Electronic Supplementary Information (ESI) for

Design and construction of competitive, noncompetitive and uncompetitive nano inhibitors of enzymes

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Table of contents

1 Experimental section

1.1 Materials

Methanol (CH_3OH), acetic acid (CH_3COOH), sodium hydroxide (NaOH), sodium chloride (NaCl), sodium borohydride (NaBH4), disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Sinopharm Chemical Reagent Co., Ltd.. Alpha-amylase (Amy), trypsin (Trp), para-mercaptobenzoic acid (*p*MBA) and acetylcholinesterase (AChE) from electric electricus were purchased from Shanghai Macklin Biochemical Co., Ltd.. Bovine α-chymotrypsin (ChT) was purchased from Tokyo Chemical Industry Co., Ltd.. Gold(III) chloride hydrate ($HAuCl_4 \cdot nH_2O$) was purchased from Energy Chemicals. GOD/POD glucose detection kit was purchased from Shanghai Yuanye Bio-Technology Co., Ltd.. Lipoic acid (LA) was purchased from Accela ChemBio Co., Ltd.. Rivastigmine, N-*α*-benzoyl-L-arginine-4-nitroanilide hydrochloride (L-BA*p*NA), *N*-succinyl-$_L$-phenylalanine-*p*-nitroanilide (SPNA) were purchased from Sigma-Aldrich. Ortho-nitrophenyl acetate (*o*-NPA) and 4,6-diamino-2-pyrimidinethiol were purchased from Bide Pharmatech Ltd.. Soluble starch was purchased from Chengdu Hongbo Industry Co., Ltd.. Maltose was purchased from Beijing Biodee Biotechnolgoy Co., Ltd.. Human serum albumin (HSA) was purchased from Shanghai regal Biology Technology Co, Ltd.. Tween 80 was purchased from Shanghai Enox Co, Ltd.. 0.01 M phosphate buffer solution (pH = 7.4) prepared with deionized water (Millipore) was used in all experiments.

1.2 Methods

1.2.1 Preparation of gold nanoparticles

DAPT-AuNPs were synthesized with a modified method according to the literature.[1] 1.4 mg DAPT was dissolved in 3 mL methanol with 20 μL acetic acid and 4 mg Tween 80. Then 0.1 mL $HAuCl_4$ (100 mM) solution was added into the DAPT solution with adequate stirring for 10 min in the ice-water bath. $NaBH_4$ solution (1.2 mg in 0.5 mL methanol) was then added dropwise. The mixture was stirred for another 1 h in the ice-water bath.

*p*MBA-AuNCs were synthesized with a modified method according to the reference.[2] 0.1 M NaOH was added dropwise to *p*MBA solution (49.4 mM) to reach a complete dissolution. 0.8 mL *p*MBA solution was then added to 10 mL $HAuCl_4$ (2 mM) under vigorous stirring. After reaction for 0.5 h, the pH of the solution was adjusted to 11.0 with 1 M NaOH. After that, 900 μL $NaBH_4$ (0.88 mM) was added dropwise to the solution. After stirring for 5 min, the solution was heated to 40 ℃ for 24 h.

The synthesis method was modified on the basis of reference.[3] 1.6 mg LA was added to 5 mL deionized water with appropriate NaOH to help dissolve it. 78 μL $HAuCl_4$ (100 mM) and 2.5 mL LA solution were added to 10 mL deionized water successively. After stirring for 0.5 h, 1.4 mL $NaBH_4$ (66 μM) was added dropwise to the solution. The reaction was then stirred at room temperature for 6 h.

DHLA-AuNCs were synthesized with the modeified method of reference.[4] Forty μL NaOH (1 M) was added to help the dissolution of LA (2.6 mg, 8 mL). The newly-prepared LA solution was then added to 40 μL $HAuCl_4$ (0.1 M) to react for 30 min under vigorous stirring. After that, 160 μL $NaBH_4$ (50 mM) was added dropwise to the solution to react for another 6 h.

All the gold nanoparticles were dialyzed twice in deionized water for 2 h each time, followed by once in PBS.

1.2.2 Characterizations of gold nanoparticles

TEM images were recorded on a JEOL JEM-2100 transmission electron microscope. UV-vis absorption spectra were recorded by Cary 100 UV-vis spectrophotometer (Agilent). A Cary Eclipse fluorophotometer (Agilent) was used to perform the fluorescence properties of AuNCs.

