Supplementary Materials

Application of Peroxidase-Mimic Mn₂BPMP Boosted by ADP to Enzyme Cascade Assay for Glucose and Cholesterol

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Experimental section

Synthesis of 2,6-bis(chloromethyl)-4-methylphenol

2,6-Bis(chloromethyl)-4-methylphenol was synthesized following the procedure previously described.[1] Briefly, 2,6-bis(hydroxymethyl)-*p*-cresol (2.5 g, 15 mmol) in DCM (30 mL) was added to thionyl chloride (10 mL, 140 mmol). After stirring for 12 h, the yellow mixture was washed with water and brine. The organic layer was dried with anhydrous sodium sulfate and concentrated under the reduced pressure to give a white solid. Yield 2.8 g (92%). ^{1}H-NMR (400 MHz, CHLOROFORM-D): δ 7.09 (s, 2H), 5.52 (s, 1H), 4.66 (s, 4H), and 2.29 (s, 3H).

Synthesis of 2,6-bis{[bis(2-pyridylmethyl)amino]methyl}-4-methylphenol (H-BPMP)

H-BPMP was synthesized following the procedure previously described.[1] Briefly, 2,6-bis(chloromethyl)-4-methylphenol (2.8 g, 14 mmol) was dissolved in 30 mL of THF in an ice bath. Then, a solution of 2,2'-dipicolylamine (4.9 mL, 27 mmol) and triethylamine (11.6 mL, 55 mmol) in 30 mL of THF was added dropwise. After stirring for 5 days at room temperature, the mixture was filtered and concentrated under a reduced pressure. The mixture was dissolved in DCM then washed three times with brine. The organic layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The mixture was hot filtered with diethyl ether to give a pale-yellow solid. Yield 6.2 g (85%). ^{1}H-NMR (400 MHz, CHLOROFORM-D): δ 10.77 (s, 1H), 8.51 (dt, *J* = 4.9, 0.9 Hz, 4H), 7.60 (td, *J* = 7.6, 1.8 Hz, 4H), 7.50 (d, *J* = 7.9 Hz, 4H), 7.14-7.09 (m, 4H), 6.99 (s, 2H), 3.86 (s, 8H), 3.77 (s, 4H), and 2.23 (s, 4H). ^{13}C-NMR (400 MHz, CHLOROFORM-D): δ 159.3, 153.6, 148.9, 136.5, 129.8, 127.3, 123.8, 122.9, 122.0, 59.8, 54.8, and 20.6. mp at 105-107 ℃.

Preparation of the Mn₂BPMP

Mn₂BPMP was prepared following the procedure previously described.[1] H-BPMP (0.42 g, 0.80 mmol), Mn(OAc)₂·4H₂O (0.39 g, 1.6 mmol), and NaOAc·3H₂O (0.33g, 2.4 mmol) were dissolved in 18 mL of aqueous methanol solution (MeOH to H₂O *v/v* = 8:1). After stirring for 30 min at room temperature, 1 mL of NaClO₄ solution (aq., 1.6 M) was added dropwise. After stirring for 1 h at room temperature, the mixture was cooled for a day at 0 °C. The mixture was filtered with an aqueous methanol solution (MeOH to H₂O *v/v* = 16:3) to give a pale-yellow, needle-like crystal. Yield 0.54 g (67%). MS (ESI): [Mn₂(BPMP)(OAc)₂]⁺ *m/z* calculated for $C_{37}H_{39}Mn_2N_6O_5^+$: 757.1737; found: 757.1715.

Limit of detection (LOD; S/N=3)

The LOD was calculated on the basis of the absorbance titration experiments. The absorbance spectra of the blank samples were measured 10 times to calculate the standard deviation of the blank measurement. In addition, to measure the slope of the calibration curve, the absorbance at 420 nm versus the concentration of the analyte was plotted. The LOD was calculated according to the following equation: LOD = $3\sigma/k$, where σ is the standard deviation of the blank measurement and k is the slope of the calibration curve.

