Electronic Supplementary Material (ESI) for Sensors & Diagnostics.

Supplementary Information

for

Single-step colorimetric detection of acid phosphatase in human urine using an oxidase-mimic platinum nanozyme

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1. Materials and methods

1.1 Materials

Chloroplatinic acid hexahydrate (8 wt.% in H_2O), sodium citrate, citric acid, ascorbic acid, 2-phospho-L-ascorbic acid (AAP), acid phosphatase from potato (ACP), sulphuric acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), o-phenylenediamine dihydrochloride (OPD), 3,3'5,5'-tetramethylbenzidine (TMB), sodium acetate anhydrous, acetic acid, and 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) were purchased from Sigma-Aldrich, Australia. Milli-Q water (18.2 MΩ cm) was obtained from a Millipore Milli-Q water purification system.

1.2 Synthesis of platinum nanoparticles

The PtNPs were synthesised by a multistep seed-mediated growth procedure as described by Bigall *et al.* (Bigall et al. 2008). PtNP seeds of 5 nm were prepared by adding 36 mL solution of 0.2% chloroplatinic acid hexahydrate to 464 mL of boiling deionised water. After one minute, 11 mL of a solution containing 1% sodium citrate and 0.05% citric acid was added. Then half a minute later, 5.5 mL of a freshly prepared solution of 0.08% sodium borohydride containing 1% sodium citrate and 0.05% citric acid was quickly injected and allowed to react for 10 min. The product was then cooled down to room temperature (Brown et al. 2000).

The next step in the synthesis process is growing the Pt seeds to ~25 nm in size. This is done by first diluting 1 mL of the Pt seed solution in 29 mL of deionised water at room temperature. To this, 45 μL of 0.4 M chloroplatinic acid solution (VEB Bergbau- and Hüttenkombinat "Albert Funk", Hexachloroplatin(IV)-säure, reinst) was added, followed by the addition of 0.5 mL solution containing 1% sodium citrate and 1.25% L-ascorbic acid. While under stirring conditions, the temperature was slowly increased (at the rate of ~10 °C/min) to the boiling point. The reaction time was 30 min in total. The synthesised PtNPs were then

washed three times by a precipitation method. This involved centrifugation of the sample at 1000 RPM, followed by redispersing the nanoparticles (pellet) in deionised water (Bigall et al. 2008).

1.3 Characterization of the PtNPs

The concentration of PtNPs was quantified by first dissolving the nanoparticles in concentrated nitric acid followed by measuring the Pt ion concentration using atomic emission spectroscopy (AES). The imaging for shape and size characterization of the PtNPs was done by first drop casting a solution containing the PtNPs onto a carbon coated copper grid followed by transmission electron microscopy (TEM) analysis using JEOL 1010 TEM instrument operating at an accelerating voltage of 100 kV. X-ray diffraction measurements were performed on Bruker AXS D4 Endeavour wide-angle XRD instrument operated with Cu Kα radiation (λ = 1.54 Å). Hydrodynamic diameter measurement through dynamic light scattering (DLS) and zeta potential measurements were carried out on the Malvern Nano ZS instrument at 25 °C.

1.4 Oxidase-mimic nanozyme activity of PtNPs

The inherent oxidase-mimic catalytic activity of PtNPs was assessed using TMB as the chromogenic substrate that is oxidised to a blue colored product. The reaction was carried out in 50 mM NaAc buffer of pH 5 at 37 °C with 0.2 mM TMB and 11.8 µM PtNPs. The blue-coloured oxidised TMB product was measured at 652 nm using a CLARIOstar plate reader (BMG Labtech). The substrate specificity of the PtNPs was assessed by exposing the nanozyme to two other colorimetric substrates such as OPD and ABTS, followed by spectroscopic measurements of their respective oxidation products. The intrinsic oxidase-mimic activity of the PtNPs was established by testing the activity of potentially leached metal ions in solution. This was done by first incubating the PtNPs in 50 mM NaAc buffer (pH 5) for 15 min at 37 °C, followed by centrifuging the solution for 15 min at 14,500 RPM. To the supernatant that

contained the potentially leached metal ions, 0.2 mM TMB as added and allowed to react at 37 °C. The optimal assay conditions for the oxidase-mimic catalytic activity of the PtNPs were determined by individually varying the buffer pH of the reaction from 2 to 10, the temperature at which the reaction occurs from 25 to 60 °C, and the concentration of the nanozyme from 2.9 µM to 20.6 µM.

