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Neutral DNA-avidin nanoparticles as ultrasensitive reporters in immuno-PCR

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KEYWORDS: protein detection; DNA-avidin nanoparticles; PEG coating; DNA reporters; immuno-polymerase chain reaction.

ABSTRACT

We have developed an immuno-PCR based diagnostic platform which couples detection antibodies to self-assembled, ultra-detectable DNA-avidin nanoparticles stabilized with poly(ethylene glycol) to link DNA amplification to target protein concentration. Electrostatic neutralization and cloaking of the PCR-amplifiable DNA labels by avidin and PEG coating reduces

non-specific "stickiness" and enhances assay sensitivity. We further optimized the detectability of the nanoparticles by incorporating four repeats of a unique synthetic DNA PCR target into each nanoparticle. Using human chorionic gonadotropin hormone (hCG) as a model analyte, this platform was able to quantitate the target hCG protein in femtomolar concentrations using only standard laboratory equipment.

INTRODUCTION

The need for ultrasensitive protein detection has challenged the scientific community for many years, with a notable example being the introduction of radio-immunoassay by Yalow et al. in 1959.[1] The gold standard for detecting protein molecules has been ELISA (enzyme-linked immunosorbent assay) in which an analyte is captured on the surface of a microplate well by immobilized antibodies and recognized by an antibody conjugated to a signal-generating enzyme reporter. Various technical innovations (e.g., miniaturization,[2] single-molecule counting,[3] microfluidics and automation,[4] engineered reporters[5,6] and substrates[7]) have improved the performance of immunoassays. Of particular note is immuno-PCR (iPCR; introduced by Sano et al. in 1992[8]), which combines the versatility and specificity of antibody recognition in immunoassays with the exponential signal-amplifying power of PCR, promising a wide dynamic range and dramatically-enhanced sensitivity.[9] Immuno-PCR uses an antibody conjugated to an amplifiable DNA reporter which can be detected very sensitively by PCR, but its great promise has been compromised by various technical difficulties.[10] First, naked DNA molecules non-specifically bind to various surfaces[11–13] and biomolecules,[14–16] increasing iPCR background signal. Second, iPCR requires often-complicated preparation of specific DNA-antibody conjugates.[9,17,18] To address these challenges, a variety of alternative biological or chemical

nanostructures, including liposomes[19] and bacteriophage virus nanoparticles[20–22] have been explored in an effort to "shield" the DNA reporters and reduce non-specific binding.

We have previously explored M13 bacteriophage as a reporter in iPCR.[20] Although the no-target background was greatly reduced, we found weak dependence of the signal on analyte concentration, likely due to steric interference of the large viral particles. Another drawback to using naturally-occurring DNA reporter sequences (e.g., M13 gDNA) in iPCR assays is their possible adventitious presence in biological samples. Inspired by an alternative immunoassay reporter with low nonspecific binding, a protein-DNA core-shell nanoparticle,[23–26] in which avidin and polyethylene glycol (PEG) are used to condense and stabilize plasmid DNA, we incorporated multiple, *de novo* designed, repetitive PCR templates into the plasmid DNA and enhanced the PCR detectability of these custom-designed nanoparticles (Figure 1). We also demonstrated the use of these custom-designed iPCR reporter nanoparticles in the detection of human chorionic gonadotropin (hCG), a glycoprotein hormone and a novel biomarker for pregnancy[27] and testicular cancer.[28] We were able to quantitate hCG as low as 660 fM using our iPCR reporter nanoparticles and standard laboratory equipment.

