Electronic Supplementary Material (ESI) for Chemical Communications

A fluorescent peroxidase probe increases the sensitivity of commercial ELISAs by two orders of magnitude

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Supplementary Information

1. Materials

Materials were purchased from Corning, Sigma Aldrich, AK Scientific Inc., Thermo Scientific Inc., eBiosciences Inc. and Abcam and were utilized as received. All reactions dealing with air- or moisture-sensitive compounds were carried out in oven dried glass round bottom flasks under nitrogen. Dichloromethane and methanol were evaporated using a rotary evaporator (Heidolph Inc.). Fluorescence measurements were performed using a Tecan i4 microplate reader.

2. Synthesis of Hydrocyanines

Hydrocyanines were generated according to Kundu et al.[1] Briefly, 3 μmoles of IR780 (Sigma Aldrich) and 30 μmoles of sodium borohydride (Sigma Aldrich) or 5.1 μmoles of cyanine3 (AK Scientific Inc.) and 51 μmoles of sodium borohydride (Sigma Aldrich) were mixed in 5 mL methanol in a 100 mL round bottom flask, for 30 minutes at room temperature under stirring. The methanol (Fisher Scientific) was then evaporated using a rotary evaporator and the hydrocyanines were extracted into dichloromethane (Fisher Scientific) (dichloromethane (5mL) from ultrapure water (Millipore) (5mL)). The dichloromethane was evaporated to completion and the hydrocyanines were resuspended at 74 μM in the buffer used to measure HRP activity (11.1 mM sodium citrate buffer, 180 μM H_2O_2 (Fisher Scientific), 0.50 μM 4-hydroxy-4-biphenyl carboxylic acid (ROS stabilizer – Sigma Aldrich),[3,4] 20.6% dimethyl sulfoxide (DMSO - VWR) , and 0.01% triton x-100 (Sigma Aldrich)) and used immediately. Hydrocyanines were prepared fresh for each experiment.

3. Effect of triton x-100 on the quantum yield (Φ) of cyanine dyes. Triton x-100 has been shown to improve the quantum yield of fluorescent dyes.[2] We therefore investigated if triton x-100 can increase the quantum yield of IR780. 1.5 μM solution of IR780 in phosphate buffer saline (PBS) and a 1.5 μM solution of IR780 containing 0.01% (v/v) triton x-100 in PBS were made and 50 μL of these solutions were added to the wells of a 96-well plate, and the absorbance (780 nm) and the fluorescence were measured (Ex/Em = 780/820 nm). The quantum yield was obtained by using the formula described below and was determined to be 0.09 in triton x-100 buffer versus 0.001 in PBS. Triton x-100 was therefore added to the hydrocyanine buffer solutions used to measure HRP activity.

$$\Phi_{triton} = \Phi_{PBS} \times \left(\frac{Fluorescence_{triton}}{Fluorescence_{PBS}}\right) \times \left(\frac{Absorbance_{PBS}}{Absorbance_{triton}}\right)$$

4. Effects of DMSO and triton x-100 on HRP activity

The hydrocyanine buffer used to test HRP activity contains DMSO and triton x-100, which are needed to solubilize hydrocyanines and increase the quantum yield of the cyanine dye generated from oxidation. We performed experiments to determine the effects of DMSO and triton x-100 on HRP activity.

A) **HRP activity in varying concentrations of DMSO.** We performed experiments to determine the effects of DMSO on HRP activity. 50 μL of HRP solutions in PBS containing 1000 pM, 500 pM, 250 pM, 125 pM and 62.5 pM were added to the wells of a 96-well plate. Commercial TMB substrate solution (Thermo Scientific Inc.) was diluted with

DMSO to obtain a final concentration of 40%, 20% or 10% DMSO. As controls, equivalent volumes of PBS were added to the commercial TMB substrate solution. 50 µL of commercial TMB substrate solution either diluted in DMSO or PBS was added to the wells containing HRP. After 5 min of reaction, 50 µL of 0.2 M sulfuric acid was added to the wells and the absorbance at 450 nm was read using a plate reader. Figures S1A, B and C demonstrate that the activity of HRP is reduced in the presence of 40% DMSO, whereas in the presence of 20% and 10% DMSO, the HRP activity is the same as that of the control.

