fluorescence probe based on carbon dots for discriminative and highly sensitive detection of acetylcholinesterase and butyrylcholinesterase in human whole blood

Xiaoman Xu[1], Yao Cen[*,1], Guanhong Xu, Fangdi Wei, Menglan Shi, Qin Hu[*]

School of Pharmacy, Nanjing Medical University, Nanjing 211166, PR China

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ABSTRACT

A ratiometric fluorescence probe based on carbon dots (CDs) was developed for discriminative and highly sensitive detection of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in human whole blood. When *o*-phenylenediamine (OPD) was oxidized by Cu^{2+}, the product 2,3-diaminophenazine (oxOPD) could effectively quench the fluorescence of CDs at 460 nm due to the inner filter effect and gave rise to a new emission peak at 570 nm. The AChE or BChE catalyzed hydrolysis reaction of acetylthiocholine or butyrylthiocholine to generate thiocholine, whose sulfhydryl group strongly captured Cu^{2+} to inhibit the oxidization of OPD, thus effectively preserving the natural fluorescence emission of CDs. The resulting fluorescence intensity ratio served as the signal output of the probe for cholinesterases (ChEs) activity sensing. The activities of AChE and BChE were determined to range from 0.2 to 14.0 U L^{-1} and from 0.1 to 5.0 U L^{-1}, with detection limits of 0.1 U L^{-1} and 0.04 U L^{-1}, respectively. Additionally, the IC_{50} of tacrine and ethopropazine for the inhibition of AChE and BChE were estimated to be 29.8 nM and 132.6 nM, respectively. Moreover, the probe was successfully applied to the discriminative determination of AChE and BChE in human whole blood without any pretreatment. These results suggested that the proposed strategy provided a discriminative, sensitive and robust analytical platform for ChEs clinical diagnostics and drug screening.

1. Introduction

Cholinesterases (ChEs) are critical enzymes in the central nervous system that catalyze the hydrolysis reaction of choline esters to choline (Mikalsen et al., 1986). There are two major ChE isoenzymes, namely acetylcholinesterase (AChE) which mainly binds to the erythrocyte membrane, and butyrylcholinesterase (BChE) which mainly presents in plasma (Miao et al., 2010). AChE plays vital roles in maintaining the levels of the neurotransmitter acetylcholine (Soreq and Seidman, 2001). Moreover, AChE and BChE activities are recognized as diagnostic biomarkers in organophosphate poisoning, and nerve agent exposure (Santarpia et al., 2013). In this regard, the development of discriminative and sensitive detection methods for both AChE and BChE activities in whole blood is of significant importance.

Currently, the most widely used approach for ChEs activities is Ellman's colorimetric method (Ellman et al., 1961). However, the widespread application of this method is limited by its shortcomings. For instance, its sensitivity is poor due to the colorimetric method, the thiol groups in samples react with Ellman's reagent to introduce false-positive effect, and the massive Soret absorbance of hemoglobin interferes with the absorbance of the reaction product, and whole blood samples need laboring pretreatment (Miao et al., 2010). The choline oxidase coupled multienzyme assay is an effective method for monitoring ChE activities (Gill et al., 2008); unfortunately, the process requires time-consuming procedures. While there have been several alternative methods, including mass spectrometry (de Jong et al., 2006), and radiometry (Winteringham and Disney, 1962), but they generally suffer from the requirement of special instruments or radiolabeling. Recently, several fluorescent nanomaterials including semiconductor quantum dots (Saa et al., 2010), silver/gold nanoclusters (Li et al., 2013), and carbon dots (Qian et al., 2016; Chen et al., 2018) have been reported for ChEs activities sensing. However, such probes based on a single fluorescent intensity change cannot avoid the interferences from probe concentrations, light source, and measurement conditions. Importantly, all of these methods are challenged by discriminatively determining both AChE and BChE activities owing to the coexistence of

* Corresponding authors.
E-mail addresses: yaocen@njmu.edu.cn (Y. Cen), huqin@njmu.edu.cn (Q. Hu).
[1] These authors contributed equally to this work.

