ULTRASENSITIVE MULTIPLEXED QUANTIFICATION OF MICRORNA AND PROTEIN PANELS ON ENCODED GEL MICROPARTICLES

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ABSTRACT

We report a versatile, microparticle-based molecular detection platform for the highly sensitive, multiplexed quantification of both microRNA (miRNA) and proteins in complex biological media. In addition to establishing our gel-particle platform as a powerful tool for rapid profiling of cytokine targets, this work significantly enhances a previous miRNA detection protocol by demonstrating rolling-circle amplification (RCA) on encoded microparticles to achieve single-molecule reporting resolution for miRNA targets. This RCA scheme provides miRNA sensitivity far superior to existing particle-based arrays and enables the direct detection of miRNA in unprocessed human serum (*i.e.*, no RNA extraction/isolation) without the need for target-amplification steps.

KEYWORDS: Biosensor, Multiplexed Analysis, Hydrogel, MicroRNA, Cytokine

INTRODUCTION

Advances in molecular diagnostics and personalized medicine require flexible detection systems with simple workflows that can accurately and efficiently quantify biological molecules in a wide range of complex media. Ideally, these systems would be versatile enough to measure a diverse range of biomarkers, including both nucleic acids and proteins. MicroRNAs (miRNAs) and cytokines are two classes of biomolecules that offer a wealth of information on disease state, and the ability to rapidly measure the expression levels of focused panels of these entities will be crucial to the development of next-generation diagnostic tools [1, 2]. Existing methods for miRNA quantification all have shortcomings, and innovative, highly sensitive detection technologies are needed to handle emerging applications, such as the serum-based assay of focused target panels for cancer diagnosis [3-6]. Meanwhile, cytokines and other proteins are typically quantified using time- and labor-intensive enzyme linked immunosorbent assays (ELISAs), which are not easily adapted to multiplexed measurements or high-throughput processing [7, 8]. In this paper, we significantly enhance the utility of a graphically-encoded gel particle-based detection platform by demonstrating the efficient multiplexed analysis of minute amounts of miRNA and cytokines using two distinct assay workflows. Microfluidic stop-flow lithography (SFL) is employed to create multifunctional poly(ethylene glycol) (PEG)-based microparticles bearing distinct chemical regions for encoding and target capture (Figure 1) [4, 9, 10]. miRNA and cytokine target panels can be quantified in model buffers as well as serum through the addition of the appropriate collection of single-probe particles, or alternatively, through the addition of particles bearing multiple spatially-segregated probe regions. We previously reported a novel labeling scheme for rapid, multiplexed miRNA profiling on our gel particles for four human cancer types using low-input total RNA and shorter assay times than existing approaches [4]. We now present an isothermal signal-amplification strategy that lowers the particles' limit of detection of miRNA to 250 aM, enabling the direct detection of miRNA in human serum. Furthermore, we demonstrate the three-dimensional immobilization of antibodies within the probe regions of our particles for the multiplexed measurement of three protein targets using a sandwich assay format. The hydrated gel environment provides higher probe immobilization efficiency than surface-functionalized protein detection platforms, while also preventing non-specific adsorption of non-target proteins in complex biological media.

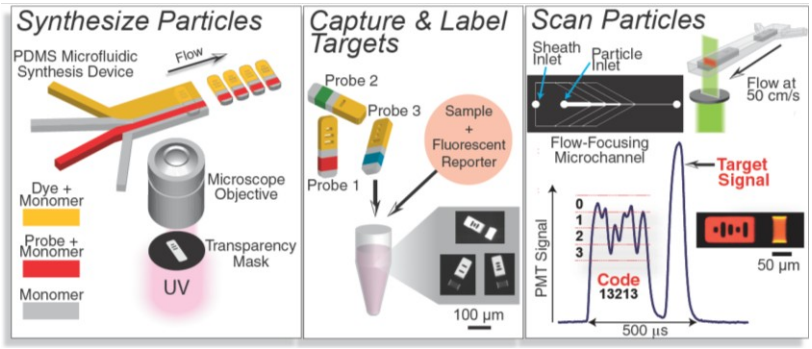

Figure 1: Gel particles are simultaneously synthesized, encoded, and functionalized via stop-flow lithography in a PDMS microfluidic device. For multiplexing, each particle has a fluorescently-doped graphical barcode that corresponds to the identity of the probe(s) covalently immobilized in a separate region. Codes and target levels can be rapidly read in a microfluidic scanning device at a rate of 25 particles/s.