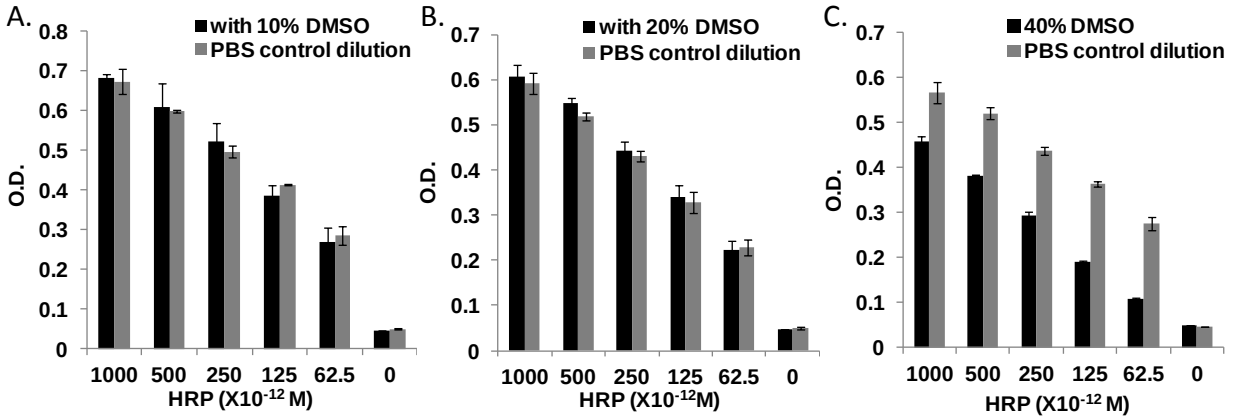

Figure S1: HRP activity at different concentrations of DMSO. HRP activity was determined by the absorbance of oxidized TMB in commercial pre-mixed TMB buffer in the presence of varying concentrations of DMSO. HRP retains its activity in the presence of 10% DMSO (**A**) and 20% DMSO (**B**), whereas in the presence of 40% DMSO (**C**) HRP activity is significantly decreased.

B) **HRP activity in varying concentrations of triton x-100.** We performed experiments to determine the effects of triton x-100 on HRP activity. 50 µL of HRP solutions in PBS containing 0.25%, 0.1% and 0.01% were added to the wells of a 96-well plate. Next, commercial TMB substrate solution was diluted with 0.5% triton x-100 to obtain final concentration of 0.25%, 0.1% and 0.01% triton x-100. As controls, equivalent volumes of PBS were added to the commercial TMB substrate solution. 50 µL of commercial TMB substrate solution either diluted with triton x-100 or PBS was added to the wells containing HRP. After 5 min of reaction, 50 µL of 0.2 M sulfuric acid was added to the wells and the absorbance at 450 nm was read using a plate reader. Figures S2A, B and C demonstrate that the activity of HRP is reduced in the presence of 0.25% and 0.1% triton x-100, whereas in the presence of 0.01% triton x-100, the HRP activity is the same as that of the control.