1.5 Steady-state kinetic parameters of PtNPs

Steady-state kinetic parameters of the nanozyme were determined by maintaining a fixed concentration of the PtNPs (11.8 µM) while varying the concentration of TMB from 50 µM to 800 µM. The colorimetric response was then fitted to the Lineweaver-Burk plot using OriginPro 2016 and enzyme-kinetic parameters – Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were calculated using **Equation 1**.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]} + \frac{1}{K_m}\right) \qquad \textit{Equation 1}$$

V_0 corresponds to the initial reaction velocity, K_m is the Michaelis-Menten constant, V_{max} denotes the maximum reaction velocity, and [S] is the substrate concentration.

1.6 Mechanism of oxidase-mimicking catalytic activity of PtNPs

The mechanism of the oxidase mimic catalytic activity of PtNPs was assessed using probes that specifically bind to hydroxyl radicals, superoxide radicals and singlet oxygen radicals. These include terephthalic acid (TA), Hydroethidine (HE) and 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA), respectively. The hydroxyl radical probing assay involved 0.5 mM TA and 11.8 µM of PtNPs in 50 mM NaAc buffer (pH 5). The reaction mixture was incubated at 37 °C, followed by exciting the fluorescent product at 315 nm. The emission spectrum was measured between 350 and 550 nm on the CLARIOstar plate reader (BMG Labtech). The superoxide probing assay involved 5 µM HE and 11.8 µM of PtNPs in

50 mM NaAc buffer (pH 5) incubated at 37 °C. The fluorescent product was excited at 510 nm followed by the measurement of the emission spectrum between 540 and 740 nm on the CLARIOstar plate reader (BMG Labtech). The singlet oxygen probing assay involved 0.2 mM ABDA and 11.8 µM of PtNPs in 50 mM NaAc buffer (pH 5) incubated at 37 °C followed by colorimetric spectrum measurement on the CLARIOstar plate reader (BMG Labtech).

1.7 Colorimetric detection of ACP

The intrinsic oxidase-mimic catalytic activity of the PtNPs was used to develop a colorimetric acid phosphatase (ACP) detection system where the quantification of ACP was carried out by three approaches.

Method 1 – Two-step reaction: a mixture of 33 µM AAP and 12 mU/mL of ACP in 50 mM NaAc buffer pH 5 were incubated at 37 °C for 30 minutes. To this mixture, 0.2 mM of TMB, 11.8 µM of PtNPs and 50 mM NaAc buffer pH 4 were added and further incubated at 37 °C for 15 min. The color generated by the oxidation TMB was measured at 652 nm.

Method 2 – Single-step reaction: all the reaction components were added to 50 mM NaAc buffer (pH 5) and incubated at 37 °C for 15 min. This was followed by the measurement of the product formed at $\lambda_{652\ nm}$.

Method 3 – Reaction termination: the reactions were first carried out as described in methods 1 and 2. This was followed by the addition of 0.1 M H_2SO_4 to the reaction mixture and measuring the absorbance at 450 nm after 5 min.

The linear dynamic detection range of each of the three methods was determined by varying the concentration of ACP in the assay from 0.5 mU/mL to 50 mU/mL, followed by performing linear regression on the plots of ACP concentration vs. $Absorbance_{\lambda 652\ nm}$ (for method 1 and 2)/$Absorbance_{\lambda 450\ nm}$ (for method 3).

1.8 Mechanism of ACP sensing using PtNP nanozyme

To understand the mechanism of how ACP modulates the oxidase-mimic nanozyme activity of the PtNPs, the effect of ascorbic acid (AA) on the PtNPs was determined. Two parallel oxidase-mimic nanozyme reactions were conducted.

Reaction 1: PtNPs (11.8 µM) and TMB (0.2 mM) were incubated in 50 mM NaAc buffer pH 5 at 37 °C. The change in absorbance at λ = 652 nm was measured over 14 minutes. This was followed by the addition of 0.1 mM ascorbic acid and further measurement of absorbance at 652 nm.