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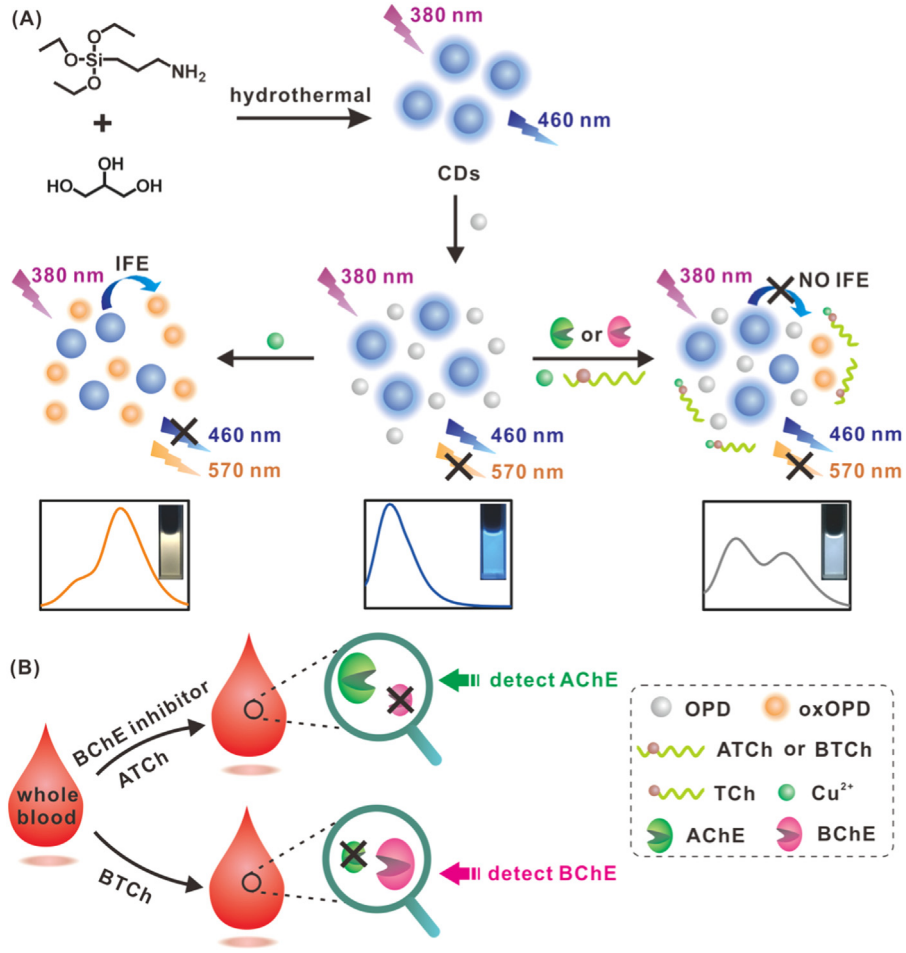

Fig. 1. Schematic illustration of the CDs-based ratiometric fluorescence probe for the detection of AChE and BChE in human whole blood.

AChE and BChE in whole blood and overlapping substrate affinities.

Carbon dots (CDs) have received great attention in the field of biosensing, bioimaging, and drug delivery due to their convenient preparation, multicolored emissions and high biocompatibility (Lim et al., 2015; Baker and Baker, 2010). In particularly, the strong photoluminescence property makes CDs an ideal choice for the development of fluorescence biosensors. Recently, several groups have exploited CDs-based fluorescence probe to detect metal ions, DNA, dopamine, and enzymes (Dong et al., 2012; Noh et al., 2013; Qu et al., 2013; Li et al., 2016). However, these biosensors exclusively depended on a single fluorescent intensity output, resulting in low signal-to-background ratios. Alternatively, several CDs-based ratiometric fluorescence biosensors have also been constructed for the detection of Cu^{2+}, Hg^{2+}, and H_2S (Zhu et al., 2012; Zhao et al., 2017; Yu et al., 2013). However, to our knowledge, the development of CDs-based probe for the ratiometric fluorescence detection of AChE and BChE remains largely unexplored.

