Electronic Supplementary Material (ESI) for ChemComm.

Experimental details

Material and Instrumentation

The sequences of all DNA oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology &Services Co., Ltd. (Shanghai, China) and purified by high-performance liquid chromatography (HPLC). T4 polynucleotide kinase (PNK), T4 DNA ligase, dNTP mixture, glucose oxidase (GOD), cytochrome C (CytC), bovine serum albumin (BSA) and dithiothreitol (DTT) were purchased from Sangon Biotech (Shanghai, China). Horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB), cellulase, acetylcholinesterase (AchE) and DNase/RNase-free water were purchased from Beijing Solarbio Science and Technology Co. Ltd. H_2O_2 (30%) was obtained from Beijing HuaTeng. Calf intestinal alkaline phosphatase (CIAP), Inorganic Pyrophosphatase (PPase), Φ29 DNA polymerase (PolΦ29) and Gibco trypsin-EDTA were from Thermo Fisher Scientific. Vivid blood separation membrane and nitrocellulose papers (HF 120 HP and NC 0.45 μm) were obtained from PALL and GE Healthcare (Chicago, IL, USA), respectively. All other chemicals were purchased from Sigma-Aldrich and used without further purification. Whole blood samples were collected from patients with liver diseases and healthy volunteers in Dalian Central Hospital (China), according to the rules of the local ethical committee.

The UV-vis absorbance was monitored on a microplate reader (Tecan, Switzerland). Scanning electron microscopy (SEM) was performed using a Hitachi UHR FE-SEM SU8010. Transmission electron microscopy (TEM), High-Angle Annular Dark Field-scanning transmission electron microscopy (HAADF-STEM) and STEM coupled with energy dispersive X-ray spectroscopy (STEM-EDS) were performed on a JEOL 2010F. Confocal fluorescence microscopy (CFM) was performed using an Olympus FV1000.

Synthesis of circular DNA.

Linear DNA precursor (300 pmol, 5'-ATTCGTGTGA GAAAACCCAA CCCGCCCTAC CCAAAAGATA TCGTCAGATG A-3'), 10 units (U) of PNK and 2 mM ATP were mixed in 50 μL of 1× PNK buffer (50 mM Tris-HCl, pH 7.6 at 25 °C, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine) and phosphorylated at 37 °C for 40 min. The obtained mixture was mixed with DNA primer (300 pmol, 5'-CTCACACGAA TTCATCTGAC-3') in 98 μL of 1× T4 DNA ligase buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8 at 25 °C), heated to 90 °C for 3 min, and then cooled at room temperature (RT) for 15 min. Subsequently, 10 U of T4 DNA ligase was added (total volume: 100 μL) and incubated at RT for 2.5 hours. The obtained circular DNAs were concentrated by standard ethanol precipitation and purified by 10% denaturing PAGE

(dPAGE, 8 M urea).

Synthesis of HRP@3D DNA

In a typical experiment, 5 μL of 10× rolling circle amplification (RCA) buffer (500 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 40 mM DTT, 100 mM $MgCl_2$, pH 7.5), 1 μL of PolΦ29 (10 U/μL), 8 μL of 2.5 mM dNTPs, 2 μL of 5 μM circular DNA, 2 μL of 10 μM DNA primer, 10 μL of 50 μM HRP, and 22 μL of DNase/RNase-Free Water (total volume: 50 μL) were incubated at 30°C for 30 min. After heating at 65°C for 10 min, PolΦ29 was deactivated. The resulting mixture was incubated at 4°C for 12 h. After centrifugation at 8000 rpm for 10 min, the obtained HRP@3D DNA particles were washed by 100 μL of DNase/RNase-free water three times and re-dispersed in 20 μL of DNase/RNase-free water.

