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Visualized Evaluation of Acetylcholinesterase Inhibition by an Easy-to-Operate Assay Based on *N*-doped Carbon Nanozyme with High Stability and Oxidase-Like Activity

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Reagents and Materials

3,3',5,5'-Tetramethylbenzidine (TMB) and NaOH were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetylcholinesterase (AChE) from drosophila was purchased from Solarbio. Water (18.2 MΩ·cm) used in this work was obtained by a Milli-Q water purification system (Millipore). Acetylcholinesterase (AChE) from electric eel, Acetylthiocholine (ATCh), Pralidoxime Iodide and 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Macklin Biochemical Co. Ltd. (Shanghai, China). $Na_2HPO_4·2H2O$ and $NaH_2PO4·2H_2O$ were ordered from Sigma-Aldrich (St. Louis, MO, USA). Paraoxon, Dursban, Parathion, Phoxim, Methidathion, Tichlorfon, Dichlorvos, Carbofuran, Carbosulfan and Thiamethoxam were obtained from Tanmo Quality Inspection Technology Co. Ltd.

Apparatus

The surface morphology of the nanomaterial was characterized by TEM HT7700 (Hitachi, Japan). XPS ESCALAB 250Xi (Thermo Fisher Scientific, USA), and XRD Bruker D8 Advance X-ray diffractometer (Bruker, Germany) were employed to study the composition and valence state. UV-vis spectra were gotten from an UV-1800 spectrophotometer (Shimadzu, Japan).

Experimental Section

Synthesis of NC900

The synthesis route of the precursor Aza-CMP refers to our previous work (Small, 2022, 18, 2104993). And then, the power was pyrolyzed under N_2 atmosphere for 1 h at 900 °C with a heating rate of 3 °C/min. After cooling to room temperature, the black power (named NC900) was obtained without any post-treatment.

Ellman Method

Typically, 40 μL of various inhibitors (final concentration: 0, 0.01, 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, and 3.0 μM) were firstly mixed with 40 μL of 200 mU mL^{-1} AChE at 37 °C for 30 min. Subsequently, 40 μL of 1.0 mM ATCh for another 30 min, and then 40 μL of 0.5 mM DTNB was added, which was adjusted to 400 μL with PBS buffer (pH 8.0). After the incubation for 30 min, the absorbance at 652 nm was monitored on a UV-1800 spectrophotometer.

Inhibitors detection

Typically, 40 μL of various inhibitors (final concentration: 0, 0.01, 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, and 3.0 μM) were firstly incubated with 40 μL of 200 mU mL^{-1} AChE at 37 °C for 30 min. Subsequently, 40 μL of 1.0 mM ATCh and 40 μL of 0.2 mg mL^{-1} NC900 were added for another 30 min. After the addition of 40 μL of 5.0 mM TMB, the system was adjusted to 400 μL with NaAc-HAc buffer (200 mM, pH 4.0) and

incubated for 30 min. The UV-Vis spectra were monitored on a UV-1800 spectrophotometer, and the absorbance at 652 nm was assigned to the characteristic peak of oxTMB.

Influence of pH on the degradation behaviour of trichlorfon and carbosulfan

Trichlorfon was incubated with NaAc-HAc buffer (pH 4.0 or pH 11.0), and then, 40 μL of various concentrations (final concentration: 0.1, 0.5, 1.0, 2.0 and 3.0 μM) were mixed with 40 μL of 200 mU mL^{-1} AChE at 37 ^{o}C for 30 min. Subsequently, 40 μL of 1.0 mM ATCh and 40 μL of 0.2 mg mL^{-1} NC900 were added for another 30 min. After the addition of 40 μL of 5.0 mM TMB, the system was adjusted to 400 μL with NaAc-HAc buffer (200 mM, pH 4.0). The absorbance at 652 nm was monitored on a UV-1800 spectrophotometer. The study of carbosulfan degradation was similar, except for the incubation of carbosulfan in the NaAc-HAc buffer (pH 4.0 or pH 9.0).

Joint Inhibition

20 μL of various dursban concentrations (1.0, 3.0, 5.0, 7.0 and 10 μM) were firstly mixed with 20 μL of other pescticide with the same concentration, and then 40 μL of 200 mU mL^{-1} AChE was added. After incubation at 37 ^{o}C for 30 min, 40 μL of 1.0 mM ATCh and 40 μL of 0.2 mg mL^{-1} NC900 were added at 37 ^{o}C for 30 min. After the addition of 40 μL of 5.0 mM TMB, the system was adjusted to 400 μL with NaAc-HAc buffer (200 mM, pH 4.0) and incubated for 30 min. The absorbance at 652 nm was monitored on a UV-1800 spectrophotometer.

The theoretical absorbance $A = (A_1+A_2)/2$; A_1: the absorbance of the system based on the inhibitory ability of dursban with the same concentration on AChE; A_2: the absorbance of of the system based on the inhibitory ability of mixed pesticide with the same concentration on AChE.

Screening of AChE activator

After the above-described incubation of inhibitors and AChE, the pralidoxime iodide with various concentrations was added for 30 min. Subsequently, the similar procedure was operated. The UV-Vis spectra were monitored on a UV-1800 spectrophotometer.

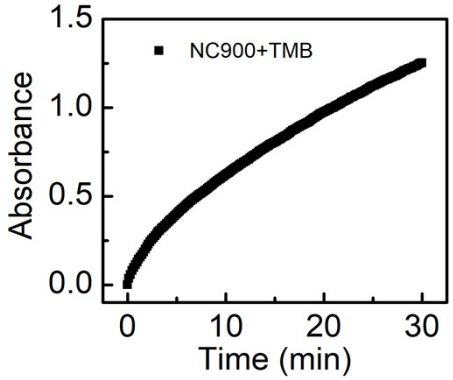

Figure S1. Time-dependent absorbance changes at 652 nm upon TMB oxidation.