Herein, we developed a ratiometric fluorescence probe based on CDs for the discriminative and highly sensitive detection of AChE and BChE in human whole blood. As shown in Fig. 1A, the CDs prepared by (3-aminopropyl)triethoxysilane and glycerol exhibited a strong fluorescence peak. *o*-Phenylenediamine (OPD) was oxidized by Cu^{2+} to generate 2,3-diaminophenazine (oxOPD), which not only effectively quenched the fluorescence of CDs due to the inner filter effect (IFE), but also yielded a new emission peak at 570 nm. In the presence of ChEs, the substrate acetylthiocholine (ATCh) or butyrylthiocholine (BTCh) was hydrolyzed into thiocholine (TCh), whose sulfhydryl group can strongly capture Cu^{2+}, and the oxidization of OPD was blocked, resulting in fluorescence changes of the system. Therefore, the fluorescence recovery at the emission peak of 460 nm together with fluorescence decrease at the emission peak of 570 nm occurred in the system, allowing the development of ratiometric fluorescence strategy for ChEs activities sensing. Importantly, as illustrated in Fig. 1B, by taking advantage of specific substrates and a selective BChE inhibitor (ethopropazine, Etho), the probe could directly detect AChE and BChE in human whole blood. On the basis of these features, the developed probe allowed for the discriminative, simple, and sensitive detection of ChEs activity with great potential for further clinical diagnosis and drug screening.

2. Experimental

2.1. Determination of AChE and BChE in aqueous solution

First, 10 μL of AChE or BChE with different activities and 0.4 mM ATCh or BTCh were added to 100 μL of Tris-HCl buffer (pH 7.5) and incubated at 37 °C for 30 min. Then, 0.07 mM Cu^{2+} and 0.5 mM OPD was added and incubated at 37 °C for 2 h. Afterwards, 20 μL of CDs was added, the solution was incubated for 20 min and subjected to fluorescence measurements. The solutions were prepared by adding other enzymes instead of AChE or BChE for the specificity assay.

2.2. Determination of AChE and BChE in human whole blood

The preparation of blood samples is described in the Supporting Information. The whole blood and erythrocytes were finally diluted 3000-fold for AChE determination, while the whole blood and plasma were finally diluted 10,000-fold for BChE determination. For the AChE

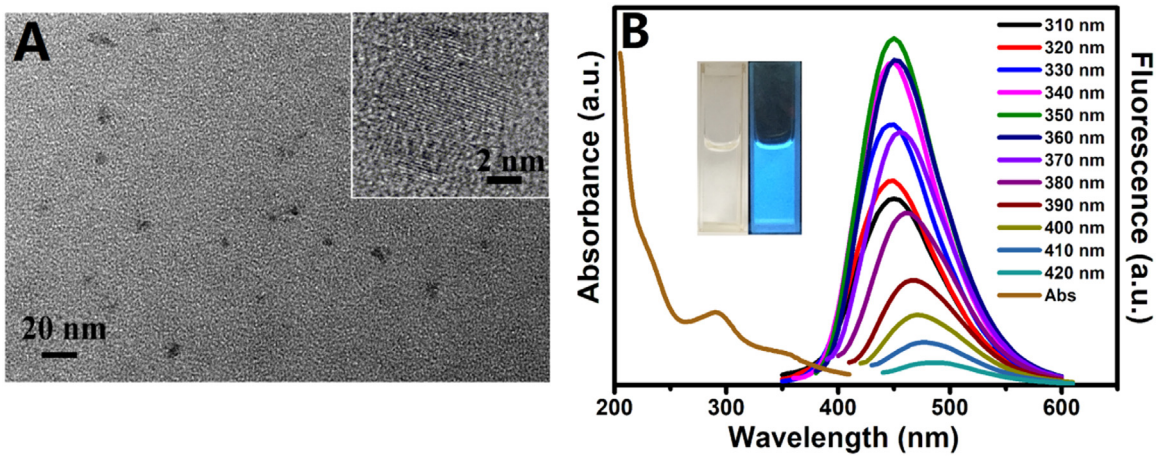

Fig. 2. (A) TEM image of CDs. Inset shows the high-resolution TEM image of CDs. (B) UV–vis absorption spectrum of CDs and fluorescence emission spectra of CDs at different excitation wavelengths. Inset: photographs of CDs under visible and UV light.

analysis, ATCh was used as the substrate and Etho as the BChE inhibitor. For the BChE analysis, BTCh was used as the substrate. The following procedures for the samples were similar to that described above.

