Supporting Information

Ultrasensitive detection of programmed death-ligand 1 (PD-L1) in whole blood using dispersible electrodes

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Contents

Materials and reagents

All the reagents used were of analytical grade or the highest purity available. Gold(III) chloride trihydrate (HAuCl$_4$ ≥ 99.9%), iron(II) sulfate (FeSO$_4$ ≥ 98.0%), potassium nitrate (KNO$_3$ ≥ 99.0%), sodium hydroxide (NaOH ≥ 96.0%), sodium borohydride (NaBH$_4$ ≥ 99.0%), polyethyleneimine (PEI branched, Mw ~ 800 Da), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC ≥ 98.0%) and *N*-hydroxy succinimide (NHS ≥ 98.0%), dopamine hydrochloride (≥ 98.0%), sodium citrate (Na$_3$C$_6$H$_5$O$_7$.H$_2$O ≥ 99.0%), hydroxylamine hydrochloride (NH$_2$OH.HCl ≥ 99.9%), hydrogen peroxide (H$_2$O$_2$, 35 wt. % in H$_2$O) and ferrocenemethanol (FcMeOH ≥ 97.0%) were purchased from Sigma-Aldrich (Sydney, Australia). All

solutions were prepared in Milli-Q water (18.2 MΩ cm) at room temperature. Human programmed death-ligand 1 (PD-L1, also known as CD274 and B7-H1) matched antibody pair (H00029126-AP41) and recombinant protein antigen (H00029126-Q01) were purchased from Abnova, US. Human PD-L1 enzyme-linked immunosorbent assay (ELISA) kit [28-8] (ab214565) was obtained from Abcam (Sydney, Australia). All solution was prepared in phosphate buffered saline (PBS, pH = 7.4) that was purchased from Thermo Fisher Scientific. Horseradish peroxidase (HRP)-streptavidin was provided from Merck Sigma-Aldrich Pty. Ltd. The blood samples used in this work were from healthy human donors (Australian Red Cross Lifeblood). All other reagents were from Sigma-Aldrich (Sydney, Australia).

Live Subject Statement
This research was approved by the UNSW Human Research Ethics Panel Executive under a Negligible Risk human ethics status at UNSW (application HC200319). This research complies with the *National Statement on Ethical Conduct in Human Research* and by State legislation, including the *Human Tissue Act 1983* (NSW), the *Privacy and Personal Information Protection Act 1998* (NSW) and the *Health Records and Information Privacy Act 2002* (NSW). Blood was purchased from the Australian Red Cross Blood Service under a material supply agreement who collected it from donations provided under informed consent.

S1 Synthesis
Synthesis of the Au@MNPs was developed initially from a co-precipitation method introduced by Goon *et al.*[1] with modifications as described below.

S1-1 Synthesis of core magnetic nanoparticles
Iron oxide cores (Fe_3O_4) with an average size of 50 nm were precipitated by the mixture of 700 mg of $FeSO_4$ in 80 mL of Milli-Q water followed by the addition of 10 mL of 2.0 M KNO_3 and 10 mL of 1.0 M NaOH respectively in an oxygen-free environment via Ar gas purge. The primary Fe $(OH)_2$ product was oxidised by heating for 2 h at 90 °C to form the Fe_3O_4 MNPs. The core nanoparticles were then separated from the supernatant solution using a strong neodymium magnet ($25\times25\times12.5$ mm^3). This magnet wash was followed by the sonication of the mixture for 2 min with an ultrasonic probe (Misonix S4000 Sonicator). The collected MNPs were incubated with 10 mg/mL PEI at 90°C for 4 h. Then the PEI-coated MNPs were magnetically washed three

times with Milli-Q water. The remaining self-assembled PEI-MNPs were coated by 2 mg/mL dopamine hydrochloride in tris buffer solution (pH=8.5) over 30 min at room temperature.[2]

The self-polymerisation of dopamine produced a layer of polydopamine (PDA) coating layer deposited on PEI-coated MNPs. The PDA/PEI coating can facilitate gold seeding and coating steps at the same time acts as stabiliser. The modified nanoparticles (PDA/PEI-MNPs) were magnetically washed with Milli-Q water for five times and then suspended in 80 mL of Milli-Q water.

