ENCODED GEL PARTICLE ARRAY FOR RAPID, MULTIPLEXED PROTEIN DETECTION IN COMPLEX MEDIA

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ABSTRACT

In this work, we use stop flow lithography (SFL) and a custom microfluidic flow-through scanning system for the development, validation, and application of a sensitive and multiplexed hydrogel microparticle based protein quantification platform. Gel particles are graphically encoded and functionalized with capture probes against protein targets of interest. We take advantage of the non-fouling and flexible nature of our substrate to demonstrate rapid and robust detection of (1) a panel of 4 cytokines in HIV viral inhibition assay cell culture supernatants using antibody-based sensing and (2) thrombin, a blood clotting protein using aptamer-based sensing.

KEYWORDS

Multiplexed Protein Quantification, Cytokine Profiling, Aptamers, Hydrogels, Biosensors

INTRODUCTION

The analysis of biologically complex and clinically relevant samples requires a sensitive, multiplexed, and high throughput protein quantification platform which allows flexibility with respect to type of sample, target panel, and capture probe. Commercially available methodologies for protein detection utilize either planar arrays, such as the enzyme-linked immunosorbent assay (ELISA) or spectrally-encoded particle arrays, such as the Luminex system[1, 2]. Although ELISAs are widely used for protein quantification, they suffer from low sample throughput and inability to multiplex with ease.[3] Surface-based particle arrays such as Luminex allow higher sample throughput, but are often prohibitively expensive due to the need for a multi-color readout system and usage of thousands of beads per assay to achieve precise quantification[4]. In this work, we use stop flow lithography (SFL) for synthesis of a graphically-encoded polyethylene glycol (PEG) hydrogel particle array bearing covalently incorporated antibodies or aptamers against proteins of interest.[5, 6] Since PEG is non-fouling, particles can be used directly in raw complex media without the need for assay diluents or sample processing.

In previous work, we showed single pg/mL recovery of human cytokines spiked into 95% fetal bovine serum (FBS) using antibody-functionalized gel particles.[7, 8] We have since validated our antibody-based sensing platform against a commercially available Luminex bead assay for direct detection of cytokines in cell culture supernatant, and now utilize it to understand cytokine signatures in $CD8^+$ killer T-cell mediated human immunodeficiency virus (HIV) inhibition. HIV continues to be a global pandemic and cytokine cascades are thought to largely direct the immunological response to the virus, making it of utmost importance to understand their secretion profiles with respect to degree of viral inhibition in a range of patients.[9, 10] We use encoded gel particles bearing antibody probes to simultaneously measure a panel of 4 cytokines that are relevant to viral inhibition: interleukin-2 (IL-2), interferon-γ (IFN- γ), macrophage inflammatory protein-β (MIP1- β) and tumor necrosis factor-α (TNF-α).

We have concurrently further extended our platform to include aptamers for protein capture and labeling. Although the majority of protein detection assays employ antibody pairs, these biomolecules are expensive, vary from lot to lot, and are prone to cross-reactivity. Furthermore, production of antibodies requires *in vivo* synthesis techniques. Nucleic acid ligands called aptamers are therefore a desirable alternative for antibodies in immunoassays due to ease of synthesis, stability in storage and a selection process that confers high affinity and specificity for intended targets.[11] Using SFL techniques from prior nucleic-acid based work allowed us to easily interface aptamers with the encoded gel particle platform.[12] A well-characterized aptamer pair against a blood clotting protein thrombin was used to evaluate aptamer performance for protein detection in the hydrogel microenvironment. Inside the flexible and three-dimensional hydrogel scaffold, we were able to achieve thrombin limits of detection (LOD) that surpass most surface-based systems without needing to use long spacer sequences between the capture aptamer and the gel anchor point.[13]

EXPERIMENTAL

All particles shown in this study were encoded, functionalized, and UV-polymerized simultaneously in a microfluidic device at rates of 16,000 particles/hour using SFL (Figure 1a). Particles were designed to carry a graphical barcode that is spatially segregated from a separate probe-bearing region. The fluorescently-doped code region consists of rectangular unpolymerized holes of varying dimension, and regions that flank the probe-bearing region provide the particle with structural stability and serve as internal controls. Probes were covalently incorporated into the gel network using acrylate chemistry. Prior to synthesis, capture antibodies were concentrated to 10 mg/mL and reacted with a heterobifunctional linker molecule (Acrylate-PEG-SCM 2000) to enable addition of an acrylate group to antibody side chains. Capture aptamers were purchased with an acrylate modification. Monomer compositions were chosen such that the probe-region would have a pore-size large enough (15-20 nm) to permit diffusion of the large biomolecules while maintaining high (3-30 μM) probe incorporation. In a typical sandwich assay, 25 particles of each type were mixed either directly into