1.2.3 Calculation of the concentration of gold nanoparticles

The volume of AuNPs was calculated from the particle size using the volume formula of sphere. Then the mass of each AuNP was calculated according to the volume and density. Then the number of gold atoms contained in each AuNP was calculated by dividing the resulting mass by the mass of Au atom. Au atom concentration of AuNP solution was calculated by the determination of the digested sample by inductively coupled plasma atomic energy spectrometry (ICP-AES, IRIS Intrepid II XSP). Finally,

the concentration of AuNP was obtained by dividing Au atom concentration by the number of gold atoms contained in each AuNP.

1.2.4 Activity assays

L-BA*p*NA, SPNA and *o*-NPA were used as the substrate of Trp, ChT and AChE respectively. The hydrolysis products of L-BA*p*NA, SPNA and *o*-NPA all had a characteristic absorption peak near 405 nm. Therefore, we could use VICTOR X5 microplate reader (PerkinElmer) to record the increase of the absorbance at 405 nm (Abs405) to monitor the reaction process of Trp, ChT and AChE.

Typically, 30 μL gold nanoparticles with different concentrations were firstly added to each well, 50 μL enzyme solution (0.16 μM for Trp, 16 μM for ChT and AChE) was then added to incubate with nanoparticles for 1 h. After adding 120 μL substrate (0.5 mM), Abs405 was then recorded every 2 min for a total of 14 times.

In order to investigate the inhibition effect of DAPT-AuNPs, 30 μL DAPT-AuNPs with Au concentrations ranging from 0 - 90 μM were firstly added to each well, 50 μL AChE solution (16 μM) was then added to incubate with DAPT-AuNPs for 1 h. After adding 120 μL *o*-NPA solution (0.5 mM), Abs405 was then recorded every 2 min for a total of 14 times.

In order to investigate the effect of rivastigmine to AChE inhibition by *p*MBA-AuNCs and DHLA-AuNCs, 1.8 μM rivastigmine was added to AChE before adding different concentrations of *p*MBA-AuNCs and DHLA-AuNCs.

In order to investigate the effect of NaCl and HSA to the enzyme inhibition by AuNPs, different concentrations of NaCl (0 - 0.8 M) or HSA (0 - 512 μM) were added to the AuNPs before their incubation with enzymes.

In order to test the inhibition effect of AuNPs to GOD/POD, 30 μL AuNPs (18 μM for *p*MBA-AuNCs, 0.96 μM for DHLA-AuNPs and 4 μM for DHLA-AuNCs) were incubated with 100 μL working solution of GOD/POD detection kit for 1 h. Then the substrate, 30 μL maltose solution (0.4 mM) was added to the mixture. Similar to glucose, using maltose as the substrate could also produce a product with a characteristic absorption peak near 490 nm.[5] Therefore, Abs490 was then recorded every 2 min for a total of 14 times.

Starch can be hydrolyzed to maltose under the catalysis of Amy, therefore, GOD/POD detection kit could be used to measure the activity of Amy where the substrate was replaced from maltose to the mixture of 30 μL starch solution (20 g/L) and 40 μL Amy solution (3.2 μM) after knowing that there were no significant inhibition effects of AuNPs to GOD/POD. It was worth mentioning that starch solution needed to be dissolved in boiling water bath.

1.2.5 Inhibition kinetics assays

In a typical inhibition kinetics assay, 30 μL gold nanoparticles with different concentrations were firstly added to each well, 50 μL enzyme solution (16 μM) was then added to incubate with nanoparticles for 1 h. After adding 120 μL substrate with different concentrations (0 mM, 0.17 mM, 0.20 mM, 0.25 mM,0.33 mM 0.5 mM) , Abs405 was then recorded every 2 min for a total of 14 times.

1.2.6 Calculation of IC_{50}

The calculating methods of IC_{50} depend on the inhibition types.

For noncompetitive inhibition, IC_{50} equals to K_i which can be calculated by the following equation:

$$V_{max}^{app} = \frac{V_{max}}{1 + \frac{[I]}{K_i}}$$

For competitive inhibition, $IC_{50} = K_i \times (1+\frac{[S]}{K_M})$, and K_i could be calculated by the following equation:

$$K_M^{app} = K_M + \frac{K_M}{K_i}[I]$$

For uncompetitive inhibition, $IC_{50} = K_i$, and K_i could be calculated by the following equation:

$$V_{max}^{app} = \frac{V_{max}}{1 + \frac{[I]}{K_i}}$$

1.2.7 Zeta potential

The mixtures of 500 μL AChE (12 μM) and different volumes of *o*-NPA (0 - 900 μL) were supplemented with PBS to a total volume of 1.6 mL. The zeta potential of

each mixture was measured on a Nano-ZS90 zetasizer (Malvern).