S1-2 Synthesis of gold seeded magnetic nanoparticles

A 2 mL volume of the PDA/PEI -MNPs suspension was firstly ultrasonicated for 2 min before being added to 90 mL of a colloidal solution of gold nanoparticles (AuNP). The colloidal AuNPs were produced from 0.075% (w/v) $NaBH_4$ reduction of 1% (w/v) $HAuCl_4$ in the presence of 38.8 mM $Na_3C_6H_5O_7.H_2O$. The mixture was mechanically stirred at 400 rpm for 4 h at room temperature. These seeded MNPs were magnetically washed three times, followed by a second PEI coating using 2.5 g/L PEI via oven heating at 90°C for 2 h. The formed PEI-coated gold-seeded MNPs were magnetically separated from the excess Au colloid solution via magnetic washing using Milli-Q water over three repetitions, before being suspended in 20 mL of Milli- Q water.

S1-3 Synthesis of golf coated iron oxide magnetic nanoparticles

Full gold shell coatings were grown on iron oxide MNPs via three iterations of 1% (w/v) $HAuCl_4$ reduction. Firstly, 10 mL of the PEI-coated gold-seeded MNPs solution was sonicated for 2 min and, once well-dispersed, the nanoparticle solution was added to 110 mL of 0.01 M NaOH solution (pH ~11.5). This mixture was stirred mechanically throughout the entire gold-shell coating process at 300 rpm. Reduction of a 1% (w/v) $HAuCl_4$ solution then occurred over a three-stage process using 0.2 M $NH_2OH.HCl$. The first iterative reduction was performed by adding 0.75 mL of 1% (w/v) $HAuCl_4$ to 0.5 mL of 0.2 M $NH_2OH.HCl$, followed by a second iteration where 0.5 mL of 1% $HAuCl_4$ was added together with 0.25 mL of 0.2 M $NH_2OH.HCl$ over 5-min intervals. For the last iteration, 0.5 mL of 1% (w/v) $HAuCl_4$ and 0.25 mL of 0.2 M $NH_2OH.HCl$ were mixed over 15-min intervals. These Au@MNPs were then magnetically

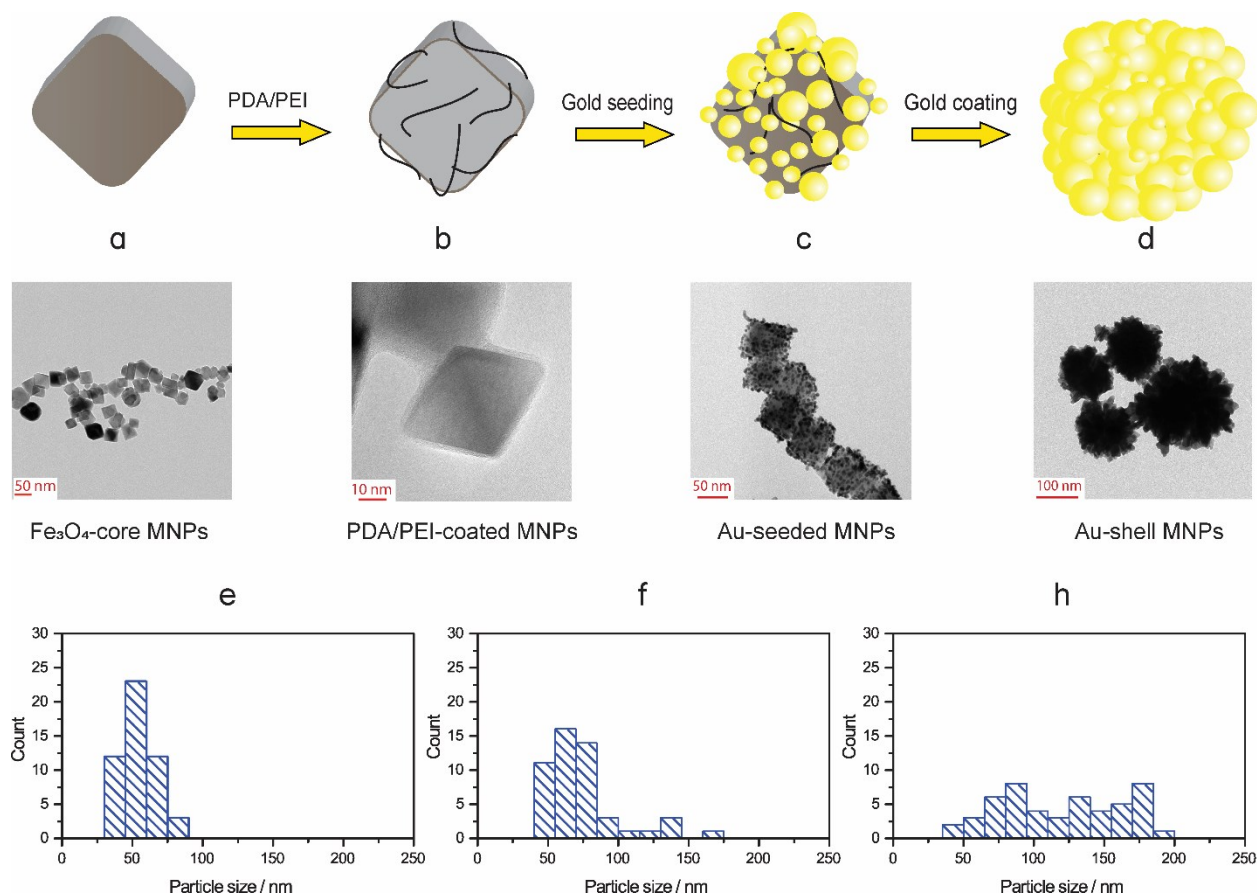separated from the solution, rinsed three times with Milli-Q water and suspended in 10 mL of Milli-Q water.