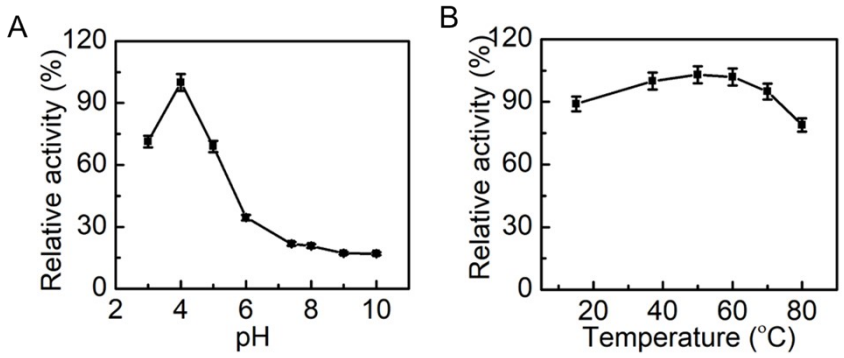

Figure S2. Influence of pH (A) and temperatures (B) on the catalytic activity of NC900.

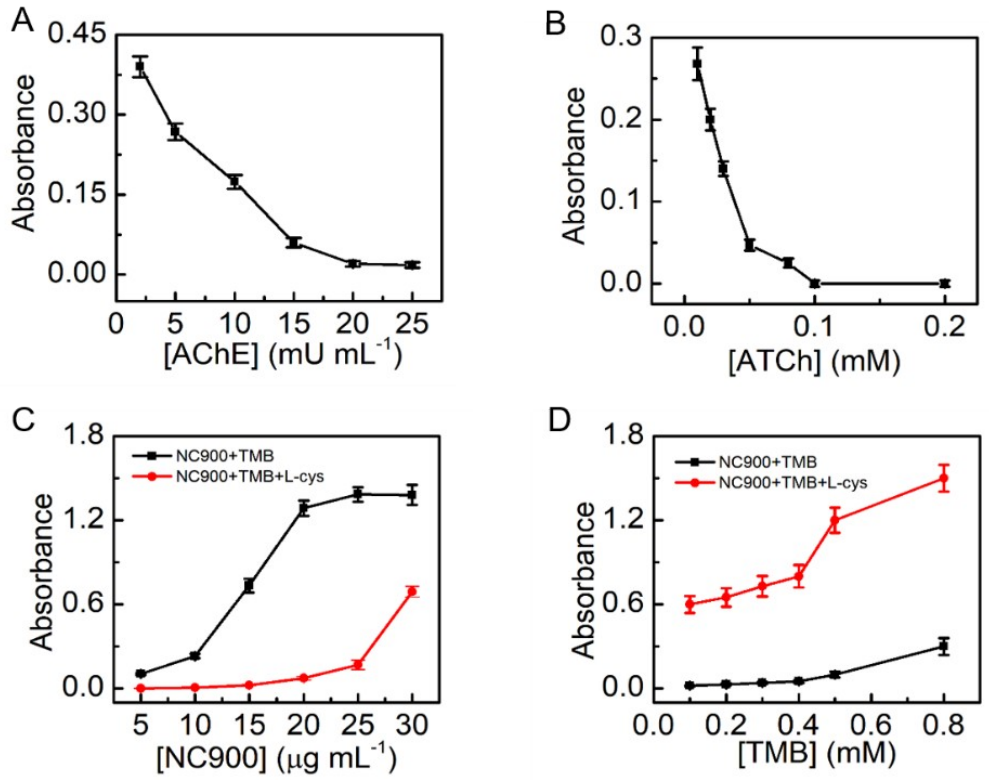

Figure S3. The influence of various factors on the assay, including AChE concentration (A); ATCh concentration (B); nanozyme concentration (C); and TMB concentration (D)