1.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out with a MINI-PROTEAN Tetra System (BIO-RAD) to verify whether AuNPs bound to enzymes. Twenty μL samples of ChT (250 μM), AChE (250 μM), different AuNPs and their mixed solution were electrophoresed at 80 V in the 1.5% gel (JY600C). The gel was visualized by a camera and Gel Doc XR+ Imaging System (BIO-RAD) before and after being stained with Coomassie blue.

1.2.9 Circular dichroism spectroscopy

CD spectra were measured on a circular dichroism photomultiplier (Applied Photophysics Limited, UK) to determine the secondary structural changes of ChT (6.4 μM) and AChE (24 μM) after 1 h of incubation with different AuNPs. Denatured ChT (DChT) and denatured AChE (DChT) obtained from a 60 ℃ water bath for 2 h were also measured as a comparison.

2. Activity Assays of DAPT-AuNPs

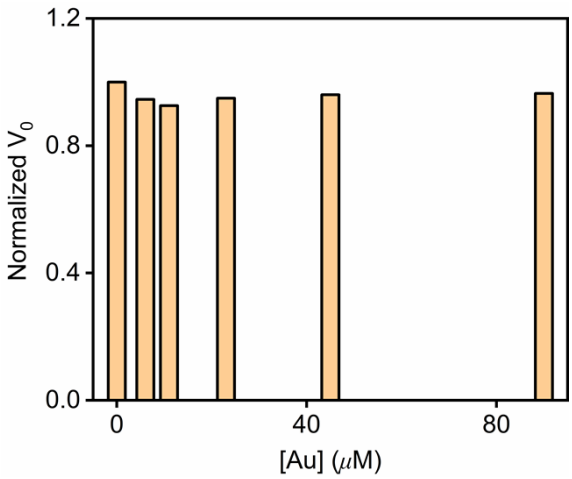


Fig. S1 Normalized initial rates (v_0) of AChE catalytic reactions under different concentrations of DAPT-AuNPs.

3. Characterizations of Gold Nanoparticles

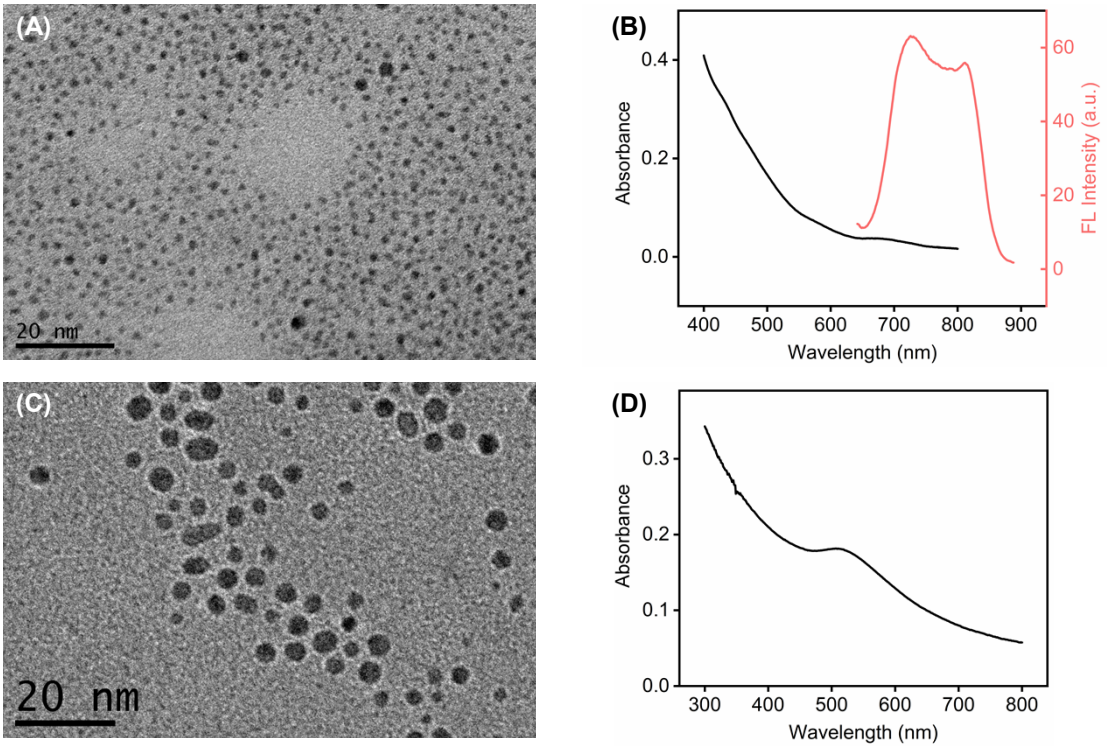


Fig. S2 TEM photographs of (A) *p*MBA-AuNCs and (B) DHLA-AuNPs and spectra of (C) *p*MBA-AuNCs and (D) DHLA-AuNPs.