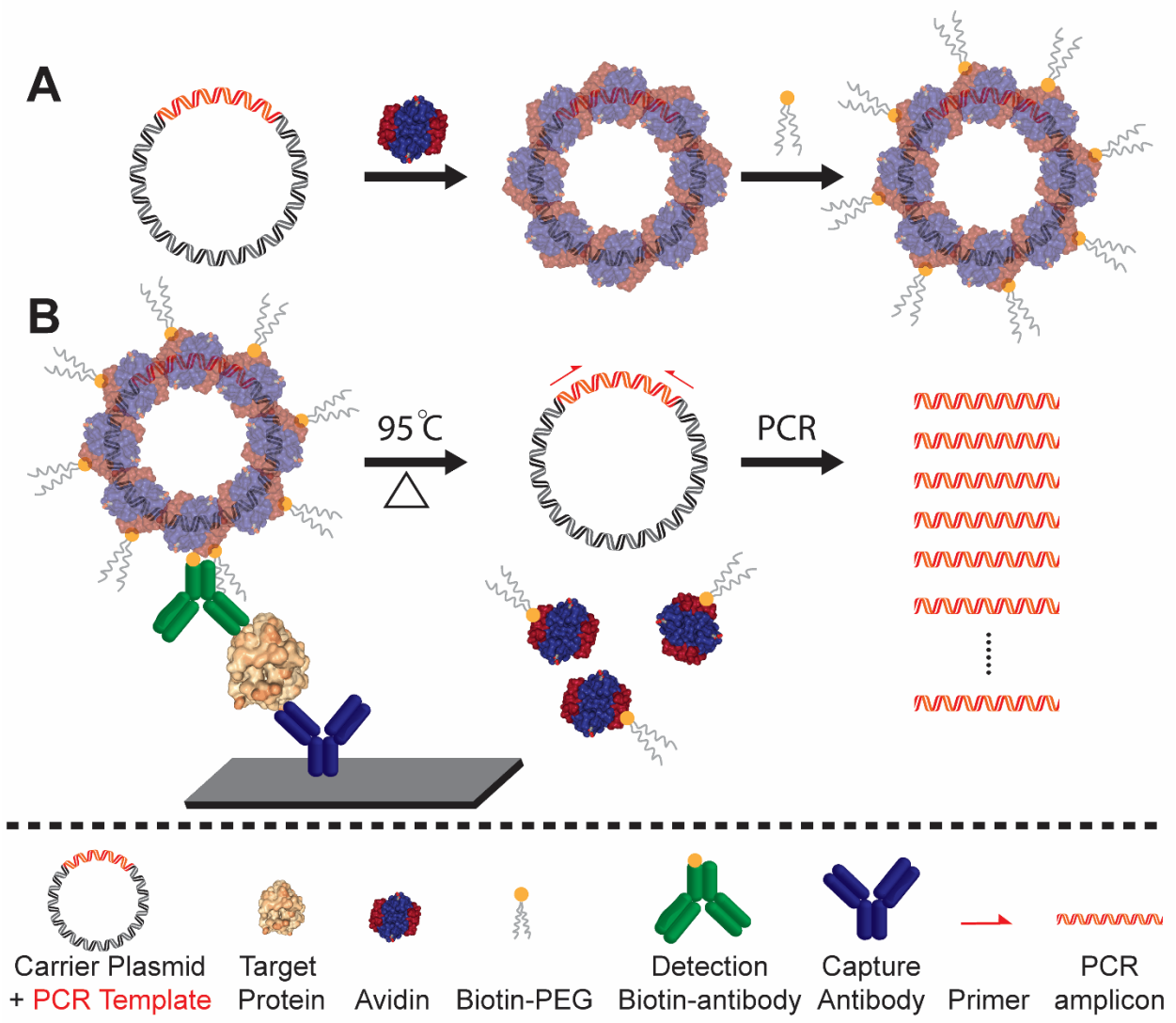

Figure 1. Schematic of the immuno-nanoparticle PCR assay. A) Assembly of DNA-avidin core-shell nanoparticles. DNA plasmids carrying the synthetic PCR template are sequentially assembled with avidin and biotin-polyethylene glycol (PEG). B) Workflow of immuno-nanoparticle PCR. Target protein molecules are captured by a capture antibody and detected with nanoparticles via a DTT-cleavable-biotin-linked detection antibody. The captured nanoparticles are disassembled by heat to expose the PCR template for PCR amplification (not to scale).