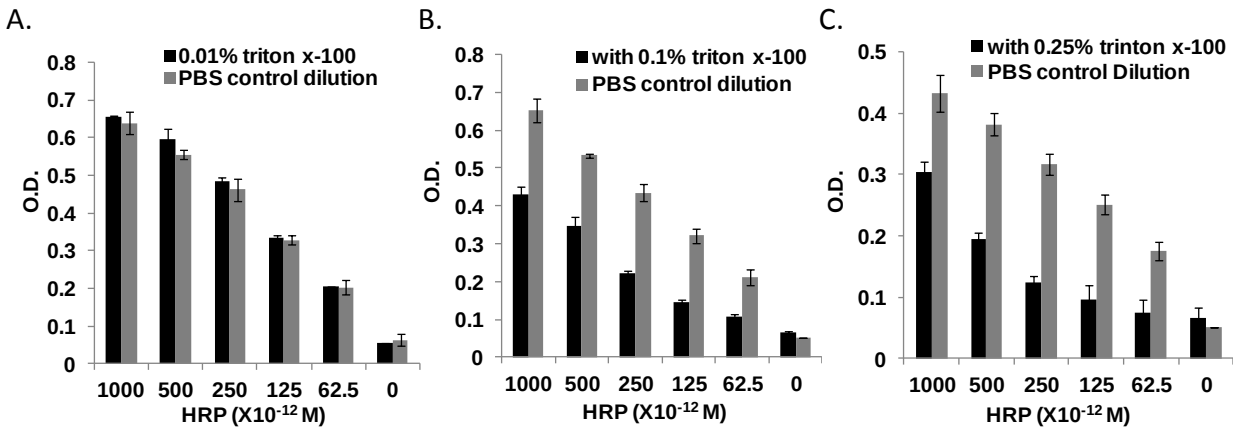

Figure S2: HRP activity at different concentrations of triton x-100. HRP activity was determined by the absorbance of oxidized TMB in commercial pre-mixed TMB buffer in the presence of varying concentrations of triton x-100. HRP retains its activity in 0.01% triton x-100 solution (**A**) whereas in the presence of 0.1% triton x-100 (**B**) and 0.25% triton x-100 (**C**) HRP activity is significantly decreased.

C) **HRP activity in hydrocyanine buffer versus commercial TMB substrate buffer.** We also determined the HRP activity in the presence of both DMSO and triton x-100. 4.5 nM of HRP in 50 µL of phosphate buffer saline (PBS) was added to the wells of a 96 well plate. 50 µL of hydrocyanine buffer containing 21 µM TMB (Sigma Aldrich) was then added to the wells containing HRP. Additionally, in separate wells, 50 µL of the commercial TMB substrate solution, pre-mixed with TMB was added to 50 µL of 4.5 nM of HRP in PBS. After 5 min of reaction, 50 µL of 0.2 M sulfuric acid was added to the wells and the absorbance at 450 nm was read using a plate reader. Figure S3 demonstrates that the absorbance from the commercial TMB substrate solution is not statistically different from the absorbance generated due to the oxidation of TMB dissolved in hydrocyanine buffer, demonstrating that the hydrocyanine buffer does not affect the activity of HRP.

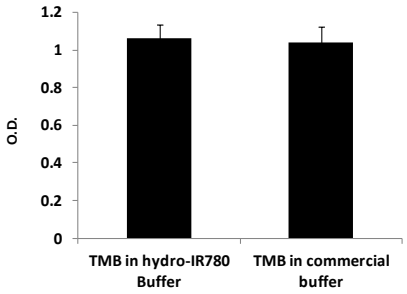

Figure S3: HRP activity is maintained in the hydrocyanine buffer. The absorbance of TMB in commercial pre-mixed TMB buffer and hydrocyanine buffer is the same, demonstrating that the activity of HRP is equivalent in the two buffers.

5. Hydrocyanine sensitivity to HRP

Horseradish Peroxidase (HRP) (Sigma Aldrich) was dissolved in pH 5.5 citrate buffer and diluted from 1000 fM to 15 fM concentrations. 50 µL of the HRP dilutions and 50 µL of the hydro-IR780, at 74 µM in a buffer composed of, 180 µM H2O2, 0.50 µM 4-hydroxy-4-biphenyl carboxylic acid (ROS stabilizer), 20.6% DMSO, and 0.01% triton x-100 in 11.1 mM

sodium citrate buffer, were added to the wells of a black 96 well plate. The fluorescent signal generated was then recorded (Ex/Em = 780/820 for hydro-IR780), after 15 min. In another set of experiments, 50 µL of the commercially obtained TMB substrate (Thermo Scientific Inc.) was incubated with the HRP dilutions in a 96 well plate, and the absorbance was recorded at 652 nm after 5 minutes of incubation. Amplification factors of hydrocyanines and TMB were determined by obtaining the ratio of oxidized hydrocyinines/TMB to HRP.