Fig. S1. Schematic representation of transmission electron micrographs of Au@MNPs with size histogram. From left to right, the schematic and micrographs of the nanoparticles after each step involved in the synthesis are shown as (a) the core MNPs formation, (b) the polydopamine/polyethyleneimine (PDA/PEI) coating layer around the core MNPs to facilitate the attachment of gold seeds in the next step and stabilise the core MNPs, (c) the gold-seeding step was defined after the attachment of gold satellite on PDA/PEI-MNPs and (d) the complete gold shell was grown around the gold-seeded MNPs with multiple iterations of gold deposited on nanoparticles surface via the reduction of HAuCl4. The final reported concentration of Au@MNPs was 1.2×10^{17} particles/mL measured by inductively coupled plasma atomic emission spectroscopy (ICP-OES). The size distribution histogram was shown for Au@MNPs after each synthesis step including (e) magnetic core nanoparticles formation with an average size of 50 nm, (f) gold-seeded MNPs with an average size of about 70 and (h) the fully gold-coated MNPs with an average size of 145 nm (n = 50).

Transmission electron microscopic samples were performed by drop-casting a nanoparticle solution dispersed in Milli-Q water onto a carbon-coated copper grid. The transmission electron micrographs were taken on a JEOL CM 200 transmission electron microscopy operating at 200 kV shown in the Fig. S1 (a, b, c and d). Size distribution was acquired from at least 50 nanoparticles using ImageJ software represented in Fig. S1 (e, f and h).

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S2 Surface modification

Surface modification of the Au@MNPs involves chemical and biological steps to make the functionalised Au@MNPs as "dispersible electrodes" to capture and detect PD-L1 target antigen.

S2-1 Chemical surface modification

Au@MNPs were first modified with a self-assembled monolayer of thioctic acid. This involved incubating 1 mL of the Au@MNPs in 100 µL of 10 mM thioctic acid solution for 4 h at room temperature. To remove the excess thioctic acid of the solution, the thioctic acid-modified Au@MNPs were magnetically separated and rinsed for three times using 75% (v/v) ethanol. The modified Au@MNPs were then activated using 1 mL of 10 mM EDC and 10 NHS coupling reaction in 75% (v/v) ethanol solution upon an hour incubation at room temperature. The EDC/NHS-activated carboxylic group of the modified Au@MNPs finally were rinsed and dispersed in PBS.