EXPERIMENTAL

Reagents

Synthetic DNA was from Integrated DNA Technologies, Inc. (Coralville, Iowa). Avidin (434401), Pierce™ premium grade Sulfo-NHS-SS-Biotin (PG82077), 4'-hydroxyazobenzene-2-carboxylic acid (HABA, 28010), Zeba™ spin desalting columns (40K MWCO, 0.5 mL, 87766), Dithiothreitol (DTT, R0861), and MediSorp clear flat-bottom immuno nonsterile 96-well plates, 400µL, (467320) were purchased from ThermoFisher Scientific. Two-arm PEG-Biotin (10 kDa, PG2A-BN-10K) was from Nanocs (Boston, Massachusetts). Amicon ultra-0.5 centrifugal filter unit (100 kDa, UFC510096), bovine serum albumin (BSA, A7906), and human chorionic gonadotropin (hCG; CG10-1VL, using the conversion factor 9.28 IU/µg from the 3rd International Standard) were from Millipore Sigma (Burlington, Massachusetts). Healthy human (male) serum was obtained from Gulf Coast Regional Blood Center, Houston, Texas 77054. Bovine serum albumin (IgG free, BSA-BAF-SMP) from Rocky Mountain Biologicals, Inc. (Missoula, Montana). Anti-hCG beta chain mAb, clone 2 (monoclonal, ABBCG-0402) and Goat anti-hCG alpha chain (polyclonal, ABACG-0500) were from Arista Biologicals, Inc. (Allentown, Pennsylvania). Phosphate-buffered saline (PBS) tablets, pH 7.4 were from Takara Bio USA Inc. (Mountainview, CA). Tween® 20, Molecular Biology Grade (H5152) was from Promega (Madison, Wisconsin). Mx3000P optical strip tubes (401428), Mx3000P optical strip caps (401425), and Brilliant III ultra-fast SYBR QPCR master mix (600882) were from Agilent Technologies, Inc. (Santa Clara, California).

A synthetic DNA template and primers were designed as previously reported.[18] Briefly, the 79-bp synthetic template 5'-TGCTGCGAGAGTATTATCTTGCACCTTATGCTACCGTGATTCA TCCAGTCTCATCGTGAAACAGACGTACTACTACCTG-3' and the 20 nt primers were

designed for both minimum similarity to any reported natural DNA sequence and optimal PCR conditions with high annealing temperature (60℃) and short extension time (30 s). DNA primers were (Forward: 5'-CAGGTAGTAGTACGTCTGTT-3', Reverse: 5'-GTGCTGCGAGAGTATTATCT-3'). QIAprep Spin Miniprep Kit (27106) was from Qiagen Inc (Germantown, Maryland).

Construction of multi-template plasmid DNA

Plasmids containing one to seven repeats of the specific 79-bp PCR target were constructed in pBC, a cloning plasmid with high copy number and chloramphenicol resistance for easy preparation and selection, as follows. For the template, the following oligos were annealed to generate the specific dsDNA template. OligoF: 5'-TCAGGTAGTAGTACGTCTGTTTCACGATGAGACTGGATGAATCACGGTAGCATAAGGTGCAAGATAATACTCTCGCAGCAGAGCTCATGTCACCAT-3' and Oligo R: 5'-CTAGATGGTGACATGAGCTCTGCTGCGAGAGTATTATCTTGCACCTTATGCTACCGTGATTCATCCAGTCTCATCGTGAAACAGACGTACTACTACCTGAAGCT-3'

Once annealed, the dsDNA template has, on its R oligo's 5' end, four overhang nucleotides (5'-GATC-3') that complement the XbaI restriction site, and on its 3' end, four overhang nucleotides (3'-AGCT-5') that complement the SacI restriction site. However, this 3'end is not recognized by the SacI enzyme since the restriction site was mutated during construction (AAGCT instead of GAGCT; this T is shown in red bold). In addition, nine nucleotides upstream of the XbaI site, a non-altered SacI restriction site, was placed (Figure 2).

Figure 2. Nucleotide sequence of the annealed dsDNA template and restriction enzyme recognition sequences. The cohesive end annealing to the compatible SacI site on the digested plasmid pBC is shown in red. Once ligated to the plasmid, the recognition sequence for SacI is abolished by a thymine-to-cytosine nucleotide change (shown in red bold). The cohesive end which anneals to the compatible, conserved XbaI site on the digested plasmid pBC is shown in blue. Shown in green is an extra SacI recognition sequence included in the dsDNA template to be used for the introduction of additional template repeats. The 79-bp PCR reporter sequence is shown in black and underlined.