3. Results and discussion

3.1. Characterization of CDs

The CDs were prepared through a facile hydrothermal method (Zou et al., 2016). The transmission electron microscope (TEM, Fig. 2A) image showed that CDs were monodispersed with a diameter of approximately 9.0 nm, which agreed with the dynamic light scattering (Fig. S1). Compared with the FT-IR spectrum of pure APTES, CDs presented new absorption bands at 1650 cm^{-1} (stretching vibration of C=O), which indicated the dehydration and carbonization (Fig. S2). The X-ray photoelectron spectroscopy survey scan verified the successful synthesis of CDs (Fig. S3). The fluorescence emission of CDs (Fig. 2B) showed a red-shift with the excitation wavelength in the range of 310–420 nm, indicating that CDs displayed an excitation-dependent fluorescence behavior, which was a trademark property of CDs (Huang et al., 2014). The fluorescence of CDs appeared strong blue upon UV light excitation (Fig. 2B, inset) and the fluorescence intensity of CDs exhibited no significant changes under different pH conditions (Fig. S4).

3.2. Construction of CDs-based ratiometric probe

The fluorescence spectra of CDs exhibited no evident changes with the addition of Cu^{2+} and other metal ions (Fig. S5). To achieve a balance between the fluorescence intensities of CDs and oxOPD, we used 380 nm as the excitation wavelength. The colorless and nonfluorescent OPD could be oxidized by Cu^{2+}, and the resultant oxOPD showed an obvious absorption peak at 415 nm, as well as a strong fluorescence emission peak at 570 nm under 380 nm excitation (Sun et al., 2016). The absorption spectrum of oxOPD overlapped well with the emission spectrum of CDs, which was a prerequisite for the IFE (Fig. S6A). After incubation of CDs with oxOPD, the fluorescence intensity of CDs was efficiently quenched (Fig. S6B). Meanwhile, no evident change in the fluorescence lifetime of the CDs was observed with oxOPD, indicating that there was no energy and/or electron transfer between CDs and oxOPD (Fig. S6C). In addition, no significant changes in the absorption spectra of oxOPD were observed with the addition of CDs, which indicated that there was a lack of complex formation and no covalent bonding between CDs and oxOPD (Fig. S6D). Meanwhile, the zeta potentials of CDs and oxOPD were all positively charged, and oxOPD could not be adsorbed on the surface of CDs via electrostatic interactions (Fig. S6E). Considering the above two points, the distance between CDs and oxOPD was hardly shorter than 10 nm (Liu et al., 2017). Therefore, the fluorescence resonance energy transfer process could not occur in this system, and the fluorescence quenching mechanism of CDs and oxOPD was caused by the IFE. The final concentration of Cu^{2+} was chosen to be 70 μM in the subsequent experiments due to the sufficient enhancement (Fig. S7).

3.3. Sensing of AChE and BChE in buffer solution

There were no significant changes in the fluorescence spectra of the system with individual substrates (ATCh or BTCh) or enzymes (AChE or BChE) (Fig. S8A). However, when ATCh was used together with CDs, OPD, Cu^{2+}, AChE or BChE, the fluorescence intensity at 460 nm was recovered, while the fluorescence intensity at 570 nm disappeared (Fig. S8B, curves c and d). Additionally, when using BTCh as the substrate, only BChE could lead to the above fluorescence spectrum change (Fig. S8B, curve f). These results demonstrated that ATCh could be hydrolyzed by AChE and BChE, while BTCh was preferentially hydrolyzed by BChE. Moreover, Etho was a selective BChE inhibitor and did not inhibit AChE activity (Worek et al., 1999). Therefore, by employing ATCh as the substrate together with Etho, or BTCh as the substrate, the changed fluorescence spectra could verify the individual activity of AChE or BChE given the coexistence of AChE and BChE (Fig. S8B, curves g and h). These results indicated that the probe can be applied to discriminatively determine AChE and BChE in human whole blood.