Reaction 2: PtNPs (11.8 µM), ascorbic acid (0.1 mM) and TMB (0.2 mM) were incubated in 50 mM NaAc buffer pH 5 at 37 °C and the absorbance at λ = 652 nm was measured.

1.9 Selectivity and Interference Study

The sensor specificity to detect ACP was assessed by exposing it to some natural enzymes such as glucose-6-phosphate dehydrogenase (G6PDH), glucose oxidase (GOX), horseradish peroxidase (HRP), pepsin, pancreatin, catalase, and biomolecules such as trypsin and glucose. A mixture of 33 µM AAP, enzyme/biomolecule and/or 10 mU/mL of ACP, 0.2 mM of TMB and 11.8 µM PtNPs were incubated in 50 mM NaAc buffer pH 5 at 37 °C for 15 minutes. This was followed by terminating the reaction by adding 0.1 M H_2SO_4 and measuring the absorbance of the product at 450 nm after 5 min.

The practical applicability of the PtNPs nanozyme system in real samples such as urine was assessed by first collecting the urine sample from a healthy volunteer. The sample was then centrifuged for 15 min at 14,500 RPM to remove any cells and other debris. This was followed by two sets of experiments, initially a proof-of-concept experiment in a pH 5.0 buffer (in which all other urine-free experiments were performed) to check the viability of the proposed concept,

and later an optimised experiment in a pH 4.0 buffer to obtain a practically viable, more sensitive detection range for ACP detection. To check the proof-of-concept at pH 5.0, urine was diluted 50 times with PBS buffer (10 mM) and mixed with ACP stock solutions to achieve the concentration ranging from 0.5 to 20 mU/mL. These ACP-spiked urine samples were then mixed with 33 µM AAP, 0.2 mM of TMB, and 11.8 µM PtNPs followed by incubation in a pH 5, 50 mM NaAc buffer at 37 °C for 15 minutes. This was followed by terminating the reaction by adding 0.1 M H_2SO_4 and measuring the absorbance of the product at 450 nm after 5 min.

Next, to assess the sensing capability of the newly developed sensor in the physiologically relevant range, the undiluted urine (post-centrifugation) was spiked with known amounts (mU) of ACP by directly dissolving the powder form of ACP in urine to achieve the ACP concentrations of 2 – 100 mU/mL. These ACP-spiked urine samples were then used for nanozyme-based assay. The total reaction volume of the assay was 200 µL that contained 25 µL of the ACP-spiked urine sample, and 175 µL made up of 50 mM NaAc buffer (pH 4.0), 33 µM AAP, 0.2 mM of TMB and 11.8 µM PtNPs. The reaction mixture was incubated at 37 °C for 15 minutes, followed by terminating the reaction by adding 0.1 M H_2SO_4 and measuring the absorbance of the product at 450 nm after 5 min. The sensor response was calculated as a function of the % change in absorbance and then fitted using a Hill function (OriginPro 2016).