Characterization of HRP@3D DNA

1.5 μL of the above as-prepared HRP@3D DNA was dropped on a clean silicon wafer or paper and dried overnight, followed by coating with platinum using Sputter Coater; To prepare TEM samples, 1.5 μL of the as-prepared HRP@3D DNA was dropped on a carbon grid and dried by solvent evaporation; 5 μL of HRP@3D DNA was mixed with 195 μL of 1×SYBR Gold in 50 mM Tris-HCl (pH 7.5) in the dark. 2 μL of the obtained mixture was dropped on glass and prepared by solvent evaporation.

Quantification of the total number of HRP per HRP@3D DNA particle

Calculation of the number of HRP@3D DNA particles. 1 μL of HRP@3D DNA (dissolved in 20 μL) was added to 199 μL of 50 mM Tris-HCl buffer solution containing 1× SYBR Gold (pH 7.5). Followed by incubation at RT for 10 min, the HRP@3D DNA particles were dyed. And then, 10 μL of the mixture was added to a hemocytometer and incubated at RT for 10 min. The Olympus IX83 inverted microscope (Olympus, Japan) equipped with a 40-fold objective was used to take bright field and fluorescent images of 80 squares on the hemocytometer. To calculate the particle number, we analyzed the obtained images using an ImageJ software. In 1 μL of sample, the total number of HRP@3D DNA particles (N) was determined using: N = [n/ (1/4000) μL] × 10 μL × 20, where n represents the average number of particles observed in each square, 1/4000 is the volume of each square, and 20 is the volume correction factor.

Calculation of the number of loaded HRP in 3D DNA. To determine the total amount of HRP encapsulated in 3D DNA, 20 μL HRP@3D DNA was firstly diluted by dephosphorylation buffer to 45 μL and then incubated with 5 μL of ALP (final activity concentration: 5000 U/L) at 37 °C for 30 min to release the HRP. After centrifugation at 14,000 rpm for 1 min, 20 μL of the obtained supernatant containing the released HRP was mixed with 80 μL of chromogenic substrate and incubated at RT for 4 min in polystyrene 96-well microtiter plates.

The change of absorbance at 652 nm (OD_{652}) was measured by a microplate reader (Tecan Spark). The loaded HRP was quantified by comparing the absorbance values of the test samples to a standard curve. The maximum number of loaded HRP (N-HRP) in 20 μL of HRP@3D DNA was calculated using: N-HRP = 0.17 μM × 6.02 × 10^{23} × 10^{-6} × (20 × 10^{-6}) L = 2.05 × 10^{12}, where 0.17 μM represents the calculated maximum concentration of N-HRP.

Calculation of the maximum number of loaded HRP per HRP@3D DNA particle.

To calculate the maximum number of loaded HRP for each HRP@3D DNA particle, the N-HRP/N ratio was used: N-HRP/N = 2.05 × 10^{12}/ {[14.1/ (1/4000) μL] ×10 μL × 20 × 20]} = 9.09 × 10^{3}, where 14.1 represents the average number of observed particles in each square.

Feasibility of ALP-mediated HRP release from HRP@3D DNA (Fig. 2A)

80 μL of HRP@3D DNA particles were firstly diluted by dephosphorylation buffer to 196 μL, and equally divided into four samples (named as No.1, 2, 3, and 4). 1 μL of DNase/RNase-free water was added to No. 1 and No. 2, while 1 μL of ALP (final activity concentration: 1000 U/L) was added to No. 3 and No. 4. After incubation at 37 °C for 20 min, each mixture was then centrifuged at 14,000 rpm for 1 min. The resultant supernatant (20 μL) was mixed with 80 μL of chromogenic substrate (2.5 μL of 20 mM TMB, 5 μL of 10% H_2O_2 and 72.5 μL of 0.1 M NaAc-HAc, pH 4.5) in a 1.5 mL microcentrifuge tube. The images were captured by iPhone 13 following 4 min incubation at RT.