The pBC plasmid was linearized with SacI and XbaI enzymes, then mixed with and ligated to the dsDNA template. To introduce the second repeat of the target sequence, the plasmid carrying one repeat of template was linearized with SacI and XbaI enzymes and again ligated to the dsDNA template. This was done sequentially until all 7 repeats were inserted into the plasmid. Plasmids were transformed into *E. coli Top10 F'* chemical competent (F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ$^-$). The size of the plasmid with one repeat of the template was 3,500 bp, and the size of plasmid increased by 85 bp for every additional repeat of the template introduced. We have deposited the plasmid with four repeats of the PCR target into the Addgene repository (#127380).

E. coli cells harboring the plasmids were grown in LB broth supplemented with 25 µg/ml chloramphenicol at 37 ºC for 14 h with shaking at 200 rpm. Plasmids were then isolated using the QIAprep Spin Miniprep Kit and were eluted in 10 mM Tris·Cl, pH 8.5 as per manufacturer's instructions.

Plasmid DNA was further purified by ethanol precipitation to reduce the ionic strength of the DNA solution, as salts could interfere with assembly of nanoparticles. To precipitate the DNA, 20 µl of 3M sodium acetate (pH 5.2) and 400 µl of ice-cold molecular biology grade absolute ethanol were added to 200 µl of plasmid DNA. The mixture was incubated at -20 ºC for 1 h and centrifuged at 14,550 rcf for 15 min, and the supernatant was discarded. The pellet was then washed with 500 µl of 70% ethanol and allowed to air-dry with the tube inverted. The DNA pellet was finally resuspended in 50 µl sterile water (typically to 160-200 ng/µl) and stored at -20 ºC until use.

Construction of DNA-avidin nanoparticles

For construction of DNA-avidin nanoparticles coated with PEG-biotin, plasmid DNA was first diluted with deionized water in a sterile microcentrifuge tube to $1x10^{12}$ DNA copies/ml based on Nanodrop A260 absorbance value. The volume of avidin and PEG-biotin mixed with the plasmid DNA was varied with the size of plasmid as discussed below. Both DNA and avidin pre-diluted in deionized water (6.1 mg/ml) were kept on ice for 15 min and plasmid DNA was then added to the avidin at a ratio of one avidin molecule for every 4 bp DNA, after which the volume was adjusted to 980 µl using deionized water. The sample was immediately vortexed for 30 sec and then allowed to incubate for 1 h at room temperature on a rotator (40 rpm, Model #RT50, Cole-Parmer, Vernon Hills, Illinois). After incubation, 10 kDa 2-arm PEG-biotin (4 mg/ml) in deionized water was added to the DNA-avidin mixture. PEG-biotin offered was 30% of the avidin biotin binding sites (7.8×10^{14} and 8.4×10^{14} biotin binding sites/ml for 1×10^{12} DNA copies/ml of plasmid 1 and plasmid 4 respectively). The final volume of the mixture was adjusted to 1 ml using deionized water. The mixture was incubated for 24 h at 4 ºC on a rotator, then divided between two Amicon Ultra-0.5, 100 kDa membrane filters and centrifuged at 10,000 x g for 15 min to remove free avidin and PEG-biotin. Approximately 20 µl of DNA-avidin nanoparticle suspension was then recovered per filter by centrifugation at 1,000 x g for 2 min, and the final volume was made up to 50 µl using deionized water. The two samples were pooled to give 100 µl stock solution of reporter particles and was stored at 4 ºC.

As an example, to construct DNA-avidin nanoparticles based on the plasmid DNA with 4 repeats of template (3,755 bp), 25 µl of plasmid DNA (167.2 µg/ml, 4×10^{13} DNA copies/ml) was mixed with 17 µl of avidin (6.1 mg/ml) and 3.5 µl of PEG-Biotin (4 mg/ml) and the final volume was adjusted to 1 ml using deionized water. This 1 ml particle suspension, after 24 h incubation, was

then filtered using two Amicon membrane filters (100 kDa) as described above, and finally pooled together to give 100 µl of stock solution of Particle 4.