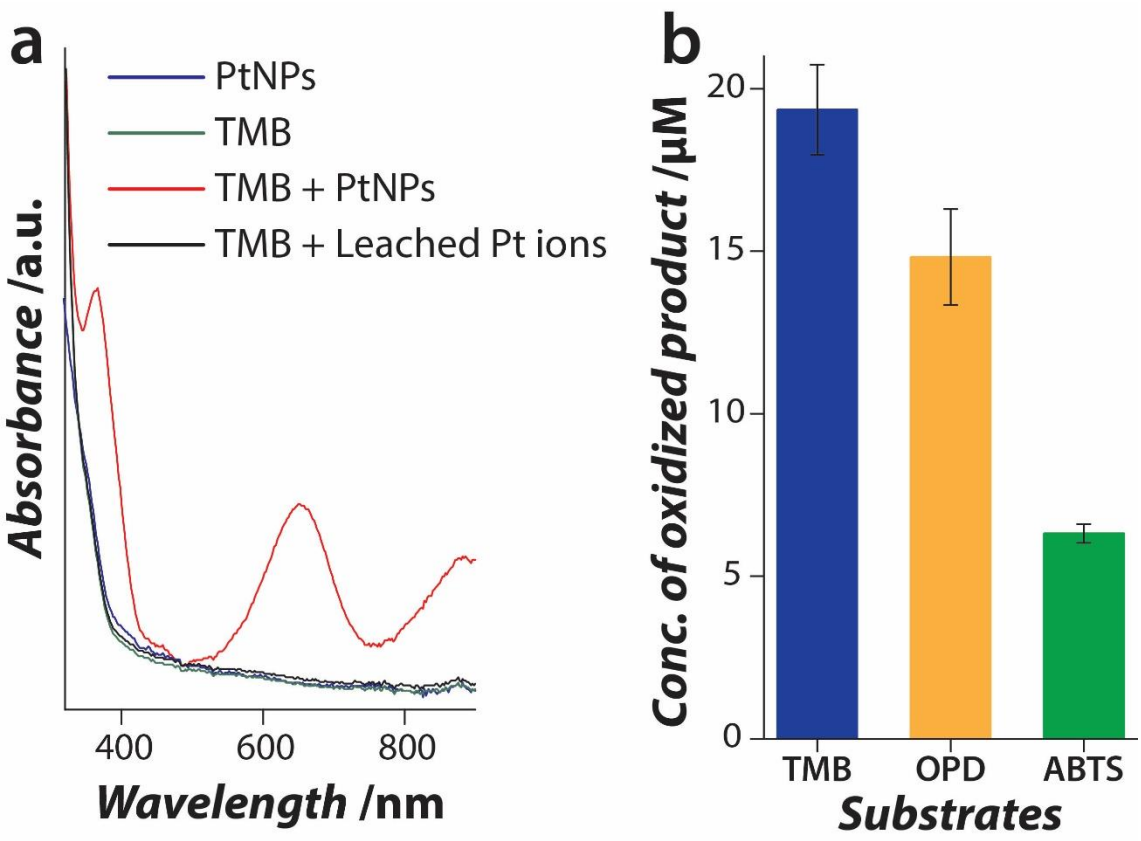

Fig S1 (a) Absorbance spectra of PtNPs catalysed oxidation product of TMB under different reaction conditions compared to using the leached metal ions as a catalyst; **(b)** Concentration of PtNPs catalysed oxidation products of colorimetric substrates – TMB, OPD, and ABTS. (Reaction conditions: pH – 5, temperature – 37 °C, concentration of colorimetric substrate – 0.2 mM, concentration of PtNPs – 11.8 µM)

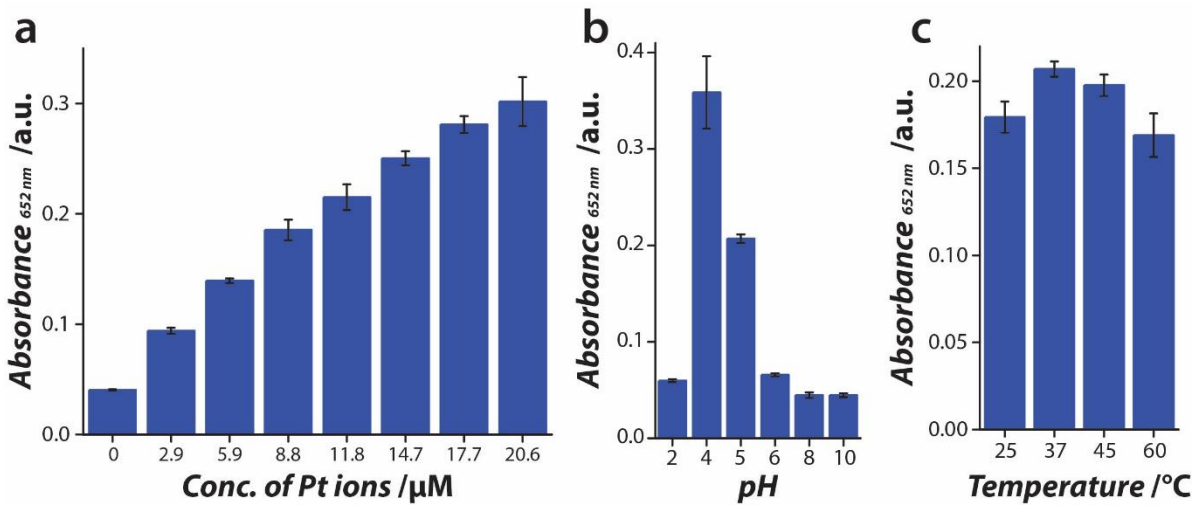

Fig S2 Effect of **(a)** nanozyme concentration, **(b)** pH, and **(c)** temperature on the oxidase-mimic catalytic activity of PtNPs. The reaction conditions that were kept consistent in **(a, b, c)** was the concentration of TMB at 0.2 mM. The reactions were carried out at pH 5 for **(a** and **c)** and a temperature of 37 °C for **(a** and **b)**. The concentration of the PtNPs in **(b** and **c)** was 11.8 µM.