Response of HRP@3D DNA-DTT to PPase and other proteins (Fig. 2B)

For the preparation of HRP@3D DNA-DTT, 10 μL of DTT with different concentrations (final concentration: 0, 5, 10, 15, 20, and 30 mM) were added during the RCA. After centrifugation at 8000 rpm for 10 min, the obtained HRP@3D DNA-DTT particles were washed by 100 μL of DNase/RNase-free water three times. 20 μL of HRP@3D DNA and 1 μL of different proteins (500 U/L ALP, 500 U/L PPase, 500 U/L AchE, 500 U/L GOD, 500 U/L CytC, 500 U/L trypsin, 0.01 mM BSA, 0.01 mM cellulase) were incubated in dephosphorylation buffer (total volume: 50 μL) at RT for 20 min. After centrifugation at 14,000 rpm for 1 min, the obtained supernatant was collected and incubated with chromogenic substrate according to the protocols described above. Quantification of HRP was achieved by comparing the initial velocity values of the test samples to a standard curve

Kinetic release of HRP from HRP@3D DNA-DTT (Fig. 2C)

20 μL of HRP@3D DNA and 1 μL of ALP (final concentration: 1000 U/L) were incubated in dephosphorylation buffer (total volume: 50 μL) at RT for different time (0, 5, 10, 15, 20, 30, and 45 min). After centrifugation at 14,000 rpm for 1 min, the obtained supernatant was collected and incubated with chromogenic substrate according to the protocols described above. Quantification of HRP was achieved by comparing the initial velocity values of the test samples to a

standard curve.

ALP concentration-dependent release of HRP (Fig. 2D)

20 μL of HRP@3D DNA and 1 μL of ALP with different concentrations (final concentration: 0, 20, 40, 100, 160, 200, 400, 800, 1000, and 2000 U/L) were incubated in dephosphorylation buffer (total volume: 50 μL) at RT for 20 min. After centrifugation at 14,000 rpm for 1 min, the obtained supernatant was collected and incubated with chromogenic substrate according to the protocols described above. Quantification of HRP was achieved by comparing the initial velocity values of the test samples to a standard curve.

Fabrication of paper device (Fig.3A)

Three paper pieces were used: PALL vivid blood separation membrane for Z1, NC 0.45 μm for Z2 and HF120 HP for Z3. Each paper was cut to the standard letter sheet size (8.5 x 11 inches) to be fed into the wax printer (Xerox ColourQube 8570N), followed by heating at 120°C for 2 min. The wax was melted into the pore structure of cellulose to form the hydrophobic barriers. To prevent nonspecific adsorption, Z2 was treated with 15 μL of 3% (w/v) BSA for 20 min and dried at RT. HRP@3D DNA was printed onto Z2 by an automated dispensing unit (Biodot XYZ3060). 5 μL of 20 mM TMB was pipetted onto Z3 and dried at RT.

Sensing performance (Fig.3C and 3D)

10 μL of ALP (final concentration: 0, 25, 50, 100, 200, 300, 500, 800 U/L) in dephosphorylation buffer was first added to Z2. Followed by incubation at RT for 20 min, Z2 was folded onto Z3. 8 μL of 0.1 M NaAc-HAc (pH 4.5) and 2 μL of 10% H_2O_2 were added onto Z3 to initiate the colorimetric reaction. After 1 min, the color was captured by an iPhone 13 placed on a 10 cm-high fixed holder under the same white light source. The image was analyzed using ImageJ software. For the selectivity test, the protocol is similar to the one described above except for: 500 U/L ALP, 500 U/L PPase, 500 U/L AchE, 500 U/L GOD, 500 U/L CytC, 500 U/L trypsin, 0.01 mM BSA, 0.01 mM cellulase were used. To eliminate the background influence, all the quantitative analysis of colorimetric samples set up with a blank control sample using dephosphorylation buffer.