S2-2 Biological surface modification

To attach the first antibodies (recognition antibodies) on the surface of the chemically modified Au@MNPs dispersed in PBS, 50 µL of stock 1.5 mg/mL recognition antibody (H00029126-AP41) was added to 100 µL of the previously modified nanoparticles. This mixture was then left to incubate using a vortex mixer for 1 h at room temperature. The formed recognition antibodies-conjugated Au@MNPs (Ab1-Au@MNPs) were magnetically separated and rinsed 3 times with PBS. The target protein PD-L1 standard solution was prepared in PBS with a serial dilution of 13.8 nM stuck (H00029126-Q01). The used PD-L1 is a recombinant protein stored at 50 mM tris-hydrochloric acid with 10 mM reduced glutathione at pH 8.0 in the elution buffer. The Ab1-Au@MNPs were incubated for 1 h at room temperature with 50 µL different concentrations of a PD-L1 target antigen (0 aM, 1.38 aM, 13.8 aM, 0.138 fM, 1.38 fM, 13.8 fM, 138 fM, and 13.8 pM) as data points shown for calibrating current vs. concentration. The formed PD-L1-Ab1-Au@MNPs were magnetically separated and then washed five times. After washing away any unattached PD-L1, the (PD-L1)-Ab1-Au@MNPs were incubated in biotinylated second reporter antibodies (biotin-conjugated detection antibodies) before adding HRP-streptavidin solution (HRP-Ab2) to give the sandwiched complex of HRP-Ab2-(PD-L1)-Ab1-Au@MNPs. In our work, 50 µL of diluted stock 1 mg/mL detection antibodies (H00029126-AP41) was diluted in PBS

mixed with formed PD-1-Ab1-Au@MNPs rest for 1 h at room temperature. This was followed by washing the Ab2-PD-L1-Au@MNPs using magnet for three times and then mixed with 1 µL of HRP-streptavidin enzyme diluted in 999 µL PBS, which was allowed to rest for 1 h at room temperature. The formed sandwiched complex was collected and rinsed 3 times by PBS.
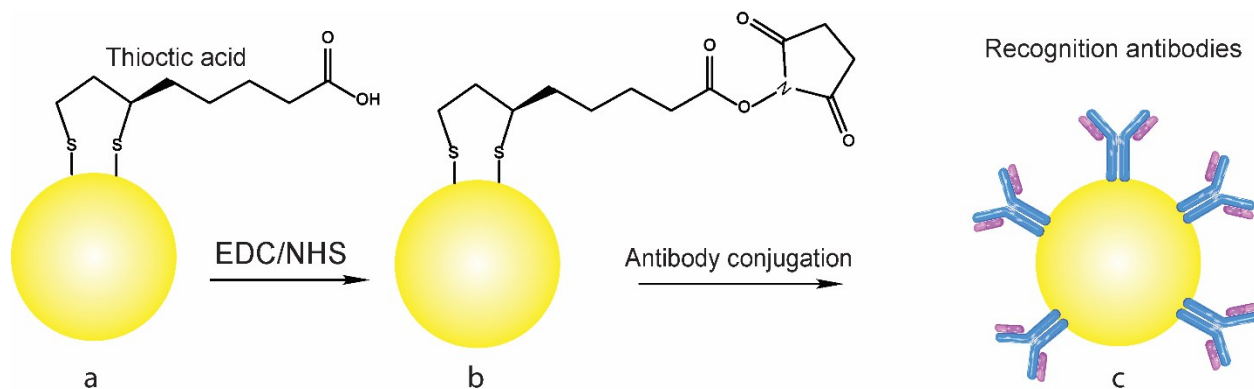

Fig. S2. Schematic representing the surface modification steps of Au@MNPs to enable conjugation of recognition antibodies (this refers to the first antibodies Ab1). The steps include chemical modification via using thioctic acid in 75% (v/v) ethanol, (a) followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxy-succinimide (EDC/NHS) activation. In this reaction, EDC converts carboxylic acid group on Au@MNPs resulted from thioctic acid modification into a reactive intermediate which is susceptible to nucleophilic attack from primary amines. EDC in presence of the NHS can produce a more stable reactive intermediate giving greater reaction yield. This reaction was done in 75% ethanol and rinsed by PBS. (b) To enable biological modification via immobilisation of the recognition antibodies onto the surface of Au@MNPs to capture and detect PD-L1 target analyte in this work.