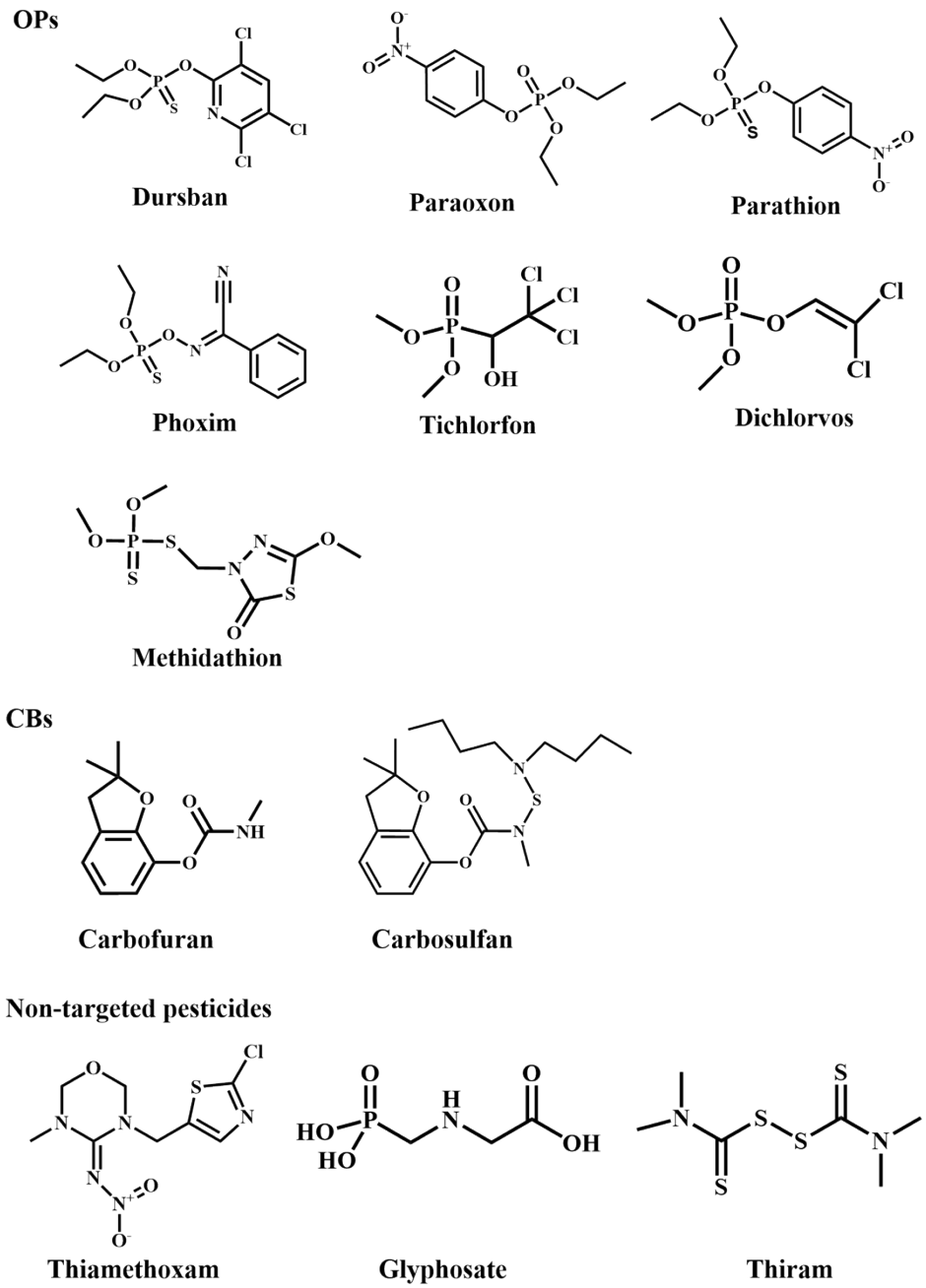

Figure S4. The chemical structures of the used pesticides in this work.

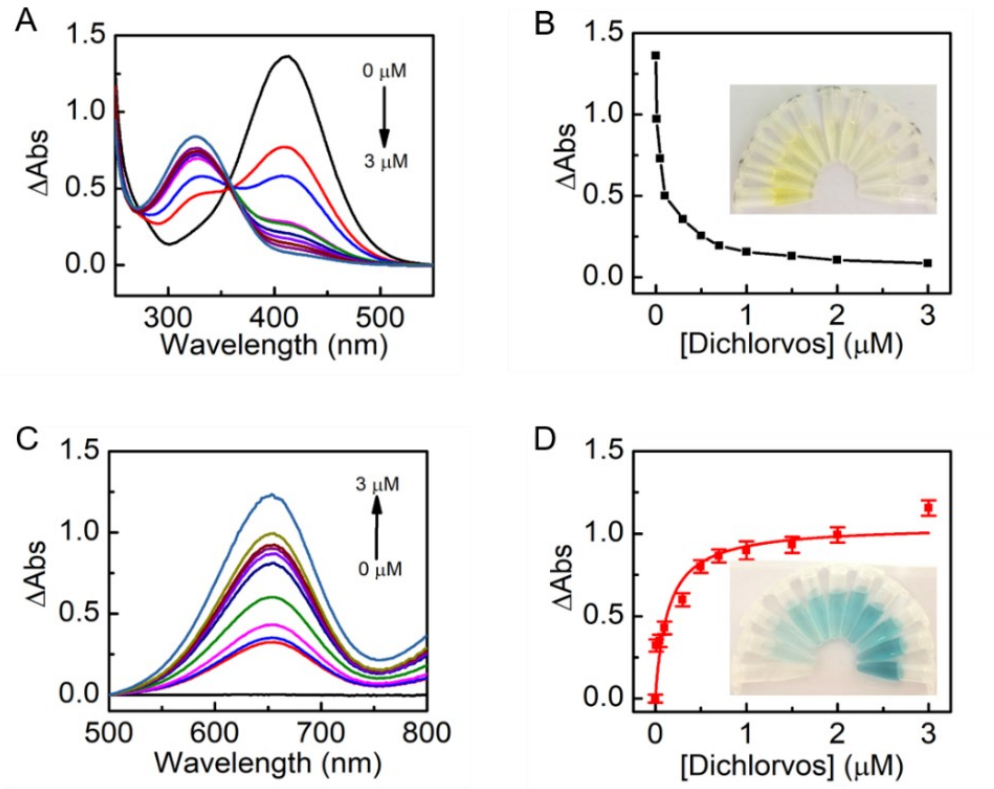

Figure S5. For Ellman's method: UV-vis absorption spectra with different dichlorvos concentration (A); the relationship between dichlorvos concentration and absorbance (B); For nanozyme-mediated assay: UV-vis absorption spectra with different dichlorvos concentration (C); the relationship between dichlorvos concentration and absorbance (D).