Traditional p-nitrophenyl phosphate (PNPP) method

140 μL of PNPP reaction solutions (25 mM Diethanolamine (DEA), 1 mM $MgCl_2$, 6.7 mM PNPP) and 10 μL of ALP with different concentrations (final concentration: 0, 20, 40, 80, 160, 320, 640, and 1280 U/L) were mixed in polystyrene 96-well microtiter plates. After incubation at 37°C for 30 min in the dark, 100 μL of 0.1 M NaOH was added to stop the reaction. The absorbance at 405 nm (OD_{405}) was measured to obtain a standard curve.

Sensitivity and selectivity for the detection of the real samples

The sensitivity and selectivity of the paper device for detecting the real samples were calculated by Eq. 1 and Eq. 2, respectively.

$$sensitivity = \frac{true\ positives}{true\ positives\ +\ false\ negatives} \quad (Eq.\ 1)$$

$$specificity = \frac{true\ negatives}{true\ negatives +\ false\ positives} \quad (Eq.\ 2)$$

Where true positives and true negatives are obtained by the reference standard, i.e., the PNPP method.

Ethics Approval and Consent to Participate.

This study was done after agreement from the Ethics Committee of Dalian University of Technology. Experiments of the human whole blood samples were carried out in accordance with the approved guidelines of China and with the patients' informed consent.

Supplementary Figures and Tables.

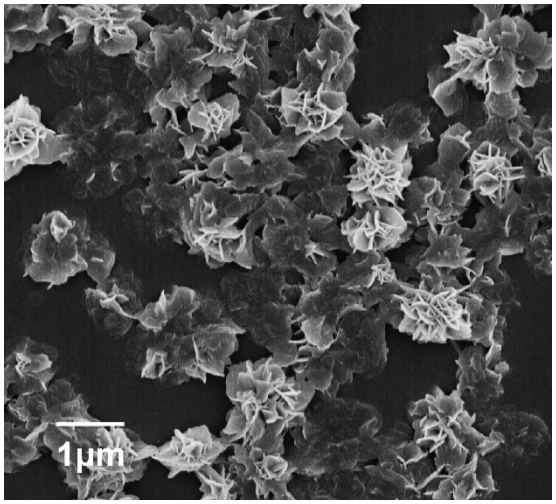

Fig. S1. SEM image of HRP@3D DNA after the ALP treatment (1000 U/L). Treatment time: 5 min.

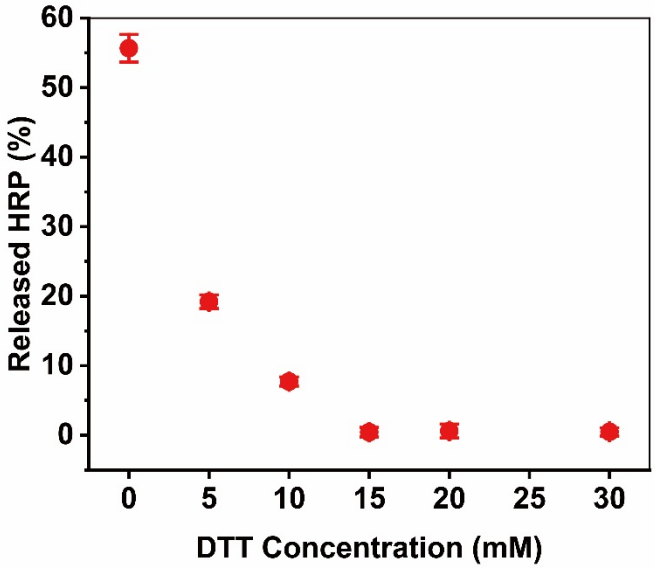

Fig. S2. Relative released HRP (%) from HRP@3D DNA-DTT with varying concentrations of DTT (0, 5, 10, 15, 20, and 30 mM) in the presence of PPase (1000 U/L).