4. The Effect of NaCl

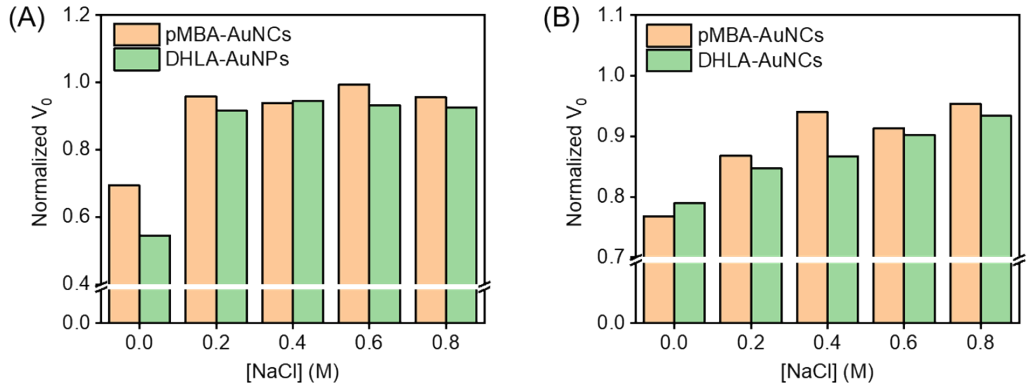


Fig. S3 Normalized initial rates (v_0) of (A) ChT in the presence of 0.27 μM *p*MBA-AuNCs and 72 nM DHLA-AuNPs respectively and (B) AChE in the presence of 2.7 μM *p*MBA-AuNCs and 0.72 μM DHLA-AuNCs respectively under different concentrations of NaCl.

5. Inhibition Kinetics Assays

Table S1 Apparent Maximum Rate (v_{max}^{app}) and the Apparent Michaelis Constant (K_M^{app}) of ChT under Different Concentrations of *p*MBA-AuNCs

[*p*MBA-AuNCs] (μM)	0	0.09	0.11	0.14	0.18	0.27
K_M^{app} (mM)	0.75	0.71	0.71	0.71	0.73	0.74
v_{max}^{app} (nmol/min)	0.16	0.13	0.12	0.12	0.12	0.11
R^2	0.988	0.974	0.967	0.962	0.990	0.987

Table S2 Apparent Maximum Rate (v_{max}^{app}) and the Apparent Michaelis Constant (K_M^{app}) of ChT under Different Concentrations of DHLA-AuNPs

[DHLA-AuNPs] (nM)	0	9	18	36	72
K_M^{app} (mM)	0.80	0.98	1.11	1.53	2.63
v_{max}^{app} (nmol/min)	0.19	0.19	0.19	0.19	0.20
R^2	0.993	0.995	0.997	0.995	0.986

Table S3 Apparent Maximum Rate (v_{max}^{app}) and the Apparent Michaelis Constant (K_M^{app}) of AChE under Different Concentrations of *p*MBA-AuNCs

[*p*MBA-AuNCs] (μM)	0	0.4	0.7	1.4	2.7
K_M^{app} (μM)	58.60	46.15	42.97	48.30	44.21
v_{max}^{app} (nmol/min)	0.98	0.81	0.76	0.76	0.70
K_M^{app}/v_{max}^{app} (min/mL)	59.94	56.68	56.77	63.89	62.97
R^2	0.988	0.992	0.998	0.997	0.993

Table S4 Apparent Maximum Rate (v_{max}^{app}) and the Apparent Michaelis Constant (K_M^{app}) of AChE under Different Concentrations of DHLA-AuNCs

[DHLA-AuNCs] (μM)	0	0.24	0.48	0.72
K_M^{app} (μM)	151.83	129.11	127.86	133.08
v_{max}^{app} (nmol/min)	1.58	1.35	1.28	1.25
K_M^{app}/v_{max}^{app} (min/mL)	95.90	95.48	100.08	106.55
R^2	0.998	0.998	0.998	0.997