Supplementary Results

NMR and Mass Spectra

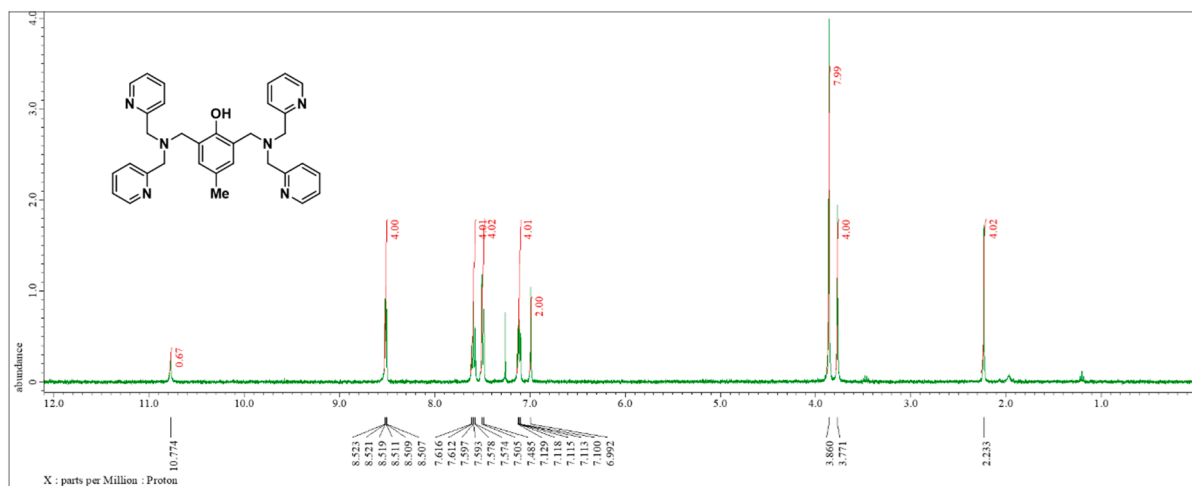**Figure S1.** ^{1}H-NMR spectrum of 2,6-bis(chloromethyl)-4-methylphenol.
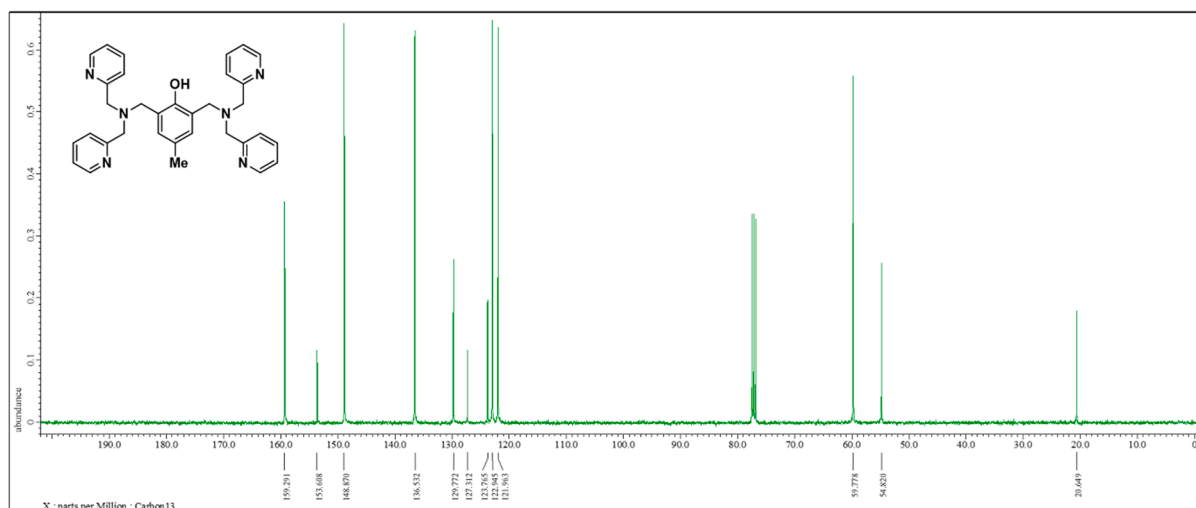