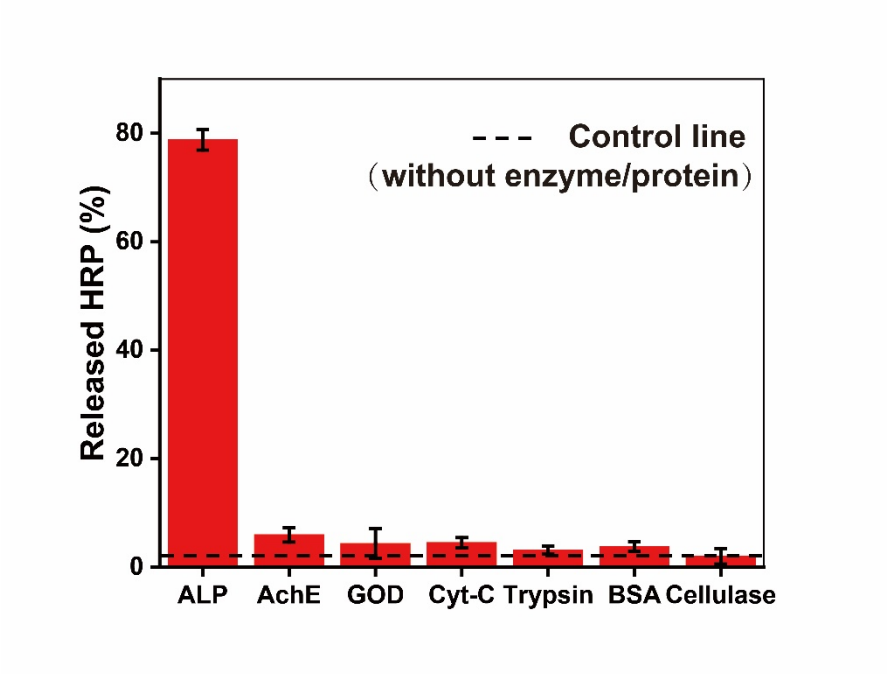

Fig. S3. Relative released HRP (%) of HRP@3D DNA in the presence of different proteins (500 U/L ALP, 500 U/L AchE, 500 U/L GOD, 500 U/L CytC, 500 U/L trypsin, 0.01 mM BSA, 0.01 mM cellulase). The error bars represent standard deviations of three independent experiments.

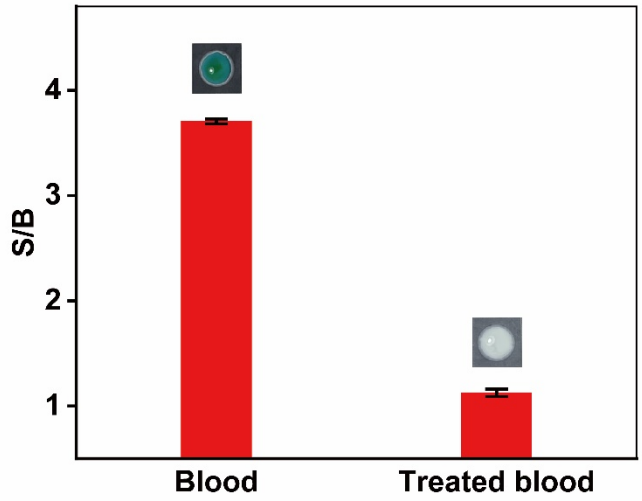

Fig. S4. S/B values for paper devices after adding the blood sample, and the treated blood sample using the whole blood separation membrane. Inset: Images of Z3 zones indicated in the figure. The error bars represent standard deviations of three independent experiments.

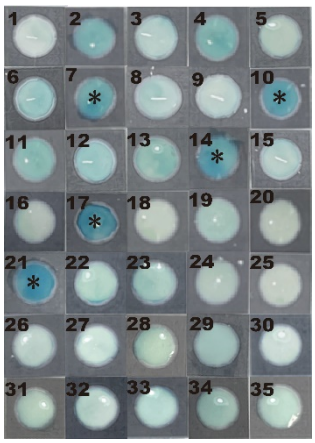

Fig. S5. Color changes of the paper device for 35 clinical blood samples.