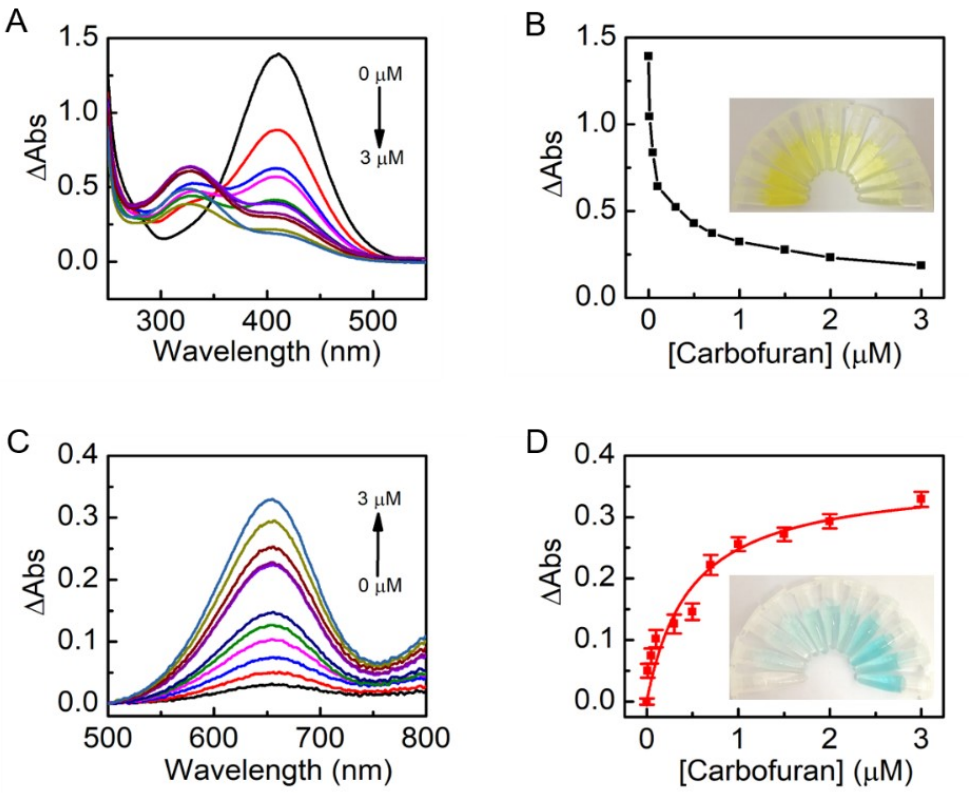

Figure S6. For Ellman's method: UV-vis spectra with different carbofuran concentration (A); the relationship between carbofuran concentration and absorbance (B); For nanozyme-mediated assay: UV-vis spectra with different carbofuran concentration (C); the relationship between carbofuran concentration and absorbance (D).

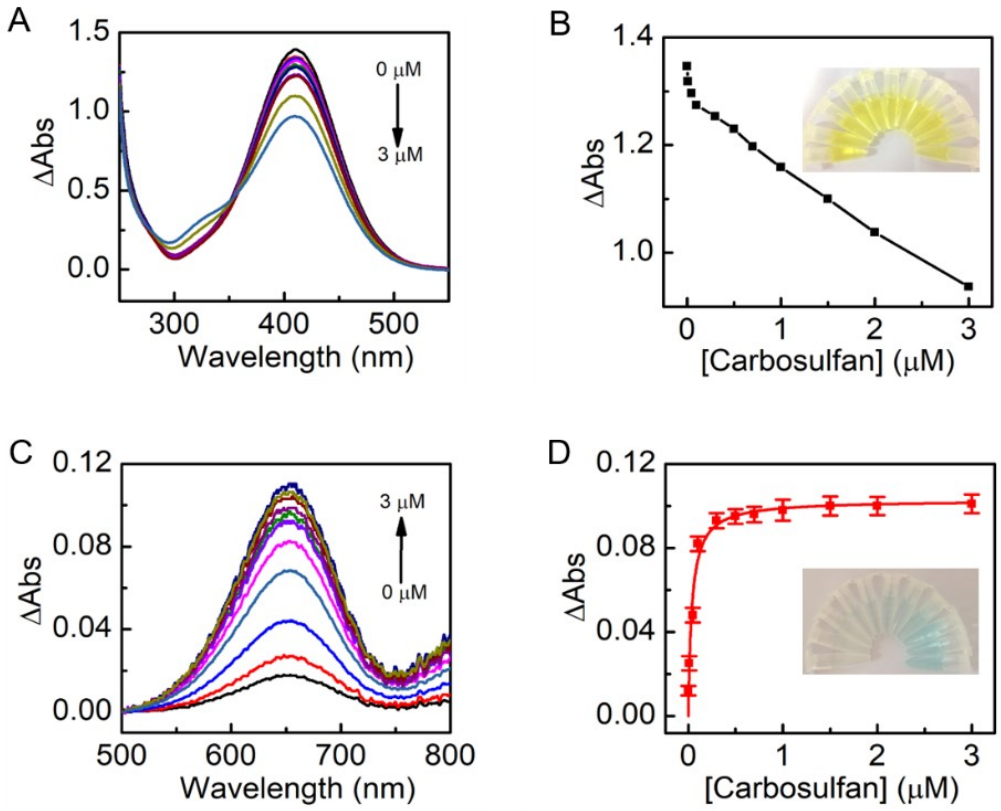

Figure S7. For Ellman's method: UV-vis spectra with different carbosulfan concentration (A); the relationship between carbosulfan concentration and absorbance (B); For nanozyme-mediated assay: UV-vis spectra with different carbosulfan concentration (C); the relationship between carbosulfan concentration and absorbance (D).