Figure S2. ^{1}H-NMR spectrum of H-BPMP.
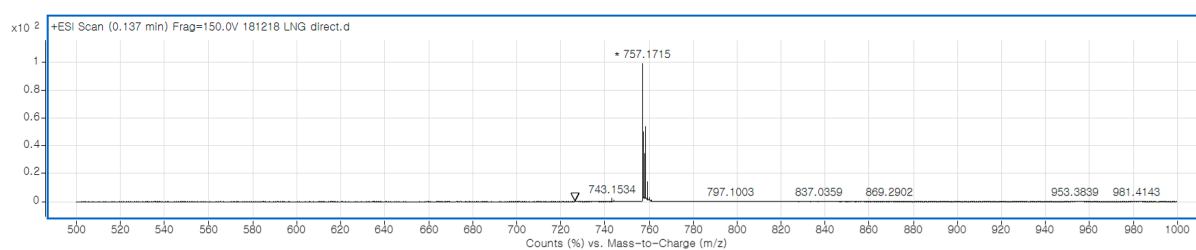
Figure S3. ^{13}C-NMR spectrum of H-BPMP.

Figure S4. Mass spectrum of Mn2BPMP.

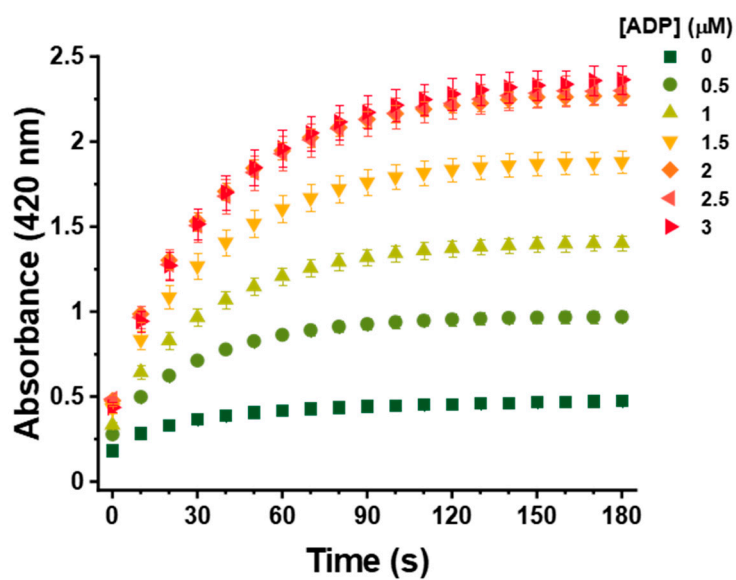*Optimization of ADP Concentration in the Mn2BPMP/ABTS/H2O2 System*

Figure S5. The plot of absorbance at 420 nm versus the time with different concentrations of ADP from 0 to 3 μM. Mn2BPMP = 1 μM, ABTS = 1 mM, and H2O2 = 5 mM in a buffer solution (Tris, 20 mM, pH 7.0).
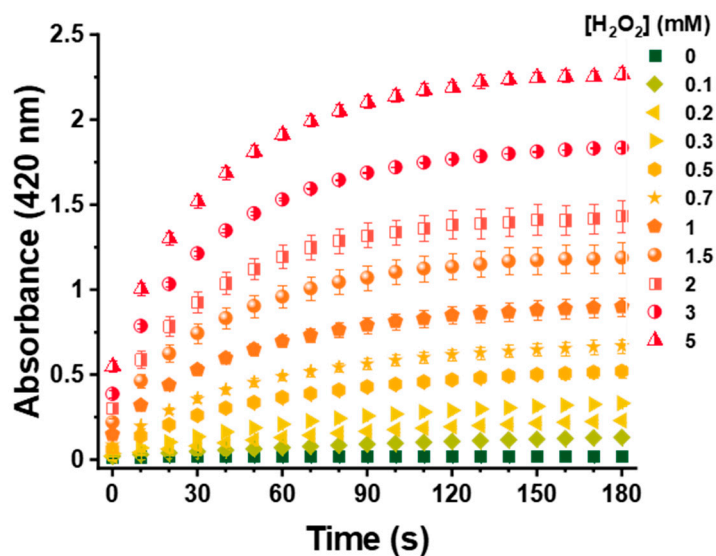
Figure S6. The plot of absorbance at 420 nm versus the time with different concentrations of H_2O_2 from 0 to 5 mM. Mn₂BPMP = 1 µM, ADP = 2 µM, and ABTS = 1 mM in a buffer solution (Tris, 20 mM, pH 7.0).
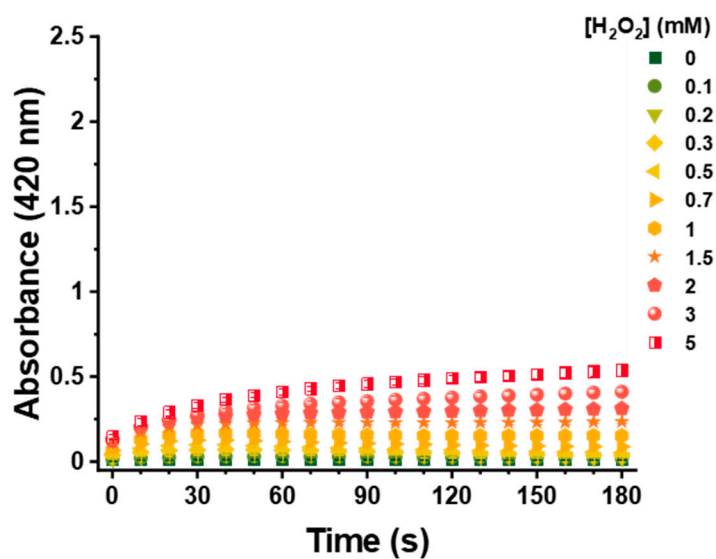