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complex media or buffer of interest (Figure 1b). Particles were incubated with target for 2-4 hours to ensure thorough diffusion and reaction of target throughout the particle scaffold, and labeled using a biotinylated reporter molecule (either an antibody or an aptamer). A fluorescent reporter conjugated to streptavidin was then used to visualize binding events. (Figures 1b& 2) Post assay, particles were loaded into a flow-focusing device for rapid (25 particles/second) decoding and target quantification. Due to spatial separation between the code region and the probe region of the particles, a single color (532 nm) excitation source was sufficient for all analysis, as compared to spectrally encoded bead arrays such as Luminex (Figure 1c).

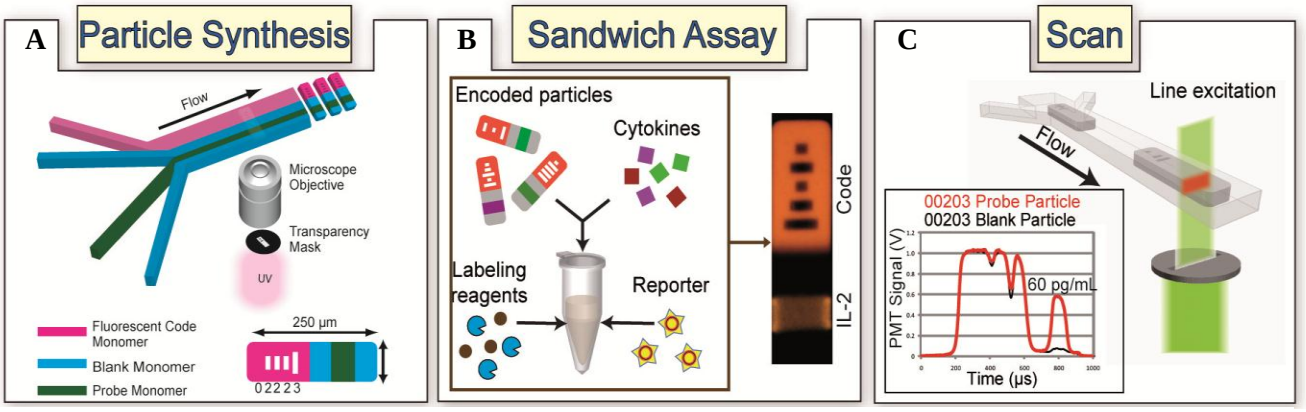

Figure 1: Assay workflow depicting (a) particle synthesis using SFL in a microfluidic device, (b) typical sandwich assay in complex media or buffer, and (c) microfluidic scanning system for particle decoding and target quantification.

Cell cultures for cytokine analysis were prepared in one of two ways. For platform validation, peripheral blood mononuclear cells (PBMCs) were externally stimulated using PHA/ionomycin to ensure release of a subset of the 4-plex panel (IL-2, IFN-γ and TNF-α). Meanwhile, viral inhibition was assessed by infecting populations of $CD4^+$ T-cells isolated from HIV patients *ex-vivo* and subsequently adding $CD8^+$ T-cells that were either autologous or allogeneic to enable viral inhibition. Measurement of an HIV core protein, p24, allowed quantification of viral inhibition activity as a consequence of $CD8^+$ T-cell addition. Cell culture supernatants were then profiled for cytokine release.

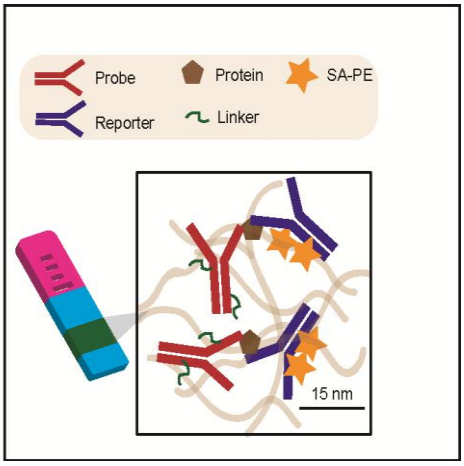

Figure 2: Post-assay depiction of gel network after a typical antibody-based protein assay. Aptamer-based assays use a similar sandwich scheme.

