Supporting Information

Real-time fluorescence turn-on assay for acetylcholinesterase activity based on the controlled release of a perylene probe from the MnO$_2$ nanosheets

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EXPERIMENTAL SECTION

Materials

P-4C+ was prepared as previously described. AChE (from Electrophorus electricus) and trypsin were purchased from Sigma (St. Louis, MO, USA). Acetylthiocholine iodide, donepezil hydrochloride and 3-hydroxycarbofuran were purchased from J&K Scientific Ltd. (Beijing, China). S1 nuclease was purchased from Fermentas Inc. (MBI, Canada). Lysozyme was purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). Alkaline phosphatase (ALP) and exonuclease I (Exo I) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). All stock and buffer solutions were prepared using water purified with a Milli-Q A10 filtration system (Millipore, Billerica, MA, USA).

Instrumentation

UV-Vis absorption spectra were obtained using a Cary 50 Bio Spectrophotometer (Varian Inc., CA, USA) equipped with a xenon flash lamp. Emission spectra were recorded using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA). Excitation wavelength was 442 nm. Quartz cuvettes with 10 mm path length and 2 mm window width were used for UV-vis and emission measurements. Zeta potential measurements were performed with a Zetasizer NanoZS (Malvern Instruments, USA). Transmission electron microscopy (TEM) images were obtained using a JEM-2100F high resolution transmission electron microscope (Philips, The Netherlands) operated at 200 kV. Atomic force microscopy (AFM) characterization was performed on a Multimode-V (Veeco Instruments, USA) using a tapping mode. Unless specified, all
concentrations of P-4C+, ATCh, MnO₂ nanosheets and buffer were those in the final assay solutions (total sample volume, 400 μL), and all spectra were taken at 37 °C in 5 mM phosphate buffer at pH 7.4.

**Preparation of the MnO₂ nanosheets**

The MnO₂ nanosheets were prepared following the reported procedures. In short, an aqueous solution comprised 20 mL of tetramethylammonium hydroxide (0.6 M) and H₂O₂ (3 wt%) was first prepared, and 10 mL of 0.3 M MnCl₂ was quickly mixed. The resulting mixture was stirred vigorously overnight in open air. The bulk MnO₂ was obtained though centrifugation, cleaned with double-distilled water and methanol and dried in an oven. MnO₂ nanosheets were obtained via ultrasonication of the aqueous solution of the bulk manganese dioxide.

**Fluorescence quenching of P-4C+ by the MnO₂ nanosheets**

A series of concentrations of the MnO₂ nanosheets (0, 8.75, 17.5, 26.25, 35, 43.75, 52.5, 61.25, and 70 μg/mL) were mixed with P-4C+ (5 μM) in 5 mM phosphate buffer (pH 7.4). Samples were mixed completely and maintained at 37 °C for 3 min. The emission intensity was then recorded.

**ATCh concentration optimization**

Thiocholine was prepared following the reported procedures and characterized by ¹H-NMR (Figure S5). Different amounts of thiocholine were mixed with 5 mM phosphate buffer solution (pH 7.4) comprising 26.25 μg/mL MnO₂ nanosheets and 50 μM PPi. The samples were
incubated at 37 °C for 60 min. 5 μM of P-4C+ was added. Samples were mixed and maintained at 37 °C for 3 min. The emission intensity was then recorded. Pyrophosphate was introduced to complex with the Mn$^{2+}$ ions, since the in situ generated Mn$^{2+}$ ions could attach to the surface of the MnO$_2$ nanosheets and caused aggregation of the nanosheets.

**AChE assay procedures**

0.3 mM ATCh (20 μL) was injected to 380 μL of 5 mM phosphate buffer solution (pH 7.4) comprising 26.25 μg/mL MnO$_2$ nanosheets, 50 μM P Pi, 5 μM P-4C+ and AChE of different concentrations. The final concentrations of AChE were 0, 5, 10, 25, 50, 100, 250, 500 and 1000 mU/mL, respectively. The temperature was stabilized at 37 °C and the emission intensity of P-4C+ at 488 nm was recorded with data points taken every 5 seconds.