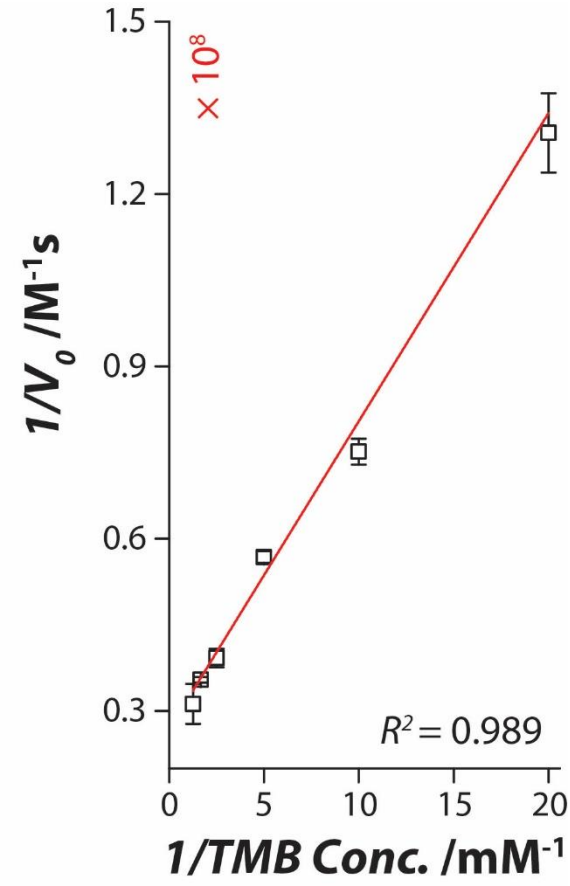

Fig S3 Steady-state kinetic parameters analysis using Lineweaver-Burk fit for the colorimetric response obtained by varying the concentration of TMB. (Reaction conditions: pH – 5, temperature – 37 °C, concentration of PtNPs – 11.8 µM)