6. Calculation of Inhibition Constants (K_i)

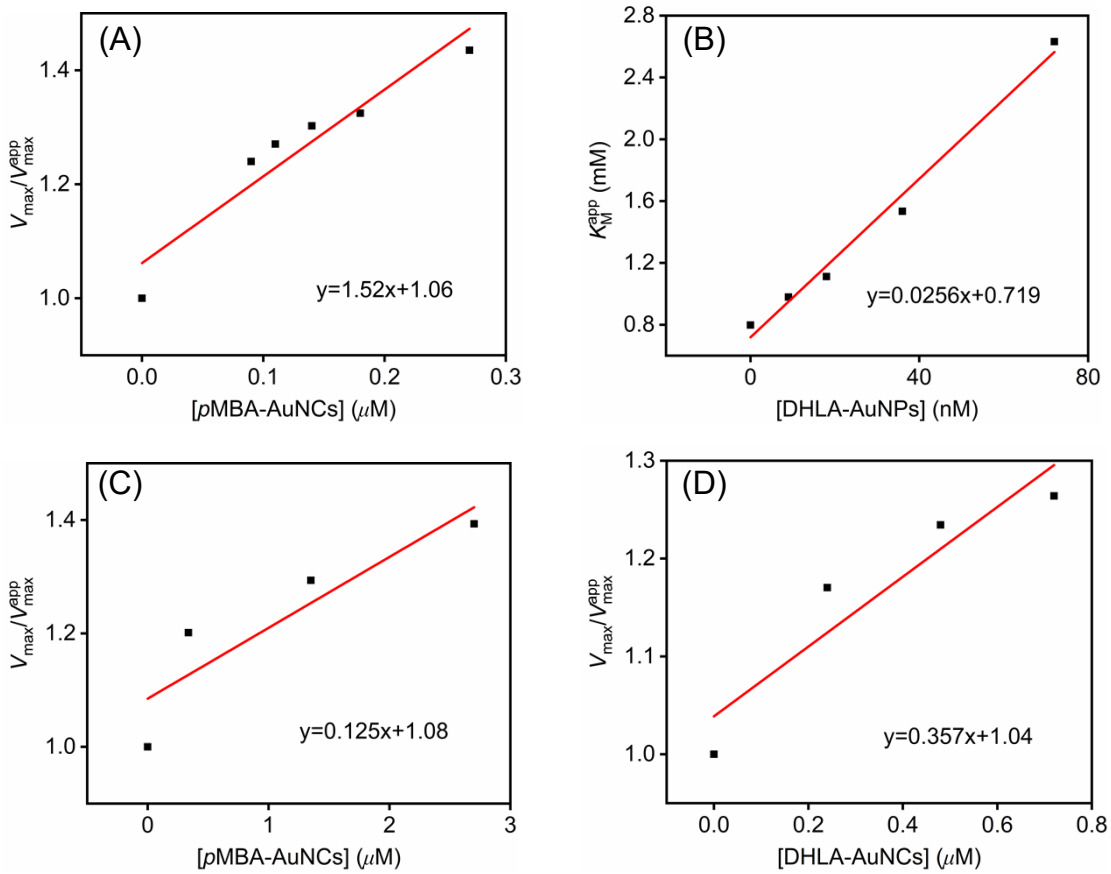


Fig. S4 Fitted lines of plots of enzymatic kinetic parameters to concentrations of nano inhibitors for calculating inhibition constants (K_i) when (A) *p*MBA-AuNCs inhibited ChT, (B) DHLA-AuNPs inhibited ChT, (C) *p*MBA-AuNCs inhibited AChE and (D) DHLA-AuNCs inhibited AChE.

7. The Effect of Rivastigmine

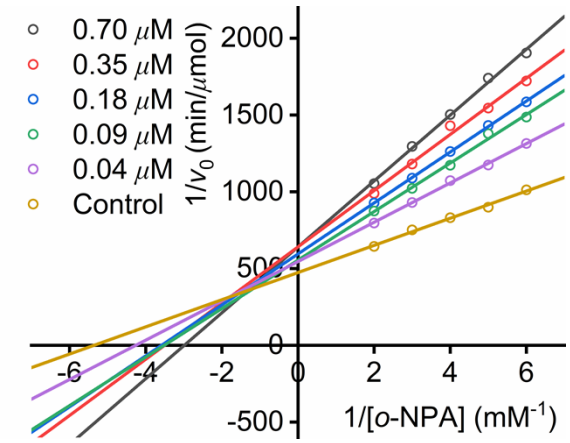


Fig. S5 Lineweaver-Burk plot for acetylcholinesterase under different concentrations of rivastigmine and *o*-NPA.