Under optimal conditions (Fig. S9), the fluorescence intensity at 460 nm gradually increased, while the fluorescence intensity at 570 nm gradually decreased with increasing AChE or BChE concentrations (Fig. 3A and B). Moreover, with the increasing AChE or BChE concentrations, the photographs exhibited not only a naked eye color change from yellow to colorless under visible light, but also a fluorescence color change from yellow to blue under UV light. The AChE or BChE activity could be determined in the range $0.2 - 14.0$ U L^{-1} or $0.1 - 5.0$ U L^{-1} with a linear range from 0.2 to 4.0 U L^{-1} or 0.1–1.2 U L^{-1} (Fig. 3C and D). The linear relationship equations were $y = 0.038\,x + 0.115$ ($r^2 = 0.990$) for AChE and $y = 0.159\,x + 0.111$ ($r^2 = 0.992$) for BChE. The detection limits of AChE and BChE were estimated to be 0.1 U L^{-1} and 0.04 U L^{-1} according to 3σ rule. These values were comparable to or lower than those of existing probes, and a detailed comparison is summarized in Table S1. Although Ellman's method had the advantages of convenience and accessibility of high-throughput analysis (Ellman et al., 1961), the developed method provided excellent selectivity and sensitivity for AChE and BChE assay and avoided the false-positive signal, background interference, and pretreatment of

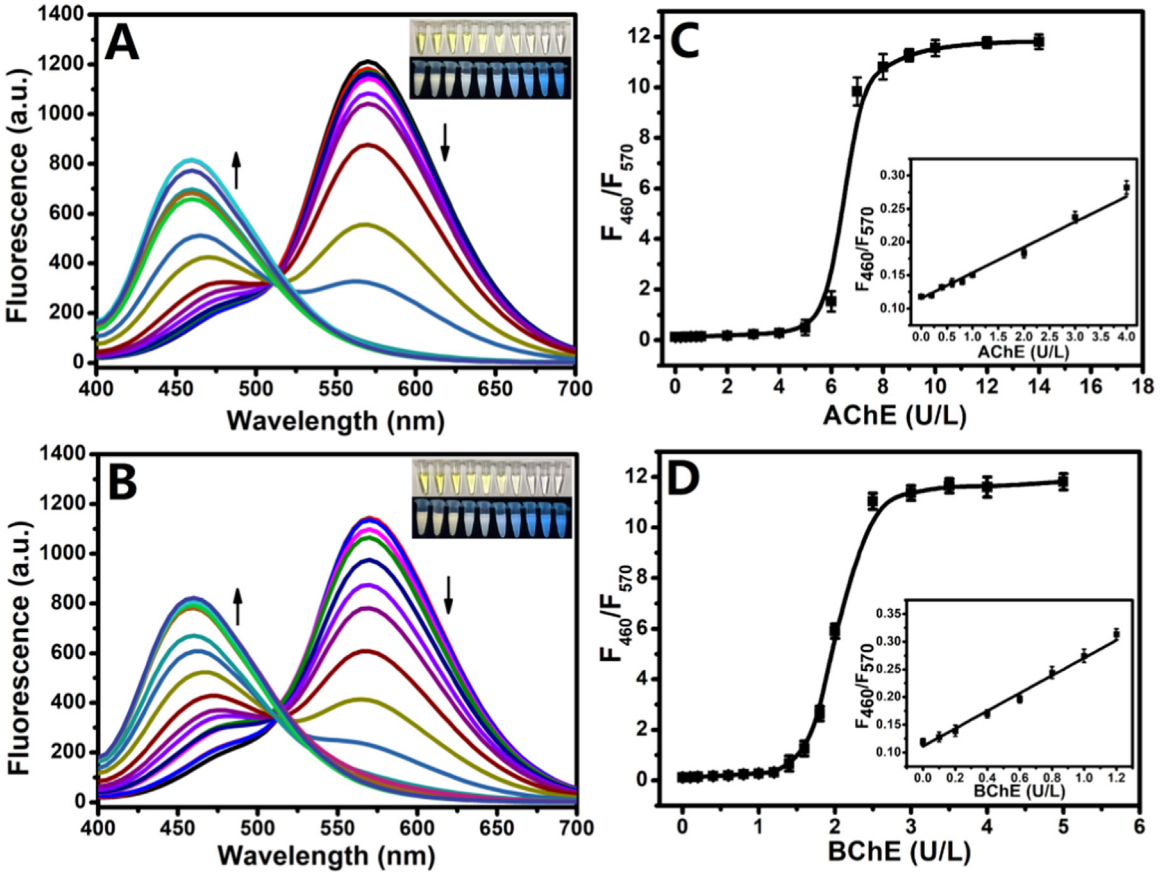

Fig. 3. Fluorescence spectra of CDs-based probe after incubation with different concentrations of (A) AChE and (B) BChE. Insets: corresponding photographs under visible (top) and UV light (bottom). The curve of F_{460}/F_{570} (ratiometric fluorescence intensity) versus the concentration of (C) AChE and (D) BChE. Insets: the linear relationship between F_{460}/F_{570} and ChEs concentration.

whole blood. These results demonstrated that the CDs-based ratiometric fluorescence probe was capable of the highly sensitive detection of AChE and BChE.