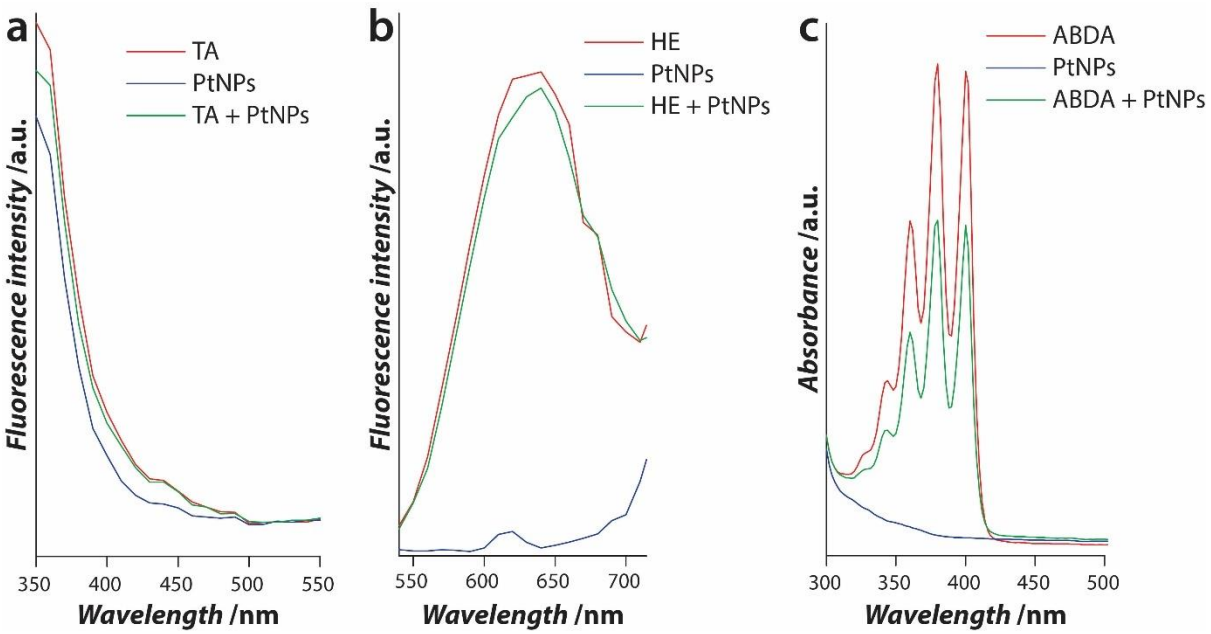

Fig S4 (a) Fluorescence emission spectra of the hydroxyl radical probe – TA ($\lambda_{excitation}$ = 315 nm), **(b)** Fluorescence emission spectra of the superoxide radical probe – HE ($\lambda_{excitation}$ = 510 nm), and **(c)** UV-vis spectra of the singlet oxygen dye – ABDA, under different reaction conditions. (Reaction conditions: pH – 5, temperature – 37 °C, TA – 0.5 mM / HE – 5 µM / ABDA – 0.2 mM, PtNPs – 11.8 µM)

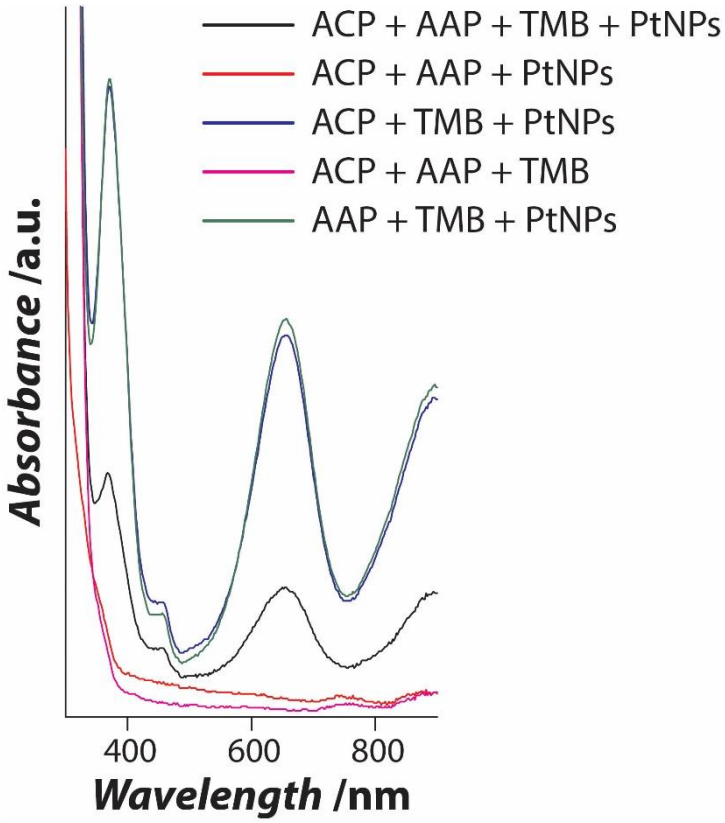

Fig S5 UV-vis spectra obtained as a result of TMB oxidation by PtNPs when exposed to different reaction conditions in an ACP sensing system. (Reaction conditions: pH – 5, temperature – 37 °C, ACP – 10 mU/mL, AAP – 33 µM, TMB – 0.2 mM, PtNPs – 11.8 µM)