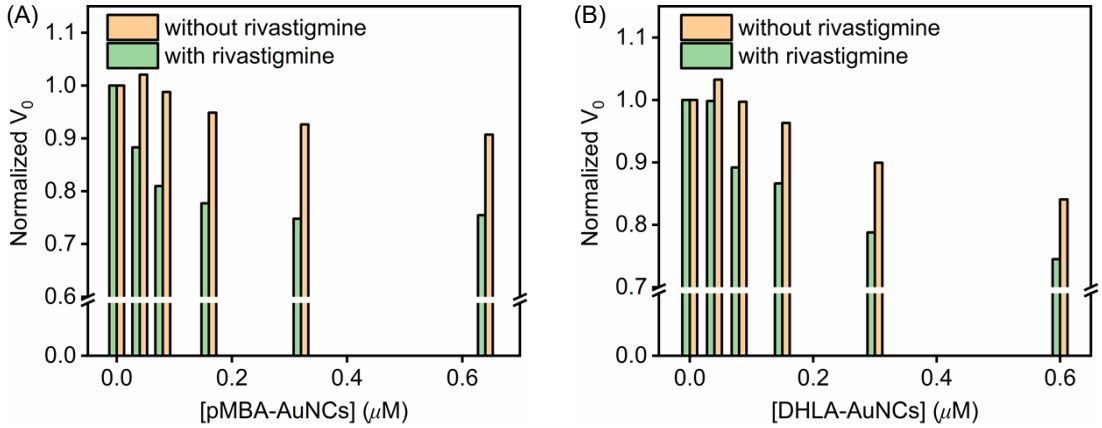


Fig. S6 Normalized initial rates (v_0) of acetylcholinesterase with or without rivastigmine under different concentrations of (A) *p*MBA-AuNCs and (B) DHLA-AuNCs.

8. Proofs and Verifications of Inhibition Models

8.1 Zeta Potential

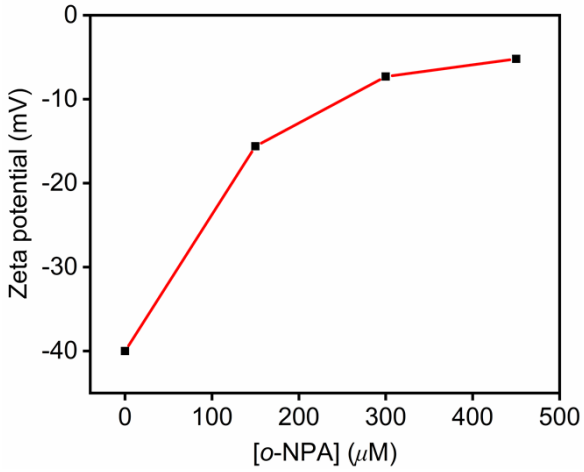
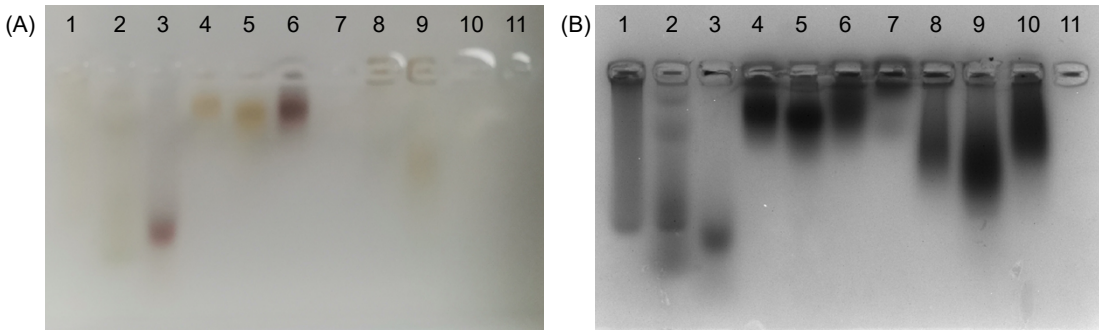


Fig. S7 Zeta potential of acetylcholinesterase under different concentrations of *o*-NPA.

8.2 Agarose Gel Electrophoresis



Lane	1	2	3	4	5	6	7	8	9	10	11
AuNPs	DHLA-AuNCs	pMBA-AuNCs	DHLA-AuNPs	DHLA-AuNCs	pMBA-AuNCs	DHLA-AuNPs		DHLA-AuNCs	pMBA-AuNCs		Blank
Enzymes				ChT	ChT	ChT	ChT	AChE	AChE	AChE	

Fig. S8 Images of agarose gel electrophoresed with DHLA-AuNCs (20 μM), *p*MBA-AuNCs (27 μM), DHLA-AuNPs (1.4 μM), ChT (250 μM), AChE (250 μM) and the mixed samples of the same concentration before (A) and after (B) being stained with Coomassie blue.