RESULTS AND DISCUSSION

Our 4-plex multiplexed cytokine assay was optimized to ensure specificity of antibody pairs and to maximize sensitivity. It was confirmed that the cell culture supernatants did not react with the particle scaffold by combining the supernatants with "blank" particles which had the same chemical composition and geometrical features of standard particles without any probe. In order to maximize signal and minimize noise, we systematically tuned the concentration of reagents that were used in the labeling steps of assays such that we maintained a 3-4 log dynamic range while obtaining high sensitivity.

All calibration curves were generated by spiking recombinant standards into cell culture media at concentrations ranging from 10 pg/mL to 1000 pg/mL. Furthermore, due to uniformity of particles during synthesis, it was necessary to only generate calibration curves once per batch of particles (enough for 350-400 assays). Target-probe specificity was confirmed through a cross-reactivity matrix assay in which each capture antibody was checked for reactivity with unintended targets and reporting antibodies. Using our sandwich assay, we were able to achieve sensitivities similar to commercially available ELISA and Luminex assays (<10 pg/mL) for most targets in cell culture supernatants without using signal amplification steps. In our platform validation experiments, the same aliquots of cell culture supernatant samples were interrogated using both Luminex and the gel particles. It was found that all measurements were within 2X of each other using both systems, indicating excellent agreement and validating the gel particle platform (Figure 3). Our work with viral inhibition assay (VIA) supernatants indicates that MIP1-β, a chemokine that is associated with anti-HIV toxicity may demonstrate differential secretion based on viral state in HIV patients.[14]

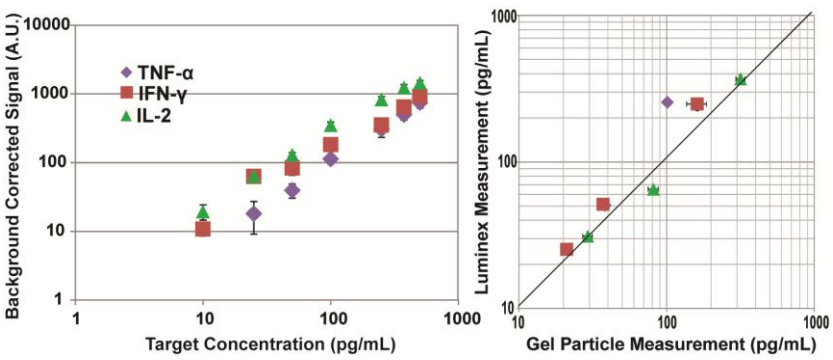

Figure 3: Calibration curves generated in cell culture media for multiplexed cytokine detection and comparison between gel particle platform and Luminex platform, showing agreement in measurement.

Using hydrogel particles functionalized with aptamers, we attained either a 4 pM thrombin detection limit (using a biotinylated reporter antibody) or a 20 pM thrombin detection limit (using a biotinylated aptamer sequence), both of which surpass what competing surface-based platforms can offer even while using capture aptamers modified with spacer molecules. In comparison to surface-based substrates, more probe can be immobilized in the projected area of a hydrogel (10^5 molecules/μm^2 in a gel versus 10^4 molecules/μm^2 on a surface) while maintaining greater distance between probe molecules (55 nm in a gel versus 10 nm on a surface). The combination of higher probe concentration and reduced steric hindrance allows aptamers to stably fold into configurations necessary for effective target binding without modifying the capture aptamer with spacers. Furthermore, even when we utilized thymine spacer molecules, we found minimal detection advantage (1-2X fold increase in signal as opposed to the 4-10X increase in signal that surface based systems often experience).[13]

CONCLUSION

In conclusion, we demonstrate a robust protein detection platform for rapid quantification in complex samples using both antibodies and aptamers. The flexibility and detection advantages of hydrogels together with our high-throughput analysis system could enable sensitive and multiplexed profiling in a range of complex media to better characterize disease state and to build non-invasive diagnostic tools.

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