EXPERIMENTAL

The miRNA and protein detection protocols described here both involve the capture and subsequent labeling of target on gel particles. Encoded particles were synthesized with SFL at a rate of 16,000 per hour with a fluorescently-doped code region (coding bits consist of unpo-

lymerized holes in the wafer structure of the particle), one or more probe-laden regions, and inert spacer regions. For RCA-based miRNA detection, each probe consisted of a DNA sequence bearing a target-specific region and an adjacent region for the hybridization of a universal adapter containing a primer site (Figure 2). Following particle exposure to sample, the universal adapter sequence was selectively ligated to miRNA targets that had been captured on the gel-embedded probes, with unligated sequences washed away via a low-salt rinse. By ligating the same primer sequence to all captured miRNA targets, we could generate long DNA extensions bearing a periodically repeated sequence from each target-binding event upon introduction of circular template, Phi29 DNA polymerase, dNTPs, and a gel-customized reaction buffer [11]. The resulting concatemers were labeled using biotinylated oligonucleotides and fluorescent streptavidin-phycoerythrin (SA-PE). Because the same primer could be used for all targets, this scheme provided signal-based amplification without the risk of sequence bias in multiplexed assays. The particles and the individual target-binding events on their surface were imaged using standard fluorescence microscopy with a 20X objective (Figure 3).

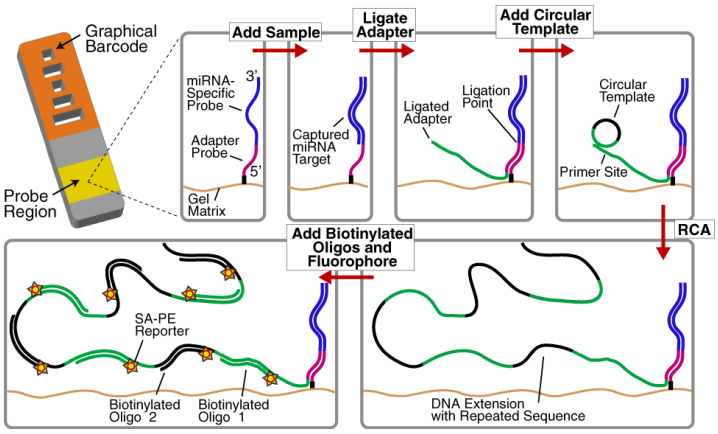

Figure 2: RCA protocol for miRNA detection. Each target-binding event can be labeled with multiple fluorophores, thereby enabling target quantification via fluorescent spot-counting. Selective labeling of only those miRNA that have been specifically captured on immobilized probes has several advantages over the bulk enzymatic and chemical methods used in other detection schemes, including higher efficiency, lower cost, and reduced likelihood of labeling bias due to secondary structure.

For protein detection, capture antibodies were covalently incorporated throughout the hydrogel matrix of the probe regions during SFL with the use of a heterobifunctional PEG linker (acrylate-PEG-succinimidyl carboxymethyl), enabling one-step synthesis, encoding, and functionalization using only small amounts of valuable reagents. Hydrogel porosity was optimized for efficient target and reagent penetration of the gel, and a sandwich assay was developed in which biotinylated reporter antibody and SA-PE were attached to a second epitope of the target protein (Figure 4). Following labeling, particles were rinsed and then scanned with a laser-induced fluorescence (LIF) setup at rates up to 25 particles/s in a microfluidic flow-focusing device to rapidly extract information regarding code identity and amount of target bound [4, 7, 12].

RESULTS AND DISCUSSION

The multiplexed detection of miR-141, miR-210, and miR-221 with the RCA-enhanced labeling scheme was investigated on three separate single-probe particle types as well as a particle type bearing all three probes (Figure 3). Through the optimization of RCA buffer composition, single-molecule reporting resolution was achieved with a polymerase extension time of 4 hr. RCA significantly enhanced the miRNA detection performance of the gel particles, lowering the limit of detection in a