Figure S7. The plot of absorbance at 420 nm versus the time with different concentrations of H_2O_2 from 0 to 5 mM. Mn₂BPMP = 1 µM, and ABTS = 1 mM in a buffer solution (Tris, 20 mM, pH 7.0).

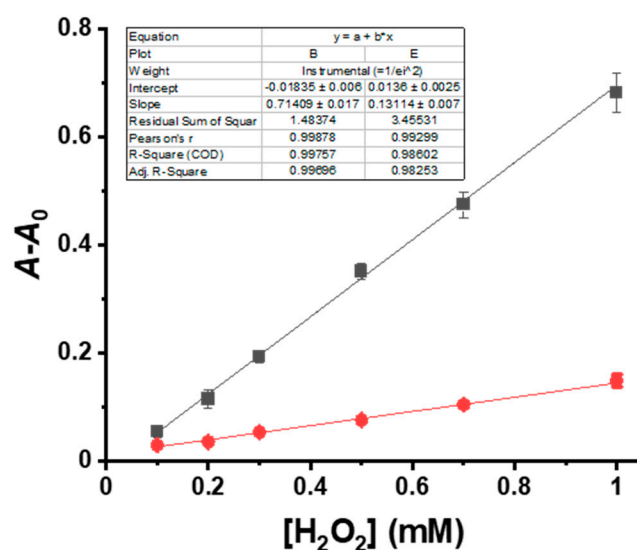| Equation	y = a + b*x	
Plot	B	E
Weight	Instrumental (=1/ei^2)	
Intercept	-0.01835 ± 0.006	0.0136 ± 0.0025
Slope	0.71409 ± 0.017	0.13114 ± 0.007
Residual Sum of Squar	1.48374	3.45531
Pearson's r	0.99878	0.99299
R-Square (COD)	0.99757	0.98602
Adj. R-Square	0.99696	0.98253

Figure S8. The plot of A-A_0 at 420 nm versus the concentration of H_2O_2 after 1 min in the presence (black line) or absence (red line) of ADP (2 μM). Mn2BPMP = 1 μM, and ABTS = 1 mM in a buffer solution (Tris, 20 mM, pH 7.0). The LOD was 9.7 μM with σ = 2.3×10^{-3} and k = 7.1×10^{-1} mM^{-1} in the presence of ADP, and The LOD was 85 μM with σ = 3.7×10^{-3}, and k = 1.3×10^{-1} mM^{-1} in the absence of ADP.

