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

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The low detection sensitivity of enzyme linked immunosorbent assays (ELISAs) is a central problem in science and limits progress in multiple areas of biology and medicine. In this report we demonstrate that the hydrocyanines, a family of fluorescent reactive oxygen species (ROS) probes, can act as turn on fluorescent horseradish peroxidase (HRP) probes and thereby increase the sensitivity of conventional ELISAs by two orders of magnitude.

The ELISA plays a central role in modern biology and is the most common method for detecting biomolecules.[1–5] However, despite its widespread use, the detection sensitivity of ELISA is in the nanomolar/picomolar range, whereas the vast majority of antigens found in clinical samples and cell lysates are in the femtomolar range. For example anti-HIV antibodies found in blood during early infections are in the femtomolar range, whereas commercial ELISA kits can detect only nanomolar levels.[6–8] The low sensitivity of ELISAs hinders progress in all areas of biomedical science, ranging from the development of new blood diagnostics to pharmaceuticals, and strategies for increasing their detection sensitivity are greatly needed.

Enzyme amplification *via* the reporter enzyme HRP plays a critical role in determining the detection sensitivity of an ELISA.[9–12] However, despite their high amplification level, HRP based ELISAs are still not sensitive, due to the lack of an effective substrate for HRP. For example, tetramethylene benzidine (TMB) is the most common HRP ELISA substrate; however, it has only nanomolar/picomolar sensitivity because it is an absorption based substrate.[1] In addition, although, two fluorescent probes for HRP have been developed, tyramide-IR800 and 10-acetyl-3,7-dihydroxyphenoxazine, their sensitivities are also in the nanomolar/picomolar range, due to their high background and instability in the presence of radical oxidants, resulting in low amplification factors.[13–15] Furthermore, although gold and DNA labels have pushed the protein detection limit to low-femtomolar ranges, these techniques cannot be routinely used in a clinic or a biology laboratory, because of their incompatibility with commercial ELISA kits and plate readers.[16–18]

In this report we demonstrate that hydrocyanines can increase the sensitivity of ELISAs by 2 orders of magnitude by acting as ''turn on'' fluorescent HRP probes. HRP generates hydroxyl radicals in the presence of H_2O_2,[12] and non-fluorescent hydrocyanines are amine-oxidized by hydroxyl radicals to fluorescent cyanine dyes (Fig. 1).[19] We therefore investigated if hydrocyanines could be used to detect HRP.

A challenge of using hydrocyanines for ELISAs is that hydrocyanines are not soluble in water and their oxidation products, cyanines, have low quantum yields in water. We therefore developed a buffer system composed of 0.01% Triton X-100 and 20% DMSO in 11.1 mM citrate, which could solubilize hydrocyanines and increase the aqueous quantum yield of the resulting cyanine dyes (quantum yield increase 0.001 to 0.09 – see ESI† – Section 3). DMSO and Triton X-100 can potentially modify the activity of HRP, we therefore performed experiments to determine the tolerance of HRP to these additives, using the commercially available HRP substrate TMB. Fig. S1–S3 (see ESI†) demonstrate that HRP retains its activity in 20% DMSO and 0.01% Triton X-100 and therefore, these additives can be used to investigate the ability of HRP to oxidize hydrocyanines.

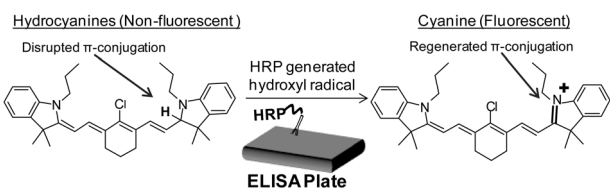

Fig. 1 Hydrocyanines increase the sensitivity of conventional ELISAs by two orders of magnitude. In this report we demonstrate that HRP generated radical oxidants can oxidize non-fluorescent hydrocyanines to fluorescent cyanine dyes, which increases the detection limits of commercial ELISAs by 2 orders of magnitude.