We further investigated possible interferents including alkaline phosphatase (ALP), human serum albumin (HSA), glucose oxidase (GOx), thrombin, trypsin and glutathione (GSH) for the specificity assay. Except for GSH, these species showed no apparent interference with the determination of ChEs (Fig. S10). However, the interference of GSH could be effectively eliminated by using the thiol scavenger *N*-ethylmaleimide. Although AChE and BChE can both catalyze the hydrolysis of ATCh, by employing ATCh as the substrate together with Etho, or BTCh as the substrate, the probe could verify the individual activtiy of either AChE or BChE, even in the coexistence of AChE and BChE. These results indicated that the probe can be applied for the selective determination of AChE and BChE activtiies.

3.4. Inhibition evaluation of AChE and BChE

Tacrine and Etho are well-known inhibitors of AChE and BChE, respectively. The ratiometric fluorescence intensity (F_{460}/F_{570}) gradually decreased with increasing concentrations of tacrine and Etho, demonstrating the inhibition of AChE and BChE catalyzed hydrolysis reactions (Fig. S11). The corresponding IC_{50} values of tacrine and Etho were estimated to be 29.8 nM and 132.6 nM, which are comparable to or lower than those existing ChEs assays (Li et al., 2013; Worek et al., 1999). These results demonstrated that the CDs-based probe holded great potential for screening ChEs inhibitors.

3.5. Determination of AChE and BChE in human whole blood

The whole blood samples were finally diluted 3000-fold and 10,000-fold for AChE and BChE determinations, respectively. For the AChE analysis, ATCh was used as the substrate and Etho used as a selective BChE inhibitor. For the BChE analysis, BTCh was used as the substrate. The mean concentrations of GSH and cysteine in the 3000-fold or 10,000-fold diluted biological sample were diluted to the nanomolar level. The additions of certain amounts of GSH, cysteine, α-lactalbumin, and β-lactoglobulin exhibited negligible interference. In addition, the fluorescence responses of CDs-based probe showed no clear changes among the different diluted biological samples. As a result, the biological samples with adequate dilutions could circumvent the background interference from GSH, cysteine, α-lactalbumin, and β-lactoglobulin and did not affect the determination of AChE and BChE activities (Fig. S12). The CDs-based probe could directly measure individual AChE and BChE activities in whole blood, while the Ellman method could not detect whole blood AChE (Table S2). The activities of AChE and BChE in whole blood were in agreement with those previously reported ranges (Qian et al., 2016; Chen et al., 2018).

4. Conclusions

We developed a ratiometric fluorescence probe based on CDs for highly sensitive detection of AChE and BChE activities. Through the inhibition of the oxidization of OPD by AChE or BChE catalyzed hydrolysis of ATCh or BTCh, the change in ratiometric fluorescence intensity served as the signal output for ChEs activity sensing and inhibitors screening. This method provided excellent selectivity and sensitivity for AChE and BChE assays with low detection limit of 0.1 U

L^{-1} and 0.04 U L^{-1}, respectively. In addition, the proposed approach avoided complex modifications of probe and expensive instrumentation. Moreover, by taking advantage of specific substrates and Etho, the probe was capable of simultaneously and discriminatively monitoring AChE and BChE in human whole blood without any pretreatment. Importantly, the proposed method could circumvent the false-positive signal and background interference in whole blood samples with adequate dilutions, indicating the potential for application in clinical diagnosis. However, the work suffered the limitation of not monitoring ChEs activities in living cells.

CRediT authorship contribution statement

Xiaoman Xu: Conceptualization, Data curation, Methodology. **Yao Cen:** Funding acquisition, Supervision, Writing - original draft. **Guanhong Xu:** Investigation, Software. **Fangdi Wei:** Resources, Validation. **Menglan Shi:** Visualization, Formal analysis. **Qin Hu:** Funding acquisition, Project administration, Writing - review & editing.

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Declaration of interest statement

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2019.02.031.

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