**Selectivity of the AChE assay**

Different enzymes including lysozyme, alkaline phosphatase (ALP), S1 nuclease, trypsin, Exo I (10.0 U/mL each) and AChE (500 mU/mL) were mixed with the solution of 0.3 mM ATCh, 50 μM P Pi and 26.25 μg/mL MnO$_2$ nanosheets in 5 mM phosphate buffer (pH 7.4). The solutions were incubated at 37 °C for 60 min. 5 μM of P-4C+ was added. Samples were mixed completely and maintained at 37 °C for 3 min. The emission intensity was then recorded.

**AChE assay in biological fluid**

Various quantities of AChE were mixed with the sample solution of 26.25 μg/mL MnO$_2$ nanosheets, 0.3 mM ATCh, 50 μM P Pi and 2.5% human serum in 5 mM phosphate buffer (pH
7.4). Samples were kept at 37 ºC for 15 min. 5 μM of P-4C+ was then mixed with the solution. Samples were mixed completely and maintained at 37 ºC for 3 min. The emission intensity was then recorded.

**AChE inhibitor screening**

Various concentrations of the inhibitors (donepezil or 3-hydroxycarbofuran) were mixed with the sample solution of 26.25 μg/mL MnO$_2$ nanosheets, 0.3 mM ATCh, 50 μM PPi and 100 mU/mL AChE in 5 mM phosphate buffer (pH 7.4). The samples were kept at 37 ºC for 15 min. 5 μM of P-4C+ was added. Samples were mixed thoroughly and maintained at 37 ºC for 3 min. The emission intensity was then recorded.

The inhibition efficiency (IE) is given by: $IE = \frac{[I - I_i]}{[I - I_0]}$

The emission intensity values of P-4C+ at 488 nm in the absence and presence of the inhibitor are symbolized as $I$ and $I_i$, $I_0$ is the emission intensity of P-4C+ at 488 nm in the absence of AChE.

**REFERENCES**


Figure S1. Tapping-mode of the AFM image of the MnO$_2$ nanosheets. Inset: the height profile of the section labeled with the white line.

Figure S2. UV-vis absorption spectrum of the MnO$_2$ nanosheets (black curve) and the fluorescence emission spectrum of P-4C+ (blue curve).
Figure S3. Zeta potential analysis: the MnO$_2$ nanosheets possess a potential value of $-26.2$ mV before (a) and $1.29$ mV after (b) mixed with P-4C$^+$.  

Figure S4. Fluorescence quenching efficiency versus MnO$_2$ nanosheets concentration (0, 8.75, 17.5, 26.25, 35, 43.75, 52.5, 61.25, and 70 $\mu$g/mL). Buffer: 5 mM phosphate buffer, pH 7.4.
Figure S5. $^1$H-NMR spectrum of thiocholine.

Figure S6. UV-vis absorption spectra: Blue curve: the reaction mixture of MnO$_2$ nanosheets and thiocholine was centrifuged, the supernatant was taken and treated with sodium periodate; Green curve: the reaction mixture of sodium periodate and MnCl$_2$; Red curve: KMnO$_4$ aqueous solution; Black curve (blank control): the MnO$_2$ nanosheets containing sample solution was centrifuged, the supernatant was taken and mixed with sodium periodate.
The reduction of \( \text{MnO}_2 \) to \( \text{Mn}^{2+} \) by thiocholine could be proven by a highly specific reaction of \( \text{Mn}^{2+} \) with sodium periodate, and the resulting sample solution shows clear color changes:

\[
2\text{Mn}^{2+} + 5\text{IO}_4^- + 3\text{H}_2\text{O} \rightarrow 2\text{MnO}_4^- + 5\text{IO}_3^- + 6\text{H}^+
\]

**Figure S7.** UV-vis absorption spectra of the \( \text{MnO}_2 \) nanosheets before (red curve) and after (black curve) the enzymatic reaction of AChE with the substrate.