6. GFP expression verification of U373-GFP with fluorescent microscopy.

U373 human glioblastoma cells were cultured according to MacKay et. al.[5] Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum, selected with 1 µg/ml puromycin, and expanded in culture with 100 ng/ml tetracycline. U373 cells that were untransfected (U373) were utilized as a control. GFP expressing cells (U373-GFP) and control cells were plated in 6-well plates at $1x10^6$ cells per well and after 2 days were imaged, in the plate, at 20X magnification using a Nikon fluorescent TE200 microscope (480 nm/ 535 nm (Ex/Em)) to determine GFP expression. Figure S4 demonstrates that U373-GFP cells express GFP.

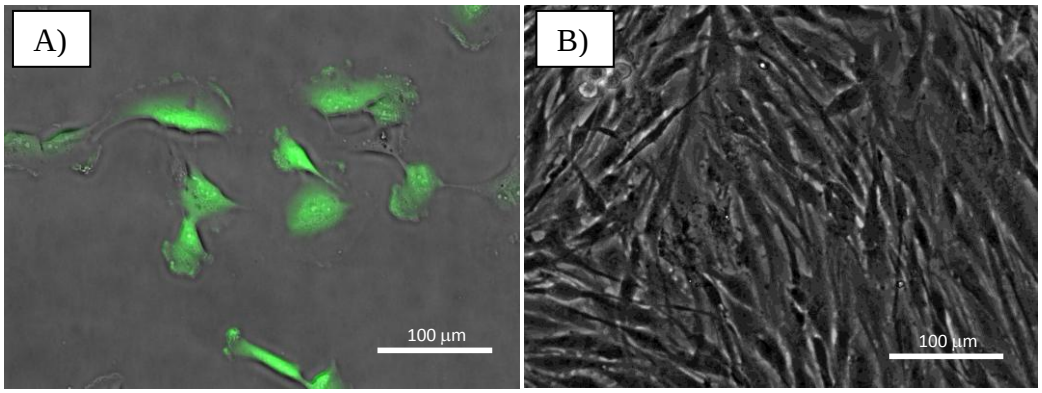

Figure S4: U373-GFP cells express GFP. Fluorescent overlay micrographs at 20X magnification show that U373-GFP cells have bright green fluorescence and express GFP (A), whereas the control untransfected cells do not express GFP (B).

7. Hydrocyanine-based ELISAs

We investigated if hydrocyanine could increase the sensitivity of commercial ELISAs. GFP ELISAs (Abcam Inc.) were performed according to the manufacturer's instructions, except that 50µL of hydro-IR780 at 74 µM was added as the HRP substrate instead of TMB, in its optimized buffer composed of, 180 µM H_2O_2, 0.50 µM 4-hydroxy-4-biphenyl carboxylic acid (ROS stabilizer), 20.6% DMSO, and 0.01% triton x-100 in 11.1 mM sodium citrate buffer, and incubated for 30 min. The fluorescence intensity was determined in a plate reader (Ex/Em = 780/820 nm) and plotted against GFP concentration; each concentration had 4 replicates to obtain statistics. A separate set of experiments were performed with TMB based GFP ELISAs for comparison. TMB utilized in these experiments was pre-mixed in the buffer provided in the commercial GFP ELISA kit.

8. Hydrocyanine-based ELISAs for quantifying intracellular GFP

We performed experiments to determine if hydrocyanines could detect intracellular GFP from a 1000 U373-GFP cells, using a commercial GFP ELISA kit (Abcam Inc.), untransfected U373 cells were used as a control. U373-GFP and