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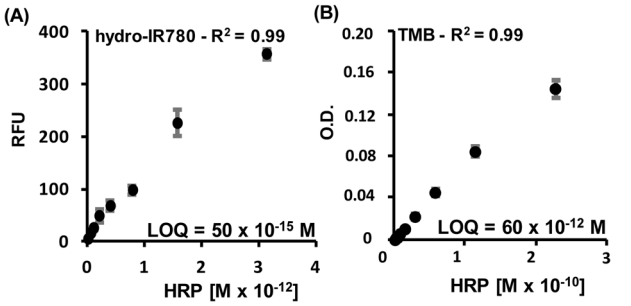


Fig. 2 Hydrocyanines are ultrasensitive HRP substrates. (A) Hydro-IR780 has a limit of quantification of 50 femtomolar for soluble HRP (*x*-axis). (B) HRP mediated oxidation of TMB has a limit of detection of 60 picomolar. Hydro-IR780 is three orders of magnitude more sensitive than TMB in detecting soluble HRP.

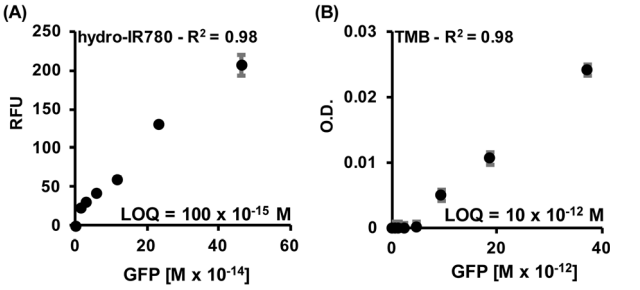


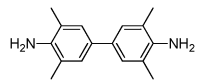
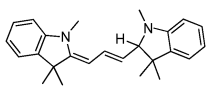
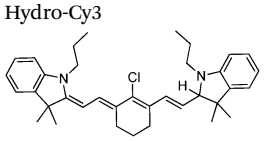
Fig. 3 Hydrocyanines enhance the sensitivity of GFP ELISAs by 2 orders of magnitude. (A) Hydro-IR780 based commercial GFP ELISA kits can quantify 100 femtomolars of GFP. (B) TMB based commercial GFP ELISA kits can quantify 10 picomolars GFP.

We determined the detection sensitivity of hydrocyanines for HRP and compared it to the currently used commercial HRP substrate TMB. Hydro-Cy3 and hydro-IR780 were synthesized according to Kundu *et al.* and dissolved in the hydrocyanine buffer described above, and were incubated with soluble HRP (H_2O_2, 180 μM) for 15 minutes at room temperature, and the resulting fluorescence was measured using a commercial plate reader.[19] As a control soluble HRP was incubated with TMB, in the TMB buffer provided by the manufacturer, and the resulting absorbance at 652 nm was measured using a commercial plate reader. Fig. 2 demonstrates that hydrocyanines are HRP substrates and have a limit of quantification of 50 femtomolar for soluble HRP, which is 3 orders of magnitude higher than that for TMB. The high sensitivity of hydrocyanines towards HRP is due to their fluorescent oxidation product, which is a cyanine dye. In contrast, TMB generates only an absorbance change after oxidation by HRP. The amplification factors of hydrocyanines and TMB were determined and found to be 22 000 and 5000, respectively, which also contribute to the high HRP sensitivity of hydrocyanines (Table 1).

