Attomolar detection of proteins in serum using single molecule enzyme-linked immunosorbent assays.

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Introduction

The clinical use of protein biomarkers for the differentiation of healthy and diseased states, and for monitoring disease progression, requires the measurement of low concentrations of protein in complex samples. Current immunoassays measure protein concentrations above 10-10 M, whereas the concentration of the majority of protein biomarkers in disease states and the early stages of disease is thought to be in the range of 10-14 to 10-10 M. The variation and complexity of protein biomarkers provide a promising approach for measuring extremely low concentrations of proteins. Here, we report an approach for detecting thousands of single protein molecules simultaneously using the same reagents as the gold standard for measuring protein concentration. The current bench-top instrument automatically seals the reaction vessel from single enzymes are generated.

Methods

Our approach makes use of arrays of hexamerized gold nanoparticles—what we term Single Molecule Arrays (SiMoA)—to isolate and detect single enzyme-labeled protein molecules. This, "digital ELISA" is shown schematically in Figure 1. A sandwich immunoassay is first formed on microscopic bead surfaces and loaded into the reporter module. When assaying samples containing extremely low concentrations of protein, a "digital" signal is generated by trapping and isolating single enzyme-labeled protein molecules. The calculated concentration of the protein is the number of signal molecules per unit area of bead surface. The number of signal molecules per unit area of bead surface is determined by the number of fluorescent product molecules. To achieve this localization in an immunoassay, beads are loaded into an antibody array containing all the antibodies required to locate a target protein and to generate a fluorescence signal. The antibodies are immobilized on the surface of the bead as a small envelope of a bead surface array (BSA). Digital ELISA is then used to measure proteins from blood using traditional enzyme label reagents and single molecule arrays has been developed.

Results

A) Enzyme label sensitivity

Initially, we assessed the potential gain in sensitivity to enzyme labels that can be achieved by singling enzyme-labeled molecules compared to a traditional, continuous-flow ELISA. We created protein standards by immobilizing protein standards with a range of concentrations of an enzyme-antibody conjugate. The two individual molecular enzyme-labeled antigen-presenting bead ensemble of beads was read out in 15 s on a Fluoroscan plate reader after 1 h of development using Enzyme-linked Digital Immunoassay (SiMoA), a fluorescent substrate for this fluorophore. The detection limit of the capture array was 10 aM of biotinylated antibody immobilized on the beads. Figure 2 shows the log plot of the percentage of beads that contain fluorescent enzyme labels at various concentrations of biotinylated antibody immobilized on the beads, from 100 aM to 100 nM biotinylated antibody immobilized on the beads. The linear dynamic range of digital detection of enzyme labels by SiMoA is from 3 nM down to 10 A, above first line.

B) Single-molecule detection of proteins and DNA

We developed digital ELISA for PSA and TIM-3, and a digital ELISA to detect DNA using weakly attached single molecule labels using SiMoA. Figure 4 shows the results from digital assays for PSA, TIM-3, and DNA. The human forms of the proteins were spiked into human serum at 10 pg/mL. The bottom panel is a log plot of the fraction of beads containing fluorescent enzyme substrates. The calculated sensitivity to enzyme labels is the number of signal molecules per unit area of bead surface. The average concentration in this cohort was 1.5 pg/mL, with the lowest concentration being 0.2 pg/mL detected using a single molecule assay.

Conclusions

An approach—called digital ELISA—for detecting single protein from blood using traditional enzyme label reagents and single molecule arrays has been developed.

The sensitivity to enzyme label of SiMoA is in the attomolar range, and is 100x more sensitive than the detection of robotic phasemask using chemiluminescence.

Digital ELISA has enabled the measurement of clinically important proteins in serum at concentrations much lower than traditional ELISA, e.g., PSA has been detected down to 1 pg/mL.

Digital ELISA enabled the detection of PSA in the serum of all samples from patients who had undergone radical prostatectomy, and thus could be used to monitor the success of detection and diagnosis of disease in a number of diseases.

References