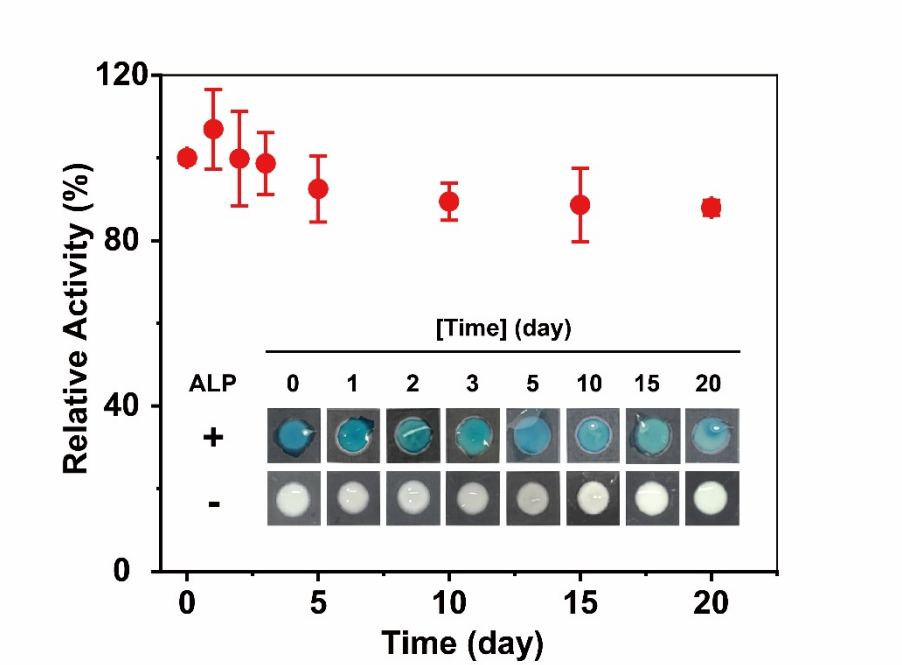

Fig. S6. Evaluation of the long-term stability of the paper device stored at room temperature.

Table S1. ALP detection using sensors

Key elements	Method	Detection limit (U/L)	Detection range (U/L)	Time (min)	Reference
Fe-N/C single-atom nanozyme	Colorimetry	0.02	0.05-100	55	1
	Colorimetry	5.4	0-120	60	2
2D-metal organic-framework-nanozymes	Colorimetry	1	1-50	70	3
Temperature Discoloration Sticker		1	1-100	95	4
Distance-based paper device	Colorimetry	5	5-200	30	5
Paper-based miniaturized immunosensor	Colorimetry	870	10^4-10^6	13	6
Personal glucose meter (PGM)	Electrochemistry	8.9	8-200	60	7
3D DNA and paper device	colorimetry	19.7 (13.4 for paper device)	0-400 (0-800 for paper device)	25 (23 for paper device)	This work

Reference

1 Q. Chen, S. Li, Y. Liu, X. Zhang, Y. Tang, H. Chai and Y. Huang, *Sens. Actuators B Chem.*, 2020, **305**, 127511.

2 D. Shi, Y. Sun, L. Lin, C. Shi, G. Wang and X. Zhang, *Analyst*, 2016, **141**, 5549.

3 X. Wang, X. Jiang and H. Wei, *J. Mater. Chem. B*, 2020, **8**, 6905.

4 X. Liu, L. Zou, X. Yang, Q. Wang, Y. Zheng, X. Geng, G. Liao, W. Nie and K. Wang, *Anal. Chem.*, 2019, **91**, 7943.

5 K. Phoonsawat, K. Khachornsakkul, N. Ratnarathorn, C. Henry and W. Dungchai, *ACS Sens.,* 2021, **6**, 3047.

6 K. Mahato and P. Chandra, *Biosens. Bioelectron.*, 2019, **128**, 9.

7 J. Ahn, H. Kim, C. Lee, K. Park and H. Park, *J. Biol. Eng.*, 2019, **13**, 51.