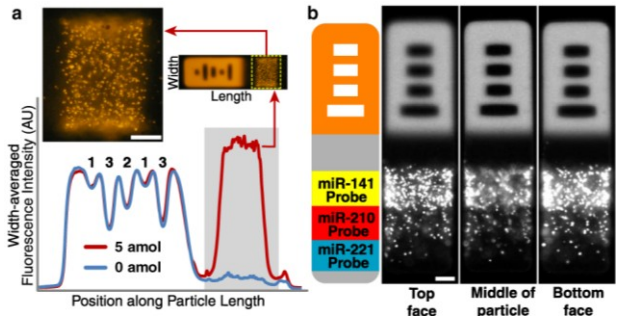

Figure 3: Single-molecule reporting resolution for miRNA analysis. (a) Overlaid stationary scans of particles exposed to either 0 or 5 amol of miR-210, with magnified color image of top face of probe region. Each fluorescent spot represents a single target-binding event. (b) Multi-probe particle exposed to solution containing 5 amol miR-141, 1 amol miR-210, and 0.2 amol miR-221. All scale bars are 20 μm.

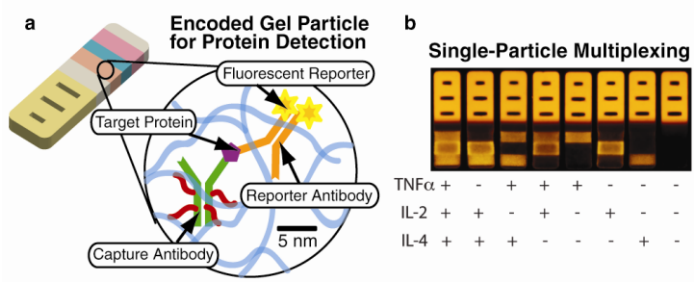

Figure 4: Protein detection on gel particles. (a) Illustration of the antibody sandwich used for protein target capture and labeling. (b) Multiplexed detection of IL-2 (120 pg/ml of target spiked in), IL-4 (110 pg/ml), and TNFα (400 pg/ml) cytokines on a multi-probe particle. Top, middle, and bottom probe strips contain capture antibodies for TNFα, IL-2, and IL-4, respectively. + denotes target has been spiked in and - denotes target has not been spiked in.

Tris-EDTA buffer (spiked with 100 ng *E. coli* total RNA to simulate complexity) from 20 fM to 250 aM. This sensitivity is far superior to existing particle arrays and also surpasses the sensitivity achieved with other RCA schemes (dumbbell, padlock blotting, branched), blot techniques, and conventional microarrays [13]. With the ultrasensitive detection afforded by RCA, we interrogated miR-141 levels in normal and cancer-associated serum and confirmed an earlier PCR-based study that showed miR-141 to be upregulated in the serum of prostate cancer patients [5]. Importantly, our multiplexed assay can detect miRNA in serum without the need for bias-prone target-amplification steps. Furthermore, our particles' non-fouling PEG scaffold enables the assay to be performed *directly* in unprocessed serum (only 25 μl required per assay), without the need for time-consuming RNA extraction/isolation, which complicates quantification on existing platforms [6].

In addition, with our antibody-sandwich assay protocol, we simultaneously detected three cytokines (tumor necrosis factor alpha [TNFα], interleukin-2 [IL-2], and interleukin-4 [IL-4]) spiked into 95% fetal bovine serum at concentrations as low as 1 pg/mL [7]. It should be noted that the non-fouling gel matrix of the particles enabled this direct serum detection without the need for blocking reagents or filtration steps. With the rapid analysis afforded by our companion microfluidic gel-particle scanner, this entire assay could be carried out in under 4 hr, providing a dynamic range of three orders of magnitude and coefficients of variation in target level less than 12%. Protein detection performance on the gel particles was more sensitive than comparable workflows on other systems. To attain the limits of detection achieved here with the gel particles, microarray formats must employ signal amplification schemes, while solid particle arrays require additional sample filtration steps [7]. Multiplexed measurement of the same cytokine panel was also performed on a single particle type (Figure 4), again demonstrating the versatility of the spatially segregated chemical regions on the encoded microgels.

CONCLUSION

In summary, we demonstrate the highly sensitive multiplexed analysis of focused panels of miRNAs and cytokines on graphically-encoded hydrogel microparticles. By also adapting the RCA scheme to reporter antibodies, the advances reported here could potentially be used for the simultaneous quantification of low-abundance miRNAs and proteins in a single assay in a variety of easily-accessible biological media, leading to faster, less invasive diagnostic assays.

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