Hydrocyanine-based HRP probes have the potential to increase the sensitivity of ELISAs, given the critical role of HRP amplification. We investigated if hydro-IR780 could increase the sensitivity of ELISAs for the green fluorescent protein (GFP). We decided to detect GFP because a well-established GFP ELISA kit is commercially-available and because GFP transfected cells can be independently validated *via* fluorescent microscopy (ESI† – Fig. S4). A GFP ELISA (Abcam Inc.) was performed according to the manufacturer's instructions, except that hydro-IR780 was used as the HRP substrate instead of TMB. Fig. 3A demonstrates that hydro-IR780 can dramatically increase the sensitivity of GFP ELISAs, and can quantify 100 femtomolar of GFP, which is 2 orders of magnitude more sensitive than the TMB based GFP ELISA, which could only quantify 10 picomolar GFP (Fig. 3A and B). We performed additional experiments to determine if hydro-IR780 could be a broadly applicable HRP substrate to increase the sensitivity of commercial ELISA kits. The ability of hydro-IR780 to increase the sensitivity of commercial IL-1β ELISA kits (eBiosciences) was therefore investigated. IL-1β was selected as a second protein for investigation because of its well established role as a biomarker for inflammatory diseases. Fig. S5 (see ESI†) demonstrates that hydro-IR780 was able to similarly increase the sensitivity of commercial IL-1β ELISA kits by a factor of 100.

We investigated if the sensitivity of hydro-IR780 based ELISAs would enable quantification of proteins directly from cell lysates and provide a quantitative high-throughput alternative to western blotting. Western blotting is the current method used to analyze proteins from cell lysates, however this method has limitations because it lacks the ability for high throughput analysis. In addition, western blots cannot precisely quantify

Table 1 Hydrocyanines are sensitive HRP substrates and have high amplification factors

HRP substrate	Amplification factor	Limit of HRP quantification [moles per liter]
 TMB	5900 ± 1300	56×10^{-12}
 Hydro-Cy3	15 300 ± 410	40×10^{-15}
 Hydro-IR780	22 000 ± 2300	50×10^{-15}

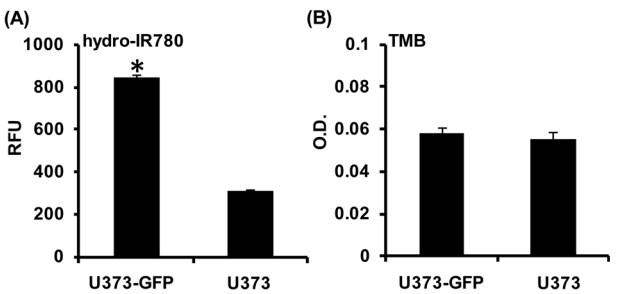


Fig. 4 Hydro-IR780 based ELISAs can detect intracellular GFP from the cell lysates of 1000 cells. (A) Cell lysates from GFP expressing cells (U373-GFP) had a two fold increase in cyanine fluorescence over control cells (U373) using hydro-IR780 based GFP ELISAs (p-value < 0.05, $n = 4$). (B) TMB based GFP ELISAs were unable to detect intracellular GFP from the cell lysates of 1000 cells.

protein concentrations. In contrast, ELISAs can be performed in a high throughput manner and accurately quantify proteins, and therefore have the potential to significantly impact cellular proteomics.

We therefore investigated if hydrocyanine based ELISAs could detect GFP expression from U373 human glioblastoma cells that had been transduced with the GFP gene[20] (U373-GFP), using a cell lysate generated from 1000 cells. As a control we also performed GFP ELISAs on untransduced cells (U373). Fig. 4A demonstrates that hydrocyanine based ELISAs can distinguish between U373-GFP and U373 cells, using as few as 1000 cells. In contrast, TMB based ELISAs were not able to detect GFP under these conditions (Fig. 4A and B).

In conclusion, we have demonstrated that hydrocyanines are fluorescent HRP substrates and can increase the sensitivity of commercial ELISAs by two orders of magnitude. Importantly, HRP based ELISAs are available for a wide variety of antigens, and hydrocyanines should be able to enhance the sensitivity of a large number of commercial kits, enabling their rapid use by the molecular biology community.

The authors declare no competing financial interest.

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