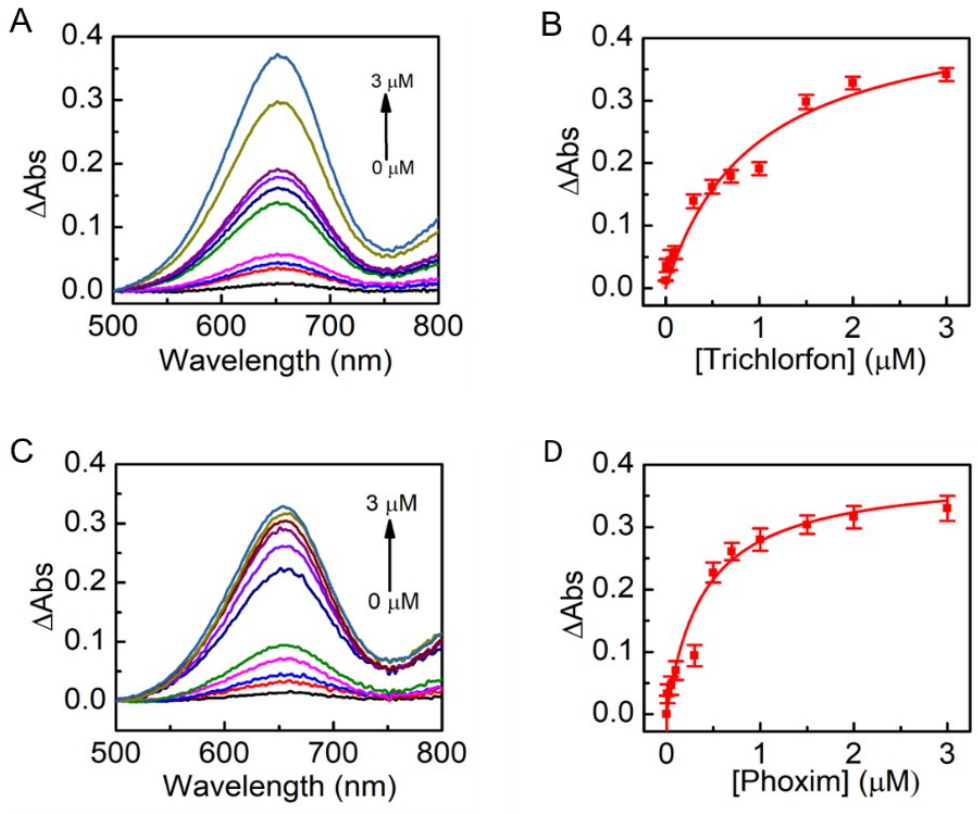

Figure S8. UV-vis spectra with different trichlorfon concentration (A); the relationship between trichlorfon concentration and absorbance (B); UV-vis spectra with different phioxm concentration (C); the relationship between phioxm concentration and absorbance (D).

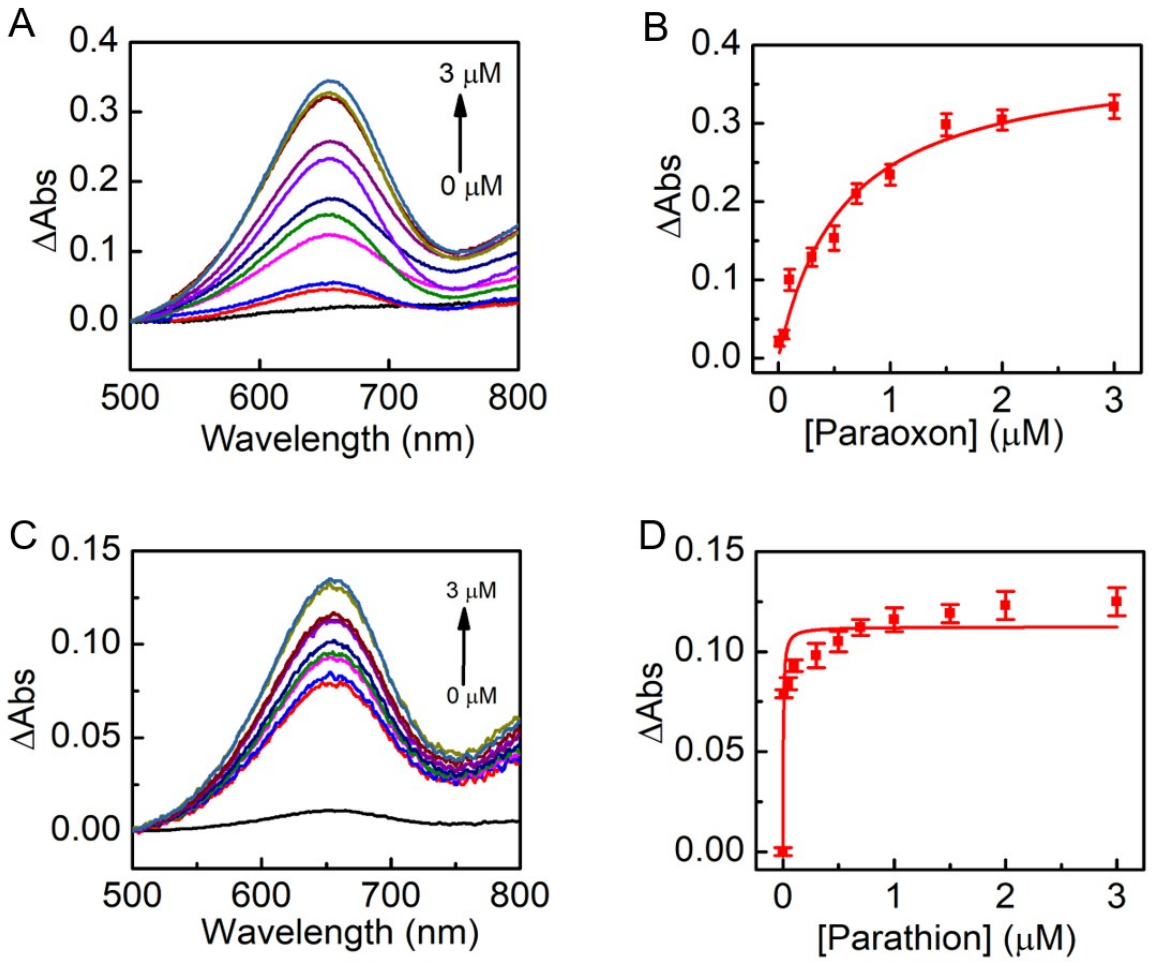

Figure S9. UV-vis spectra with different paraoxon concentration (A); the relationship between paraoxon concentration and absorbance (B); UV-vis spectra with different parathion concentration (C); the relationship between parathion concentration and absorbance (D).