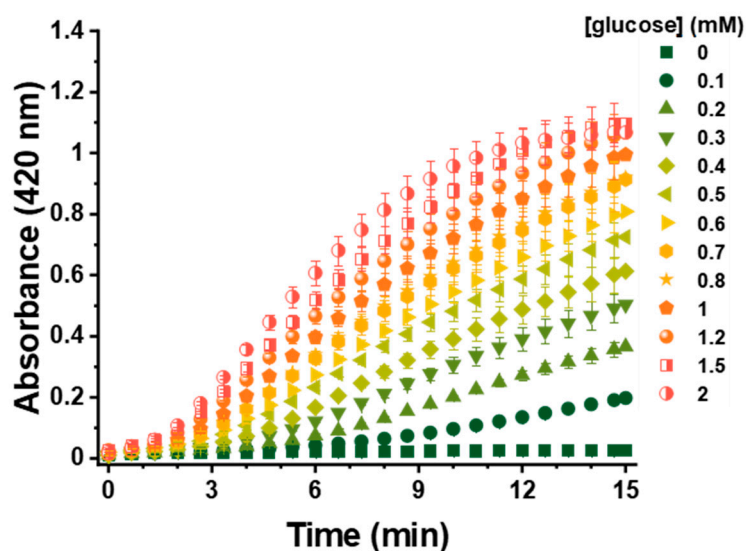*Glucose Titration Using the Mn2BPMP/ABTS/GOx System in the Presence or Absence of ADP*

Figure S9. The plot of absorbance at 420 nm versus the time with different concentrations of glucose from 0 to 5 mM. Mn2BPMP = 2 μM, ADP = 4 μM, ABTS = 1 mM, and GOx = 1 U/mL in a buffer solution (Tris, 20 mM, pH 7.0).
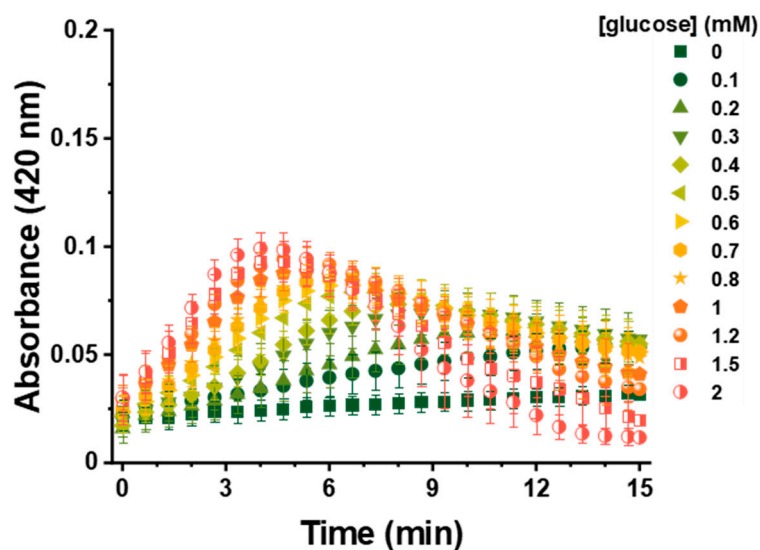
Figure S10. The plot of absorbance at 420 nm versus the time with different concentrations of glucose from 0 to 5 mM. Mn_2BPMP = 2 μM, ABTS = 1 mM, and GOx = 1 U/mL in a buffer solution (Tris, 20 mM, pH 7.0).
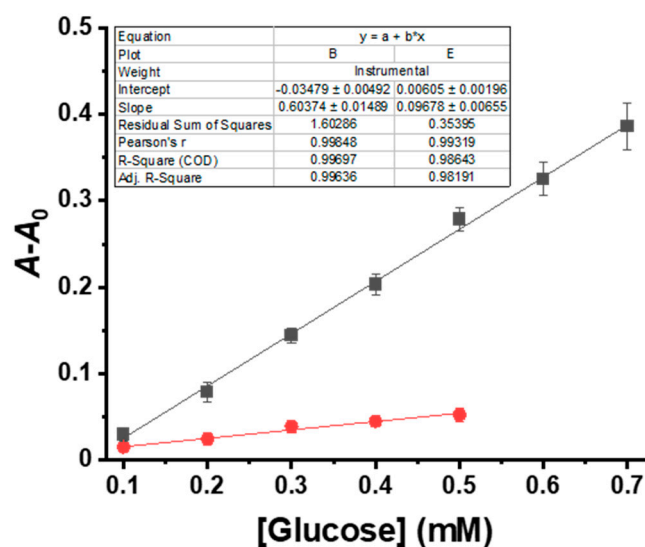