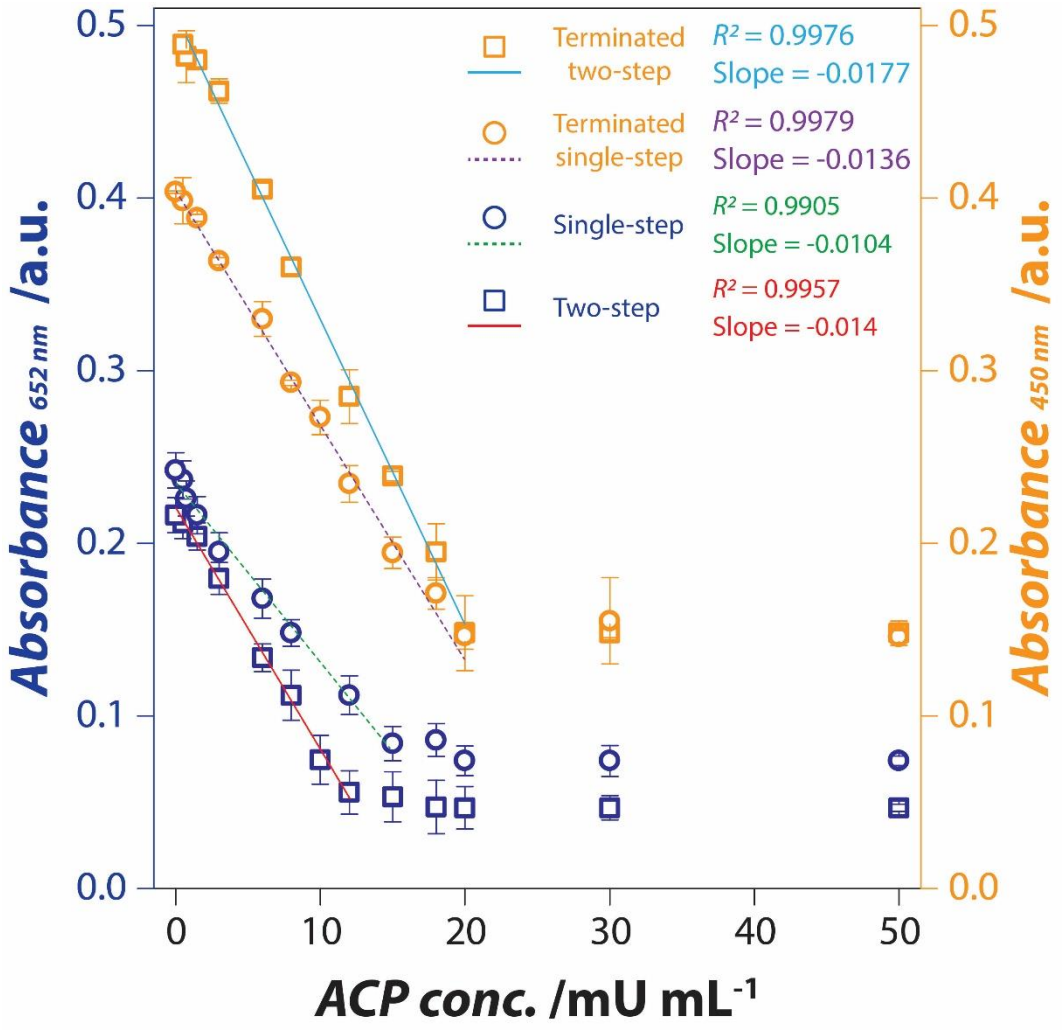

Figure S6 Colorimetric response of the Pt nanozyme based ACP sensor when exposed to different concentrations of ACP (0.5 – 50 mU/mL). Figure 2 in the main manuscript shows the linear dynamic range of the sensor.

Table S1. *ACP estimation using PtNP nanozyme (terminated single-step method) in urine samples spiked with known concentrations of ACP. Experiments were performed at pH 5.0.*

ACP spiked [mU mL^{-1}]	Estimated ACP conc. [mU mL^{-1}] ± SD [a]	Recovery [%] [b]
0	0 ± 0.00	100
0.5	0.5 ± 0.0	97-102
1	1.0 ± 0.0	98-104
5	4.9 ± 0.1	96-99
10	10.0 ± 0.1	99-101
20	19.7 ± 0.1	98-99

[a] Standard deviation calculated from three independent experiments; [b] Recovery calculated using (Measured concentration / Expected concentration) × 100.

References

Bigall, N.C., Härtling, T., Klose, M., Simon, P., Eng, L.M., Eychmüller, A., 2008. Nano Letters 8(12), 4588-4592.

Brown, K.R., Walter, D.G., Natan, M.J., 2000. Chemistry of Materials 12(2), 306-313.