Ultra-sensitive protein detection using single molecule arrays (SiMoA): the potential for detecting single molecules of botulinum toxin

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Abstract: Single molecule arrays (SiMoA) make it possible to detect subfemtomolar concentrations of proteins in complex samples. We describe the basis of SiMoA and its potential application in the detection of proteins, such as botulinum neurotoxin (BoNT), from pathogenic bacteria. We also provide a concept for an integrated handheld SiMoA device for the detection of very low concentrations of BoNT in the field.

Keywords: single molecule arrays; SiMoA; single molecules; proteins; digital ELISA; botulinum toxin; microfluidics.

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The detection of biomolecules – and, by inference, pathogenic agents – is driven by the sensitivity of the available analytical techniques. The detection of nucleic acids (DNA and RNA) associated with pathogenic bacteria and viruses is well served by extremely sensitive analytical techniques, such as the polymerase chain reaction (PCR). PCR, for example, can routinely detect down to 50 viruses per mL of HIV based on the detection of RNA. The detection of proteins associated with bacteria and viruses, however, is limited by lack of sensitivity of the current analytical methods. For example, the enzyme-linked immunosorbent assay (ELISA) – which has been the gold standard of protein detection for 40 years – routinely detects down to subpicomolar concentration of proteins, i.e., about 120 million molecules of the p24 capsid protein of HIV or ~60,000 viruses per mL. With improved sensitivity, however, immunoassays could complement nucleic acid testing because they are lower cost, less complex, easier to automate, not prone to 'spoofing' by synthetic molecules, and they detect protein

pathogens directly. Sensitive analytical methods for detecting proteins, such as p24 and the botulinum neurotoxin (BoNT) produced by the bacterium *Clostridium botulinum* are, therefore, needed.

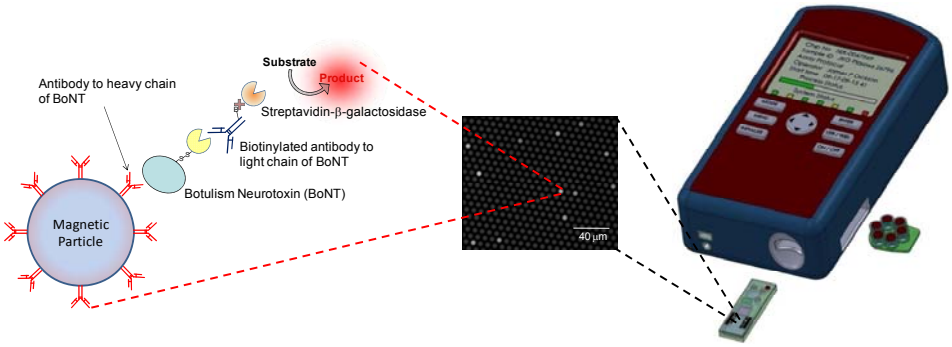
Sensitive analytical methods have benefits other than detection of low numbers of biomolecules in a sample. Sensitivity can be used to allow dilution of samples to minimise interference from matrix effects, e.g., for the detection of proteins in complex samples such as food. Sensitive methods can also be leveraged to test low volume sample (~1−10 μL) by allowing dilution of samples while maintaining the ability to detect the target molecules.

We have recently developed a method called digital ELISA (Rissin et al., 2010) based on the detection of individual protein molecules in single molecule arrays (SiMoA). This method is about 1,000 times more sensitive than conventional, analogue ELISA, and has opened the intriguing possibility of protein assays matching the sensitivity of PCR to viruses and bacteria. In digital ELISA, protein molecules are first captured on 2.7-μm-diameter paramagnetic beads that are coated in an antibody to the target protein (Figure 1). The proteins are then labelled with an enzyme by sequential incubations with biotinylated detection antibody and streptavidin-β-galactosidase. At very low concentrations (in the fM or fg/mL range) the ratio of protein molecules to beads is low, so the number of proteins captured per bead can be described by the Poisson distribution. At ratios of labelled proteins to beads of less than about 0.1, the Poisson distribution indicates that the vast majority of labelled beads only present a single, labelled protein. The single molecule sensitivity of the method derives, therefore, from the Poisson distribution statistics at low numbers of molecules. The enzyme-associated beads are then deposited into arrays of microwells (4.5-μm diam. and 3.25-μm deep) with femtoliter volumes, such that each well contains only a single bead. These arrays are then sealed in the presence of enzyme substrate and fluorescently imaged onto a CCD to determine the generation of fluorogenic product in each well. The sealing step is critical to localise the fluorescent product within the microwell (50-fL volume) making it possible to detect a single enzyme. Images are analysed to determine if a well is 'on' (contains an enzyme-associated bead) or 'off' (contains a bead not associated with an enzyme). The fraction of beads associated with an enzyme (f_{on}) is then determined and converted to average enzymes per bead (AEB) using the Poisson distribution equation (Rissin et al., 2011). The dynamic range of the method is expanded by determining the average intensity of the beads in the array once > 70% of the beads are associated with an enzyme (Rissin et al., 2011).