S3 Electrochemical measurement

The electrochemical immunoassay procedure involved the electrochemical reduction of HRP for quantitative determination of PD-L1 interacted with a sandwiched complex of enzyme via Au@MNPs network in the presence of H_2O_2 with FcMeOH as a redox mediator. As detailed in Section S2, the sandwiched complex of PD-L1 antibodies and antigen on Au@MNPs was dispersed in 1 mL of 1 mM FcMeOH (prepared in PBS) for electrochemical analysis. An electrochemical cell was made of custom glass for all electrochemical measurement involving Au@MNPs with the immobilised sandwich assay. A gold macroelectrode acting as a working electrode was made of 1 cm x 1 cm x 1 mm pure gold foil. The gold macroelectrode was first cleaned with piranha solution and then sequentially polished with 1.0, 0.3 and 0.05 alumina powder on microcloth pads for 15 min each time before being used. Any residual alumina trace on the gold working electrode was removed by sonicated in a Milli-Q water and ethanol for 15 min each one at room temperature. The cleaned and polished gold macroelectrode was shown

in Fig. S4a. The gold macroelectrode was connected to the working electrode lead of a potentiostat (CHI 600E). The gold working electrode was cleaned further electrochemically in 0.05 M sulfuric acid by scanning between 1,500 mV and −300 mV vs. Ag|AgCl 3 M at a scan rate of 100 mV s^{-1} until a reproducible cyclic voltammogram was achieved. The previously modified HRP-Ab2-(PD-L1)-Ab1-Au@MNPs dispersed in 1 mL of 1 mM FcMeOH solution in the electrochemical cell followed by applying a magnetic field using a neodymium magnet for less than 5 min to collect the sandwiched Au@MNPs on the gold macroelectrode (the working electrode surface) (see Fig S4 b, c). After this, 2 μL of 1 M H_2O_2 was added to the cell. The platinum flag acting as a counter electrode and the Ag|AgCl 3M reference electrode were both placed in the electrolyte solution to perform chronoamperometric measurements. The current was recorded over 250 s for multiple concentrations of the PD-L1 for the calibration purposes (see Fig. 1). Figure S3 displays raw data obtained from chronoamperometry whereupon potential remained unchanged (+0.15 V). The difference between current measured with and without introducing H_2O_2 into the electrolyte was then evaluated. The normalised current in the manuscript was determined with"-ΔI" symbol which refers to change in cathodic current relative to the current before adding H_2O_2.
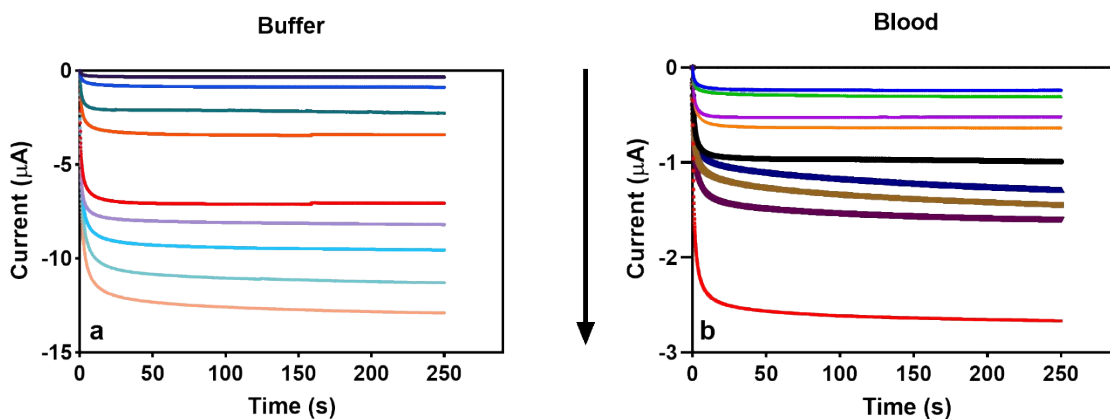

Fig. S3. Chronoamperometry plot indicating the current response vs. various PD-L1 concentration at an unchanged potential of +0.15 V vs. Ag|AgCl 3 M over 250 s time frame for 3 independent measurement in (a) 0 aM, 13.8 pM (of PD-1 incorrect antigen), 1.38 aM, 13.8 aM, 0.138 fM, 1.38 fM, 13.8 fM, 0.138 pM, 1.38 pM, and 13.8 pM concentration of PD-L1 prepared in PBS from top to bottom, (b) the undiluted whole blood in PD-L1 concentration range of 0 aM, 13.8 pM (of PD-1 incorrect antigen), 13.8 aM, 0.138 fM, 1.38 fM, 13.8 fM, 0.138 pM, 1.38 pM, 13.8 pM, and 0.138 nM from top to bottom, respectively. There is a correlation between cathodic current and PD-L1 antigen concentration. Investigation of sensor selectivity is by treating the sensor in 13.8 pM of an incorrect programmed death one (PD-1) antigen in blood and buffer.