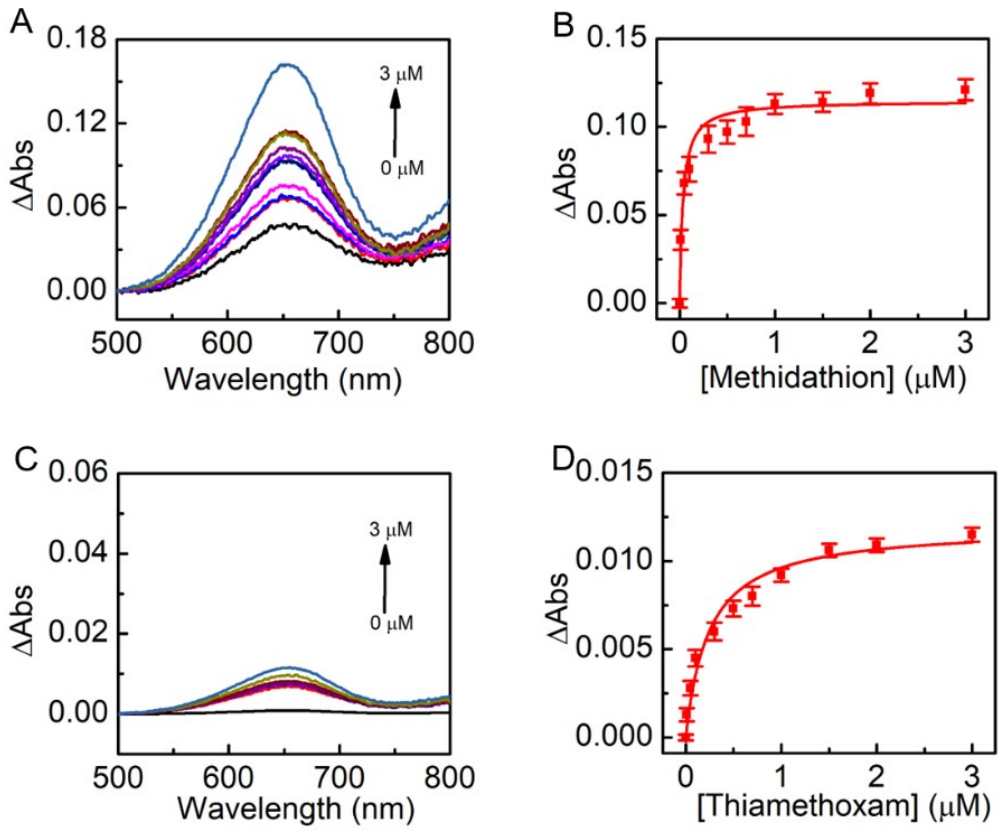

Figure S10. UV-vis spectra with different methidathion concentration (A); the relationship between methidathion concentration and absorbance (B); UV-vis spectra with different thiamethoxam concentration (C); the relationship between thiamethoxam concentration and absorbance (D).

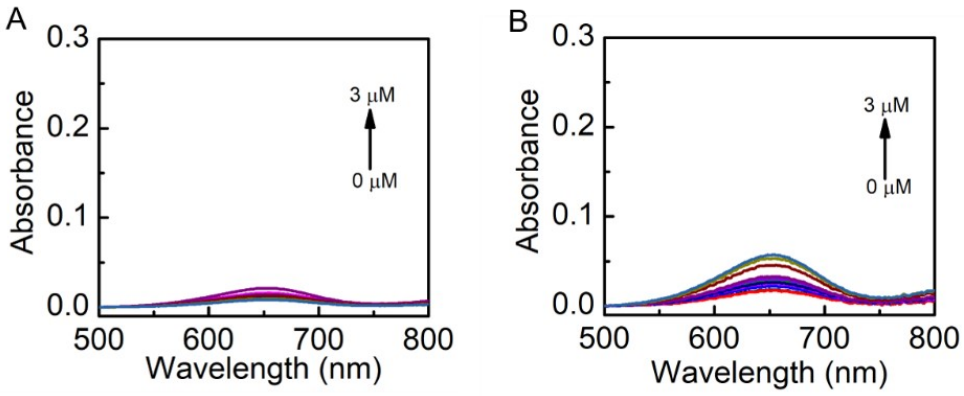

Figure S11. UV-vis spectra with different glyphosate concentration (A) and thiram concentration (B).