8.3 Circular Dichroism Spectroscopy

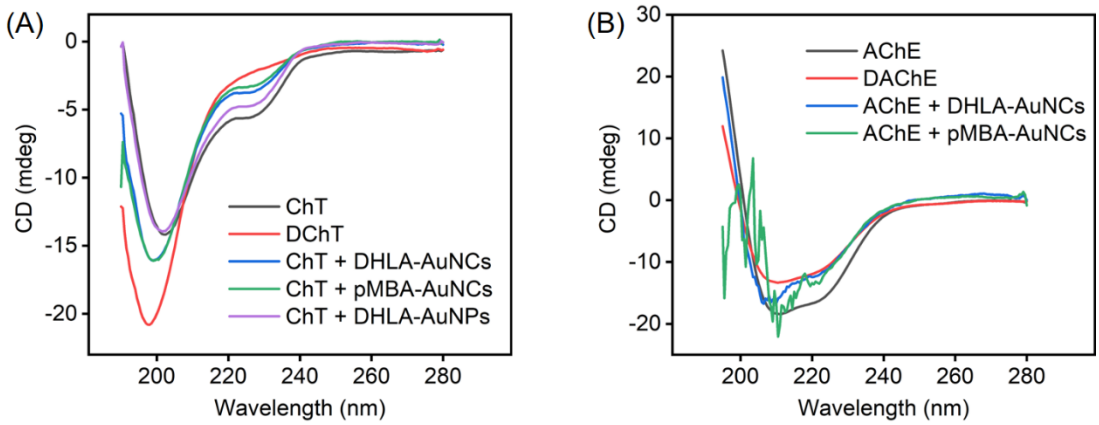


Fig. S9 CD spectra of (A) 6.4 μM ChT before and after incubation with DHLA-AuNCs (0.45 μM), *p*MBA-AuNCs (0.45 μM) and DHLA-AuNPs (0.2 μM) and (B) 24 μM AChE before and after incubation with DHLA-AuNCs (12 μM) and *p*MBA-AuNCs (12 μM). The fluctuation of the green line in figure B was caused by the strong signal interference of the high-concentration *p*MBA-AuNCs at low wavelength.