S4 Scanning electron microscopy (SEM) of Au@MNPs accumulation on the gold foil

The presence of magnetically assembled Au@MNPs on the gold macroelectrode surface after electrochemical measurement was obtained by SEM. The scanning electron micrographs of the working electrode before and after magnetically assembling Au@MNP on the gold macroelectrode are shown in Figure S4(a) and Figure S4(b), respectively. The Fig. S4(c) illustrates a higher resolution of Fig S4 (b) which is attributed to the aggregation of the sandwiched Au@MNPs network collected on the gold macroelectrode.
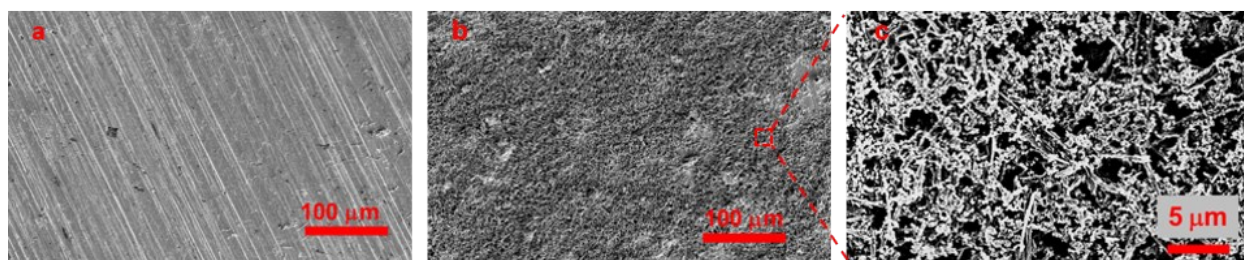

Fig. S4. Scanning electron micrographs of the gold macroelectrode before and after immobilising the sandwiched Au@MNPs. (a) The bare gold macroelectrode surface, (b) the magnetic assembled of the sandwiched Au@MNPs on in 100 μm scale bar and (c) higher resolution of the image "b" with 5 μm scale bar to show the magnetically assembled sandwiched structure of aggregated Au@MNPs on the gold macroelectrode.

S5 Human PD-L1 commercial ELISA kit

A polystyrene 96-well microplate was coated at room temperature with 50 μL of a PD-L1 protein standard solution and 50 μL antibody cocktails as described on the human PD-L1 commercial ELISA kit (ab214565). The kit contains 300 μL of the recognition antibodies and 300 μL of the detection antibodies. The mixture was incubated in the polystyrene 96-well microplate at room temperature for 1 h. Then the plate was rinsed three times with 350 μL washing buffer that came with the PD-L1 ELISA kit. Followed by the incubation of the wells at room temperature with 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) over 10 min in the dark and then 100 μL of a stop solution (2 M sulfuric acid) was added to each well and the plate was shaken for 1 min to mix well. TMB refers to the most common chromogenic substrate for HRP detection in ELISA and the stop solution is introduced to terminate the peroxidase/TMB reaction. Finally, the absorbance was measured using a microplate reader at 450 nm. The reported data were achieved with three replicates and the error bar represents the standard deviation from 3 independent experiments. To determine the limit of detection and the dynamic range of the commercial PD-L1 ELISA kit assay in the buffer sample, standard solutions

of PD-L1 concentration (17.19 pM, 34.38 pM, 68.75 pM, 137.5 pM, 275 pM, 0.55 nM, and 1.1 nM) were prepared and evaluated by the assay. A calibration plot was then constructed between the PD-L1 concentrations and the measured absorbance at 450 nm (see Fig. S5).