Figure S11. The plot of A-A_0 at 420 nm versus the concentration of glucose after 7 min in the presence (black line) or absence (red line) of ADP (4 μM). Mn_2BPMP = 2 μM, ABTS = 1 mM, and GOx = 1 U/mL in a buffer solution (Tris, 20 mM, pH 7.0). The LOD was 16 μM with σ = $3.2×10^{-3}$ and k = $6.0×10^{-1}$ mM^{-1} in the presence of ADP, and The LOD was 59 μM with σ = $1.9×10^{-3}$, and k = $9.7×10^{-2}$ mM^{-1} in the absence of ADP.

Comparison of ADP/Mn₂BPMP/ABTS/AOx System with Previous Peroxidase-Based Enzyme Cascade Assay Methods

Table S1. Pros and cons of the ADP/Mn₂BPMP/ABTS/AOx system compared to the previous peroxidase-based enzyme cascade assay methods.

	HRP-Based Enzyme Cascade Assay Method	Peroxidase-Mimics-Based Enzyme Cascade Assay Method	This Method
Peroxidases	Horseradish peroxidase (HRP; Natural enzyme)	Magnetic nanoparticles, gold nanoparticles, metal－organic frameworks, and etc. (Mainly nanomaterial-based peroxidase-mimics)	Mn₂BPMP (Small molecule-based peroxidase mimic)
Characteristics of peroxidases	Workable at neutral pH High catalytic activity	Workable at acidic pH (mainly pH 4) Poor batch to batch variation	Workable at neutral pH Easy chemical modification Great reproducibility
Pros	One-pot assay system Rapid detection time	High stability Cost-effective	Improved sensitivity by simple addition of ADP One-pot assay system High stability Rapid detection time Cost-effective
Cons	High preparation cost Low stability for environmental conditions	Multiple step assay (pH adjustment required; e.g. pH 7 to pH 4) Long detection times.	Relatively low activity compared to HRP

Serum Glucose Assay Using the ADP/Mn₂BPMP/ABTS/GOx System

Table S2. Detection of glucose in human serum.

	Added (mM)	Actual Conc. (mM)	Found (mM)	Recovery (%)	RSD (%)
	5.00	10.35	9.45	91.34	5.26
Human serum	10.00	15.35	12.71	82.79	8.80
	25.00	30.35	30.40	100.16	9.12

Figure S12. The plot of absorbance at 420 nm versus the time with different concentrations of cholesterol from 0 to 300 μM. Mn$_2$BPMP = 2 μM, ADP = 4 μM, ABTS = 1 mM, and ChOx = 1 U/mL in a buffer solution (Tris, 20 mM, pH 7.0).
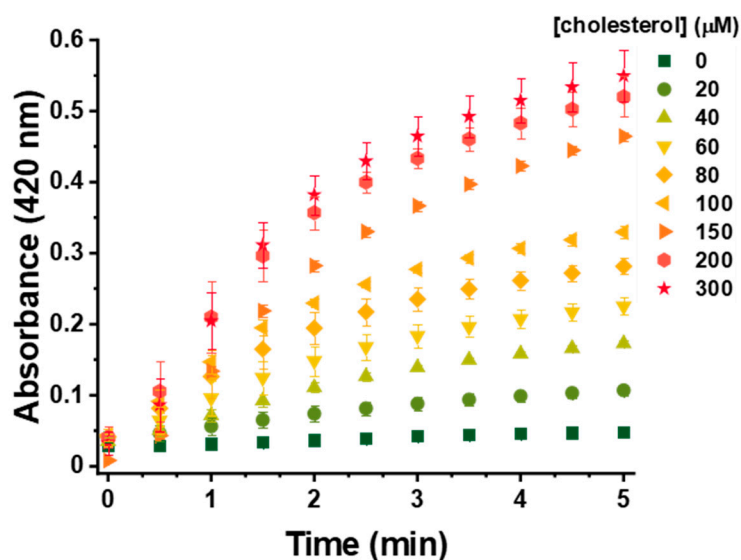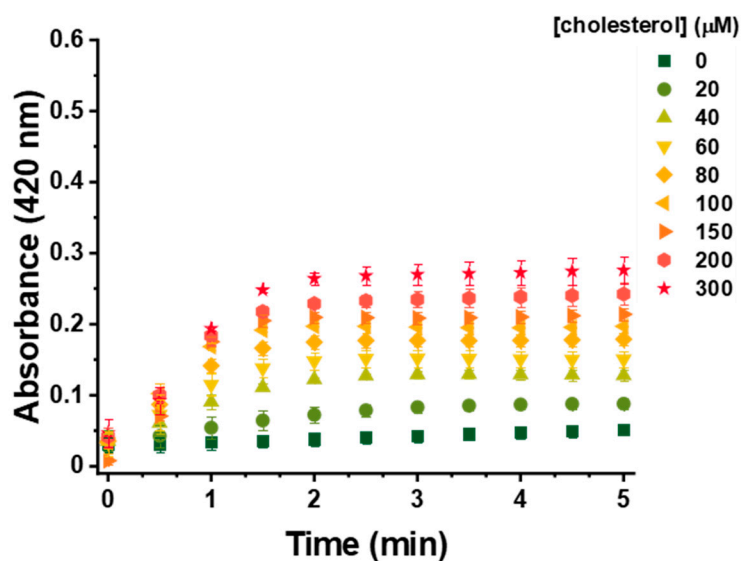