9. Selectivity

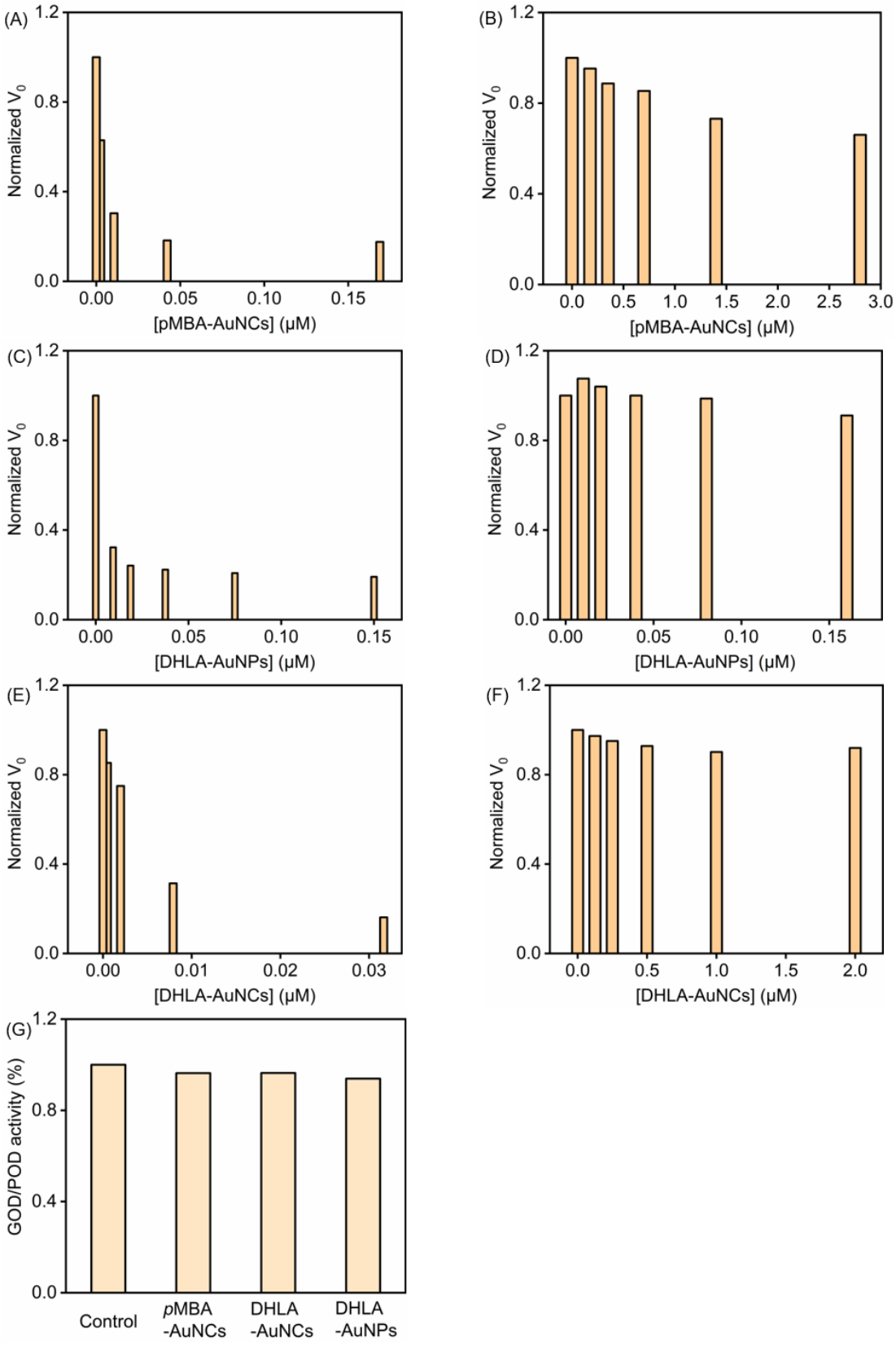


Fig. S10 Normalized initial rates of trypsin (Trp) under different concentrations of (A) *p*MBA-AuNCs, (C) DHLA-AuNPs, (E) DHLA-AuNCs, α-amylase (Amy) under

different concentrations of (B) *p*MBA-AuNCs, (D) DHLA-AuNPs, (F) DHLA-AuNCs and (G) glucose oxidase (GOD) /peroxidase (POD) in the absence and presence of 5.4 μM *p*MBA-AuNCs, 1.2 μM DHLA-AuNCs or 288 nM DHLA-AuNPs.

Table S5 Selectivity of three gold nanoparticles for enzyme inhibition

	Trp	ChT	AChE	Amy	GOD	POD
*p*MBA-AuNCs	Inh.	Inh.	Inh.	Inh.	Uninh.	Uninh.
DHLA-AuNCs	Inh.	Inh.	Inh.	Uninh.	Uninh.	Uninh.
DHLA-AuNPs	Inh.	Inh.	Uninh.	Uninh.	Uninh.	Uninh.
Isoelectric point	10.5	8.5	5.5	4.2	4.2	6.0

10. The effect of HSA

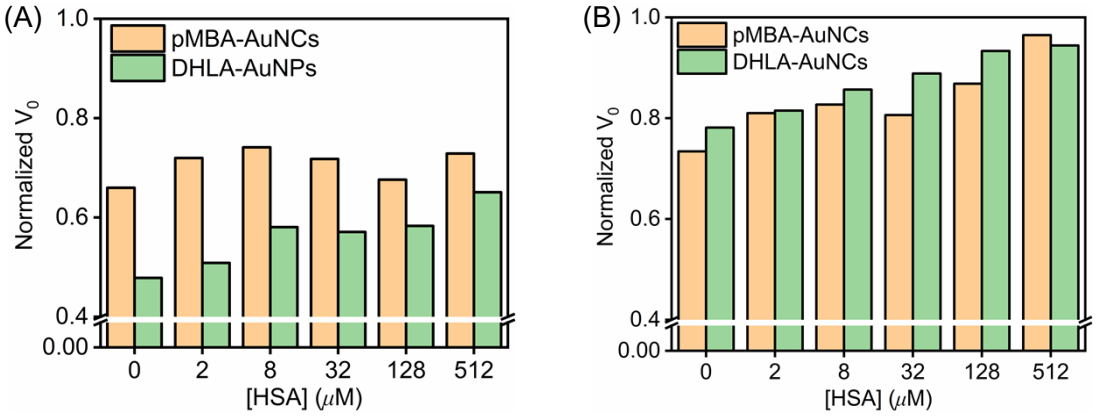


Fig. S11 Normalized initial rates (v_0) of (A) ChT in the absence or presence of 0.27 μM *p*MBA-AuNCs or 72 nM DHLA-AuNPs and (B) AChE in the absence or presence of 2.7 μM *p*MBA-AuNCs or 0.72 μM DHLA-AuNCs under different concentrations of HSA.

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