This commercial ELISA kit of human PD-L1 is a single wash over 90 min sandwich assay, which is designed for the quantitative analysis of PD-L1 protein in different types of samples including serum and plasma. The limit of detection and dynamic range of the assay was estimated in the PBS. In the studied interval, as shown in Fig. S5, there is a linear relationship between the PD-L1 concentration and absorbance at 450 nm wavelength obtained within the dynamic range from 17 pM to 1.1 nM. The PD-L1 ELISA kit limit of detection in the buffer media was determined to be 8.8 pM (standard deviation of 0.1 pM). The limit of detection was calculated by the following equation[3]

$$limit\ of\ detection = \frac{3\ (standard\ deviation\ of\ the\ blank\ sample)}{slope\ of\ the\ calibration\ plot}\ Eq.1$$

where the standard deviation of the blank sample absorbance was evaluated to be about 0.1 pM and the slope of the calibration plot from the linear equation of the calibration plot was obtained to be 3.4×10^{-3}.
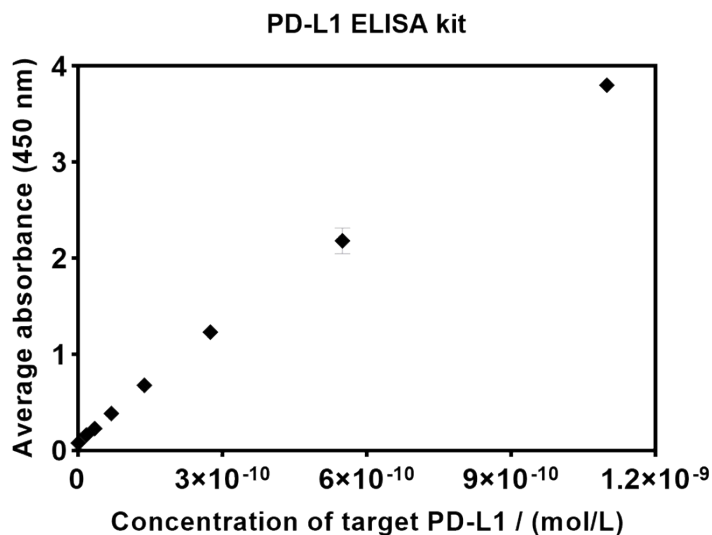

Fig. S5. The standard calibration plot of human PD-L1 ab214565 commercial ELISA kit. The plot represents the average of absorbance in 450 nm in the response of a various range of PD-L1 concentration in buffer (17.19 pM, 34.38 pM, 68.75 pM, 137.5 pM, 275 pM, 0.55 nM, and 1.1 nM). The method was based on forming a sandwich structure via adding PD-L1 standard solution into primary and secondary antibodies solution at once, before being incubated in a polystyrene 96-well microplate for 90 min at room temperature. After washing the polystyrene 96-well microplate, it was treated by a stop solution (2 M sulfuric acid) and the solution colour was observed to turn

to yellow. Error bars represent standard deviation from 3 independent measurements (N = 3). Error bars are insignificant which are not clearly distinguishable for all points of the plot except at 0.55 nM concentration of PD-L1.

Here, the performance of the dispersible electrodes was compared with formerly developed methods for PD-L1 detection in terms of technique types, limit of detection and dynamic detection range of methods. The limit of detection and dynamic range are critical sensing parameters which were reported for the standard methods such as PD-L1 ELISA kits (which can quantify protein concentration), surface enhanced Raman scattering,[4-6] surface plasmon resonance,[7] localised surface plasmon resonance,[8] and chemiluminescent immunoassay.[9] The limit of detection and dynamic range of detection for ELISA assays were reported up to pM in four different studies.[10-13] In another study, coupling commercial ELISA with PDA-modified metal–organic framework and improved limit of detection up to 1.2 pM.[14] Here immunohistochemistry and Western Blot assays are not considered as their results are mostly qualitatively with less sensitivity and require high concentrations of analytes. Our sensor can effectively improve both selectivity and sensitivity as well as a broad dynamic range from attomolar to nanomolar with detection limit of 3.4 aM in buffer and 15 aM in blood. This means limit of detection in buffer and blood are approximately 2,600,000 (2.6 million times) and 600,000-fold lower than that of commercial ELISA kit (ab214565).

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