Figure S13. The plot of absorbance at 420 nm versus the time with different concentrations of cholesterol from 0 to 300 μM. Mn$_2$BPMP = 2 μM, ABTS = 1 mM, and ChOx = 1 U/mL in a buffer solution (Tris, 20 mM, pH 7.0).

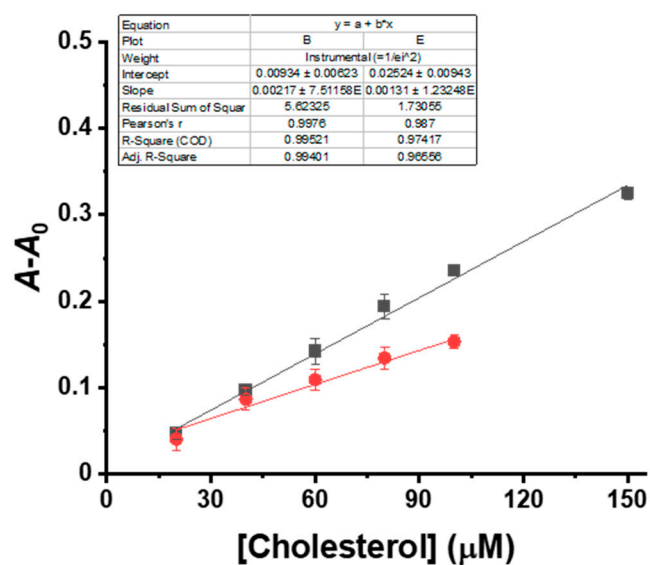| Equation	y = a + b*x	
Plot	B	E
Weight	Instrumental (=1/ei^2)	
Intercept	0.00934 ± 0.00623	0.02524 ± 0.00943
Slope	0.00217 ± 7.51158E	0.00131 ± 1.23248E
Residual Sum of Squar	5.62325	1.73055
Pearson's r	0.9976	0.987
R-Square (COD)	0.99521	0.97417
Adj. R-Square	0.99401	0.96556

Figure S14. The plot of A-A_0 at 420 nm versus the concentration of cholesterol after 3 min in the presence (black line) or absence (red line) of ADP (4 µM). Mn$_2$BPMP = 2 µM, ABTS = 1 mM, and ChOx = 1 U/mL in a buffer solution (Tris, 20 mM, pH 7.0). The LOD was 4.8 µM with $\sigma = 3.5 \times 10^{-3}$ and $k = 2.2$ mM^{-1} in the presence of ADP, and The LOD was 12 µM with $\sigma = 5.1 \times 10^{-3}$, and $k = 1.3$ mM^{-1} in the absence of ADP.

References

1.   Lee, Y.; Yoo, S.; Kang, S.; Hong, S.; Han, M.S. An [Mn$_2$(bpmp)]$^{3+}$ complex as an artificial peroxidase and its applications in colorimetric pyrophosphate sensing and cascade-type pyrophosphatase assay. *Analyst* **2018**, *143*, 1780–1785.