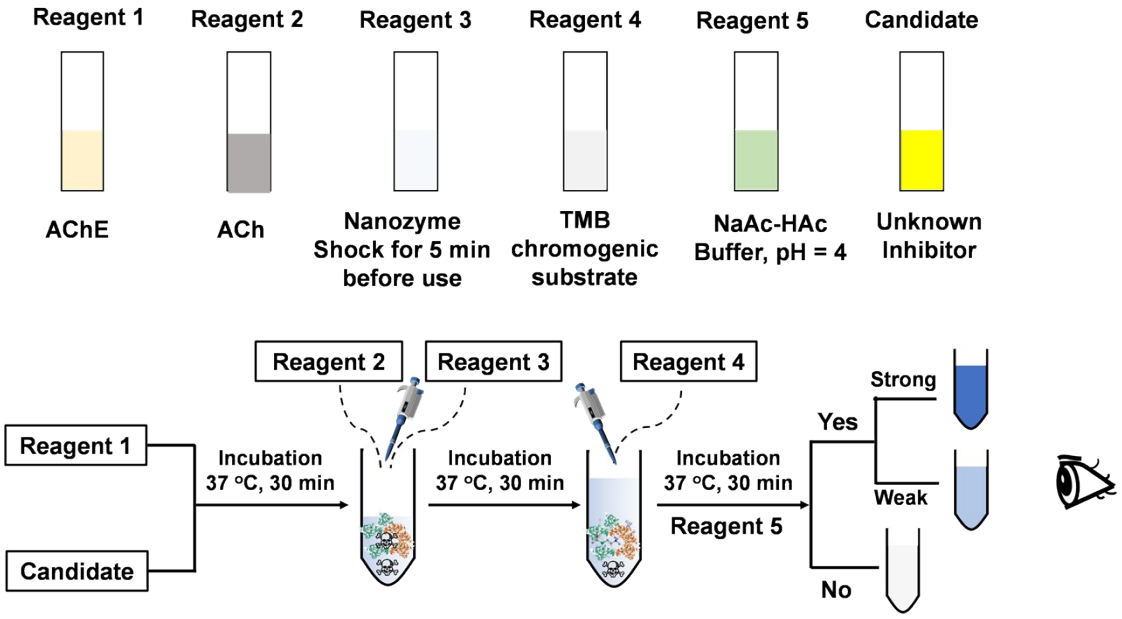

Figure 12. The proposed kit for AChE inhibitors screening.

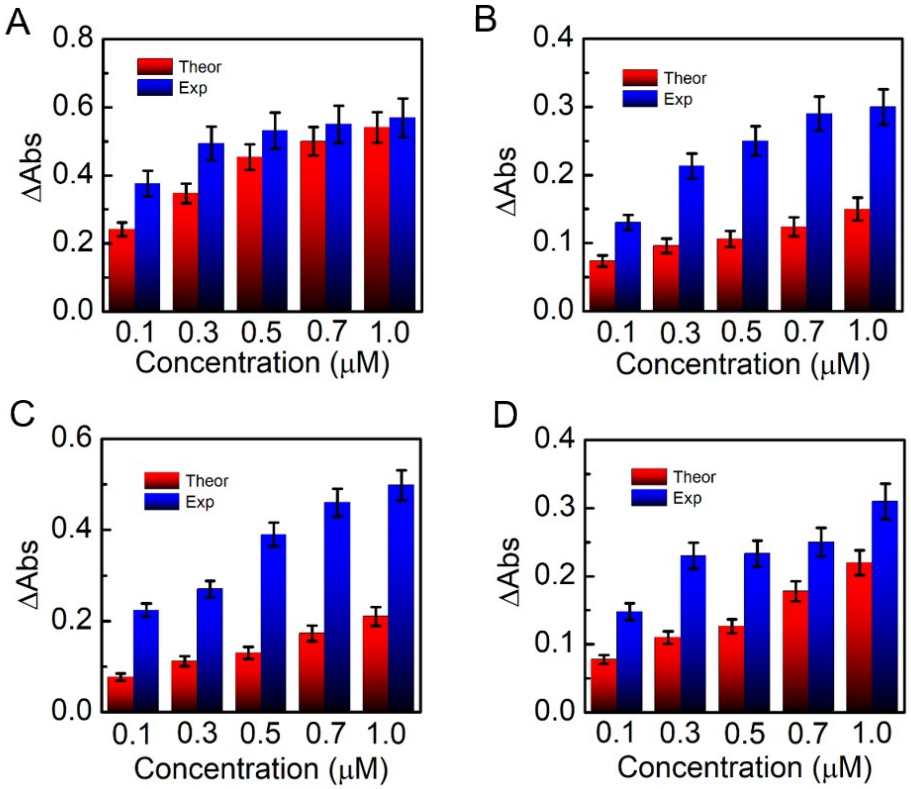

Figure S13. The absorbance changes of the system based on the mixture of dursban and other pesticide: dichlorvos (A); parathion (B); paraoxon (C); carbofuran (D).

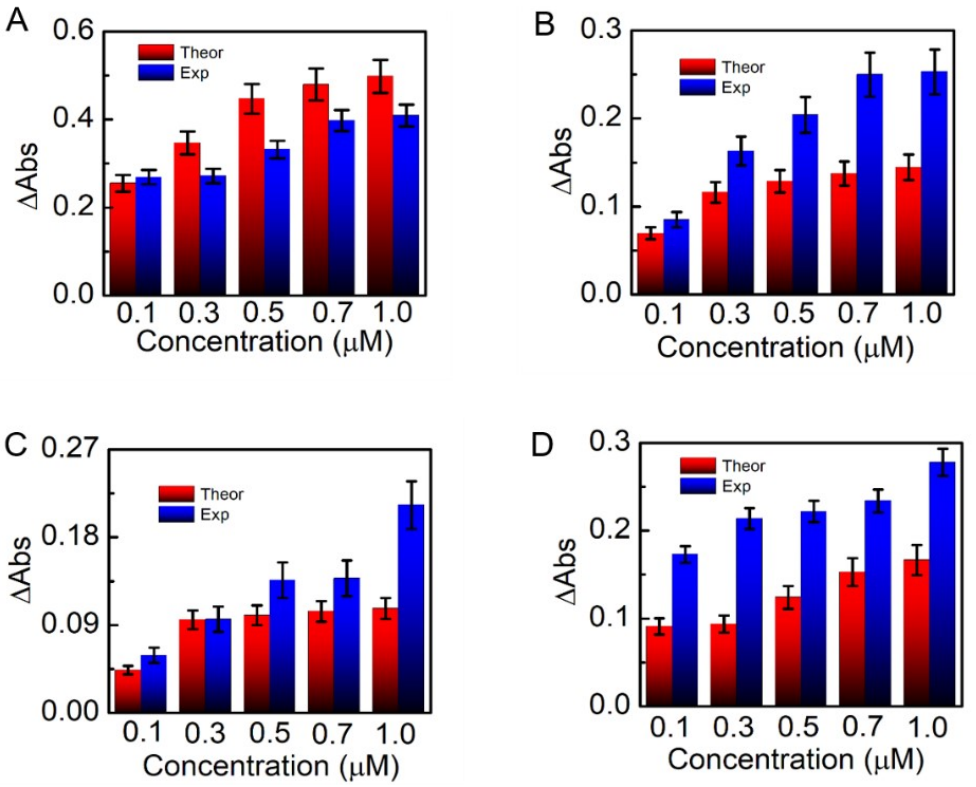

Figure S14. The absorbance change of carbosulfan mixed with different pesticides dichlorvos (A); trichlorfon (B); parathion (C); paraoxon (D).