The high sensitivity of SiMoA to enzyme label – we showed that we could detect 20 yoctomole or about ten enzymes in a 100 μL droplet (Rissin et al., 2010) – results in very sensitive immunoassays. As the method is so sensitive to label, the concentration of labelling reagents (detection antibody and enzyme) that dominate the background in these assays can be lowered > 100-fold. This reduction in turn results in a reduction in assay background to the equivalent of fM of analyte. Very low backgrounds combined with the ability to count so few labels, makes it possible to detect reliably sub-fM concentrations using SiMoA. We have demonstrated the detection of femtomolar concentrations of inflammatory cytokines (Song et al., 2011), cancer biomarkers (Rissin et al., 2010, 2011) and neurological markers (Zetterberg et al., 2011) in plasma and serum using digital ELISA. In each case, improvements in sensitive of > 1,000-fold were demonstrated over standard ELISA using the same antibody pairs. The digital nature of the method means that it is highly precise and not sensitive to variations in temperature, enzyme activity, or

enzyme substrate concentration. As a result, the precision of the detection step is Poisson noise limited, and within-run and day-to-day imprecisions of <10% can be achieved routinely (Wilson et al., 2011).

Figure 1 The components of digital ELISA, (a) Capture of single protein molecules on paramagnetic beads and labelling by enzyme (b) Fluorescence image of single, enzyme-labelled proteins captured on beads and loaded into femtoliter well arrays (c) Concept for a miniaturised digital ELISA system



(a) (b) (c)

Notes: The bright wells correspond to a single enzyme label associated with a bead. All bead incubations, washing, array loading, and imaging occurs on a microfluidic chip. A handheld reader images the arrays, and a reagent cartridge houses beads and labelling reagents.

As well as assay performance, ultra-sensitive protein detection techniques must be automated and low-cost for them to be adopted broadly by researchers and clinicians. SiMoA is well suited for fully automated, low-cost instrumentation. The dominant solid phase used in the immunodiagnostics industry for >20 years has been paramagnetic particles, and the liquid handling robots for mixing, incubating and washing these beads are well established. The microwell arrays used to confine single beads can be manufactured from a low-cost, biocompatible polymer by replica moulding of microfabricated parts using blu-ray DVD technology (Kan et al., 2012). Imaging of these arrays can be achieved using the illumination sources, filters, and CCD cameras found in typical cell biology microscopes. By combining maturing developments in the robotics, microstructured moulding, and CCD imaging industries, a system for performing digital ELISA in high throughput is being developed.

While digital ELISA has not been employed to date for the detection of bacteria, we believe that BoNT provide an excellent example of where the benefits of such a sensitive immunoassay could be employed for detecting protein pathogens. As the pathogenic agents of the bacterium, it is not possible to detect BoNT using nucleic acid technologies; an immunoassay must be employed. The high toxicity of BoNT means that it is important to have the tools to measure extremely low concentrations of the protein. Such measurements would be employed in the food industry, for national security, and to monitor clinical use of BoNT in the cosmetics industry. Current immunoassays have similar limits of detection of the gold-standard mouse bioassay (~1−10 pg/mL). More sensitive assays that could detect BoNT at the fg/mL in a variety of samples (food, water, dirt, and blood) would enable early detection of the presence of the pathogen in complex

samples. We have proposed a method for detecting BoNT using digital ELISA that is based on the same reagents that are used today to detect the molecule using standard ELISA [Figure 1 (a)]. The toxin is first captured on beads using an antibody to the heavy chain of BoNT. A biotinylated detection antibody to the catalytic portion of the toxin is then used to label the molecule with an enzyme. The single, enzyme-labelled BoNT molecules are then isolated in the arrays of microwells and imaged as usual for digital ELISA.

In the future, it should also be possible to develop miniaturised systems for performing digital ELISA for detecting BoNT using portable units (Figure 1). The microwell arrays can be readily incorporated into microfluidic systems for the automated loading and sealing of beads (Kan et al., 2012), and it should be possible to develop a microfluidic system that performs the steps for capture and labelling of proteins also. The use of LED illumination sources and CCD chips for imaging would allow a low cost reader to be developed.

In summary, digital ELISA based on the detection of single protein molecules in SiMoA enables the detection of proteins at femtomolar concentrations, about 1,000-fold lower than traditional, analogue ELISA. The application of digital ELISA to detecting protein toxins such as BoNT would provide a general approach to detecting these molecules as early as possible.

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