control cells were plated in 6-well plates at $1x10^6$ cells per well. A 0.05% trypsin solution (VWR) was added to the cells and incubated at 37 ^{o}C for 2-4 minutes. Cells were removed from the plate and added to 10 mL of cell culture media (10% calf serum in DMEM) to neutralize the trypsin and were washed 2 times with PBS by centrifuging at 300xGs for 5 min. The cell pellet was resuspended in PBS, counted using a hemocytometer (Fisher Scientific), and an aliquot containing a 1000 cells was centrifuged in an eppendorf tube at 300xGs for 5 min and resuspended in a 100 µL of GFP lysis buffer, diluted 10^5 times in PBS (Abcam catalog # ab117992). The cell lysate was kept on ice for 20 min and centrifuged at 16,000xGs for 20 min. 100 µL of this supernatant was then utilized to perform ELISAs. The ELISA was performed according to the manufacturer's instructions, except that 50µL of hydro-IR780 (74 µM) was added as the HRP substrate instead of TMB, in its optimized buffer composed of, 180 µM H_2O_2, 0.50 µM 4-hydroxy-4-biphenyl carboxylic acid (ROS stabilizer), 20.6% DMSO, and 0.01% triton x-100 in 11.1 mM sodium citrate buffer, and incubated for 30 min. The fluorescence intensity was determined in a plate reader (Ex/Em = 780/820 nm), and plotted against control U373 cells. A separate set of cell experiments were performed with TMB based GFP ELISAs for comparison. The TMB utilized in these experiments was pre-mixed in the buffer provided in the commercial GFP ELISA kit.

9. Hydrocyanine-based ELISAs for quantifying IL-1β

We performed experiments to determine if hydro-IR780 could increase the sensitivity of commercial IL-1β ELISA kits (eBiosciences). The IL-1β ELISA was performed according to the manufacturer's instructions, except that 50 µL of hydro-IR780 (74 µM) was added as the HRP substrate instead of TMB, in its optimized buffer composed of, 180 µM H_2O_2, 0.50 µM 4-hydroxy-4-biphenyl carboxylic acid (ROS stabilizer), 20.6% DMSO, and 0.01% triton x-100 in 11.1 mM sodium citrate buffer, and incubated for 30 min. The fluorescence intensity was determined in a plate reader (Ex/Em = 780/820 nm) and plotted in Figure S5A. A separate set of experiments were performed with TMB based IL-1β ELISAs for comparison (Figure S5B). TMB utilized in these experiments was pre-mixed in the buffer provided in the commercial IL-1β ELISA kit.

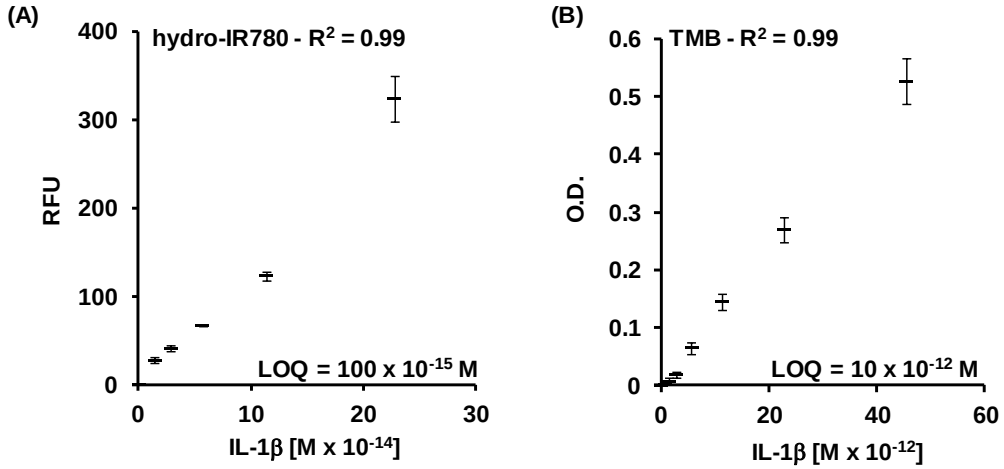

Figure S5: Hydrocyanines enhance the sensitivity of IL-1β ELISAs by 2 orders of magnitude. A) Hydro-IR780 based commercial IL-1β ELISA kits can quantify a 100 femtomolar of IL-1β. **B)** TMB based commercial IL-1β ELISA kits can quantify 10 picomolar IL-1β.

10. Statistical Analysis

Statistical analyses were performed using student's t-test and p-values for each experiment were determined. Statistically significant data ($p<0.05$) are depicted using the '*' symbol.

11. References

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