**Figure S8.** Changes in emission intensity of P-4C+ (5 \( \mu \)M) at 488 nm upon the addition of...
increasing concentrations of free thiocholine. Conditions: 5 mM phosphate buffer (pH 7.4),
26.25 μg/mL MnO\textsubscript{2} nanosheets and 50 μM PPI.

**Figure S9.** Selectivity study. Columns A–G: AChE, lysozyme, ALP, S1 nuclease, trypsin and
Exo I nuclease. AChE: 500 mU/mL; the other enzymes: 10 U/mL each. 50 μM EDTA was used
instead of pyrophosphate. I and I\textsubscript{0} represent the emission intensity of P-4C\textsuperscript{+} in the presence and
absence of the enzyme.
**Figure S10.** Probing AChE activity in the presence of different biothiols (Cys, GSH, Hcy and Na₂S). Black columns: no AChE added. Red columns: all contains 100 mU/mL AChE. Columns a – d: different concentrations of ATCh were added (0, 50, 100, 150 μM, respectively). All samples contain 5 mM phosphate buffer (pH 7.4), 5 μM P-4C+, 26.25 μg/mL MnO₂ nanosheets, 50 μM biothiol (Cys, GSH, Hcy, or Na₂S), and the emission intensity changes of P-4C+ at 488 nm were monitored.
Figure S11. Changes in emission intensity of P-4C+ at 488 nm (5 μM) in the presence of the reductive substances. Green column: background emission of P-4C+ in the presence of the MnO$_2$ nanosheets and ATCh only; Red column: emission of P-4C+ in the presence of the MnO$_2$ nanosheets, ATCh, and Cys (or GSH, Hcy, Na$_2$S); Blue column: samples of Cys, GSH, Hcy, and Na$_2$S were passed through a filter (MWCO 30K), wash with water, the supernatant was taken, and mixed with P-4C+, MnO$_2$ nanosheets, and ATCh; Black column: samples of Cys, GSH, Hcy, and Na$_2$S were mixed with AChE, passed through the filter, and mixed with P-4C+, MnO$_2$ nanosheets, and ATCh. Conditions: 5 mM phosphate buffer (pH 7.4), 26.25 μg/mL MnO$_2$ nanosheets, 0.3 mM ATCh, 0.3 mM Cys (or GSH, Hcy, Na$_2$S), 0.5 U/mL AChE.
Figure S12. Emission intensity changes of P-4C+ (5 μM) at 488 nm in dilute human serum (2.5%) with time. Conditions: 5 mM phosphate buffer (pH 7.4), 50 μM PPI, 26.25 μg/mL MnO$_2$ nanosheets. The results show that the assay mixture is stable enough for the enzyme activity quantification (Figure 3).

Figure S13. Changes in emission intensity of P-4C+ at 488 nm (5 μM) with AChE concentration in dilute human serum (2.5%). Conditions: 5 mM phosphate buffer (pH 7.4), 26.25 μg/mL MnO$_2$ nanosheets, 0.3 mM ATCh, 50 μM PPI.
Figure S14. Changes in emission spectrum (a) and the corresponding emission intensity at 488 nm (b) of P-4C+ upon the addition of increasing concentrations of donepezil (0 – 200 nM). Conditions: 5 mM phosphate buffer (pH 7.4), 26.25 μg/mL MnO$_2$ nanosheets, 0.3 mM ATCh, 50 μM PPI and 100 mU/mL AChE.
**Figure S15.** Changes in emission spectrum (a) and the corresponding emission intensity at 488 nm (b) of P-4C+ upon the addition of increasing concentrations of 3-hydroxycarbofuran (0 – 400 nM). Conditions: 5 mM phosphate buffer (pH 7.4), 26.25 μg/mL MnO$_2$ nanosheets, 0.3 mM ATCh, 50 μM PPI and 100 mU/mL AChE.