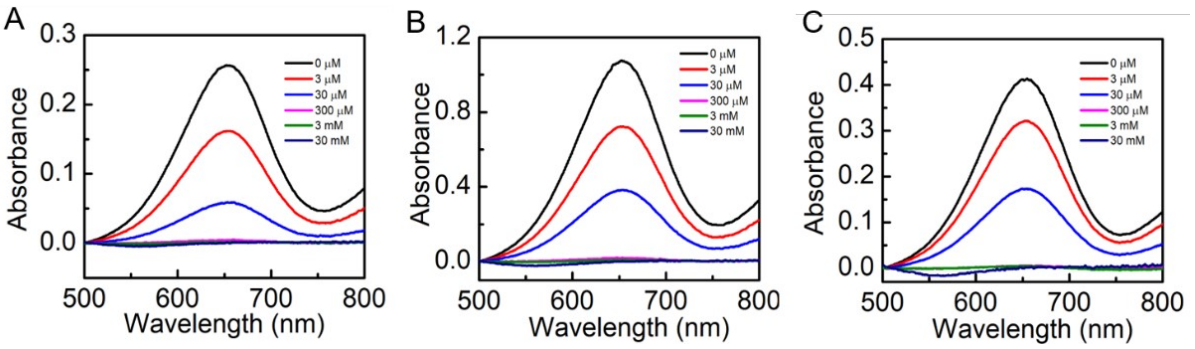

Figure S15. The influence of various pesticides mixed with pralidoxime iodide on absorbance spectra: dursban (A), dichlorvos (B), and trichlorfon (C).

Table S1. Contents of C, N, and O for NC900 from XPS characterization.

Sample	C (atomic%)	N (atomic%)	O (atomic%)
NC900	87.09	4.39	8.52

Table S2. Contents of different N species for NC900 from XPS characterization.

Sample	Pyridinic-N (%)	Pyrrolic-N (%)	Graphitic-N (%)	Oxidized-N (%)
NC900	36.0	11.5	39.8	12.7

Table S3. Comparison of the kinetic constants of oxidase-like carbon nanozymes.

Catalyst	Nanozyme (mg L^{-1})	K_m (mM)	Vmax (10^{-7} M s^{-1})	Ref
NC900	**20**	**0.26**	**1.52**	**This work**
N-PCNSs-5	25	0.095	0.027	*Nat. Commun.* 2018, 9, 1440.
N-PCNSs-3	25	0.084	0.040	
CSF-900	50	0.713	0.095	*Small* 2020, 16, 2004129
SeNPs	/	8.3	0.507	*J. Nanopart. Res.* 2016, 18,.74.
CS-SeNPs	/	0.852	0.238	*Biochem. Engin. J.* 2019, 152, 107384.
Triazine-based CTF-1	/	0.48	---	*Microchem. J.* 2017, 135, 91.
QAU-Z1	25	0.102	0.12	*Small 2022, 18, 2104993*
Fe-N-C	5	1.81	0.60	*Small* 2019, 15, 1903108.
Co-N-C	10	0.15	0.11	*Anal Bioanal Chem* 2022, 414, 1857
SA Fe-N-C	10	0.114	0.65	*Chem. Sci.* 2022,13, 4566
SA Fe nanozyme	5	0.13	0.225	*Chem. Commun.* 2019, 55 2285.
N-CDs	25	0.094	0.065	*Nano Today* 2022, 45, 101530
Fe-CDs	25	0.071	0.171	
Fe-N/C-CNTs	20	0.62	5.26	*Chem. Commun.* 2019, 55 5271.
Fe-N/C	20	0.94	5.98	*Sens. Actuators B.* 2020, 305 127511

Table S4. Summary of the relationship between inhibitor concentration and absorbance

Pesticide	M-M equation	K_m	A_m	R^2
Dursban	$A = \frac{0.21575c}{0.39369 + c}$	0.39369	0.21575	0.958
Dichlorvos	$A = \frac{1.05092c}{0.13813 + c}$	0.13813	1.05092	0.924
Carbofuran	$A = \frac{0.36658c}{0.47978 + c}$	0.47978	0.36658	0.967
Carbosulfan	$A = \frac{0.10282c}{0.03932 + c}$	0.03932	0.10282	0.959
Tichlorfon	$A = \frac{0.45607c}{0.94644 + c}$	0.94644	0.45607	0.925
Phoxim	$A = \frac{0.38818c}{0.39833 + c}$	0.39833	0.38818	0.963
Paraoxon	$A = \frac{0.38712c}{0.58014 + c}$	0.58014	0.38712	0.970
Parathion	$A = \frac{0.11241c}{0.00428 + c}$	0.00428	0.11241	0.986
Methidathion	$A = \frac{0.11474c}{0.03491 + c}$	0.03491	0.11474	0.987
Thiamethoxam	$A = \frac{0.01195c}{0.24304 + c}$	0.24304	0.01195	0.986