qPCR of plasmid DNA and DNA-avidin nanoparticles

Plasmid DNA or DNA-avidin nanoparticles were serially diluted in sterile water from 5 million DNA copies/reaction to 5 DNA copies/reaction and subjected to qPCR (Agilent Mx3005P qPCR System). For setting up qPCR reactions, 10 µl of template was mixed with 10 µL 2x qPCR Master Mix (containing 1 µM primers) and DNA was amplified using the following PCR conditions: 1 cycle at 95 °C for 10 min, then 50 cycles of 95 °C for 15 s and 60 °C for 30 s.[18]

Nanoparticle Tracking Analysis (NTA)

The size and concentration of DNA-avidin nanoparticles present in the filtered stock solution were determined using nanoparticle tracking analysis (NTA). The filtered stock solution of DNA-avidin nanoparticles was diluted 100 times with sterile 0.22 µm- filtered DI water. A NanoSight LM14 microscope (Nanosight Ltd) equipped with a 20x lens (NA. 0.4) and a 532 nm laser was employed to monitor the diffusional mobility of individual DNA–avidin nanoparticles at 10°C. Approximately 500 µl of 100X diluted DNA–avidin nanoparticle solution was injected into the NanoSight cuvette with holdup volume of 300 µl. To prevent overheating of samples due to laser irradiation, data was collected within 5 min of sample injection. A sensitive CMOS camera (Model C11440-50B, Hamamatsu Photonics K.K., Japan) supplied by NanoSight was used to record the particles' Brownian motion at 24 fps. The accompanying software package (NTA. Version 2.3 Build 0025) was used to generate particle trajectories. Three movies showing distinct fields of view were collected for each sample for over 30 s using manual shutter and gain adjustments.

Zeta potential

The zeta potential of plasmid DNA and DNA-avidin nanoparticles diluted in sterile water to 5×10^7 DNA copies/ml and 5×10^7 nanoparticles/ml was determined by running 10 cycles at 23 ºC using a Nicomp 380 ζ-potential analyzer, calibrated using a zeta potential transfer standard (-42 mV ± 4.2 mV, Malvern Instruments Ltd., UK).

Antibody biotinylation

Polyclonal Goat anti-hCG alpha chain antibody was mixed with Pierce™ premium grade Sulfo-NHS-SS-Biotin (DTT-cleavable biotin) at a 1:20 mole ratio and incubated on ice for 2 h. Biotinylated antibody was then separated from unbound biotin using Zeba™ spin desalting columns (40K). Using the HABA assay the biotinylation ratio was determined to be between 4.11-5.33 biotin molecules per antibody. Biotinylated antibody was stored in PBS (pH 7.4) at 4 ºC. The DTT-cleavable biotinylated detection antibody allowed release of the bound DNA-avidin nanoparticles in the PCR assay, as described below. We used a similar DTT-cleavable biotinylated detection antibody in a previous publication[18].

DNA-avidin nanoparticle-based iPCR

Wells of a 96 well plate were charged with 100 µl of 10 µg/ml anti-hCG beta chain monoclonal antibody in PBS, pH 7.4, incubated overnight at 4 ºC, blocked with 300 µl PBS containing 3% BSA for 2 h at 25 ºC, and washed thrice with PBS + 0.1% Tween 20 (HydroFlex microplate washer, Tecan, Co., Männedorf, Switzerland). 100 µl of hCG diluted from 10 ng/ml to 1 pg/ml in PBS containing 1% BSA was added to the wells (triplicates) and incubated for 1.5 h at 25 ºC. For no-hCG control (triplicates), 100 µl of PBS containing 1% BSA was added in the wells and

incubated for 1.5 h at 25 ºC. The remining assay steps were the same for all wells, as described below.

Wells were then washed thrice with PBS + 0.1% Tween 20. 100 µl of 100 ng/ml biotinylated detection antibody (anti-hCG alpha chain Ab conjugated with DTT-cleavable biotin) was added and incubated for 1.5 h at 25 ºC. Wells were washed thrice with PBS containing 0.1% Tween 20. 100 µl of DNA-avidin nanoparticles based on plasmid with 4 copies of template ("Particle 4"; 5×10^7 particles/ml) diluted in PBS + 2% BSA was added and incubated overnight at 4 ºC. Wells were washed five times with PBS + 0.1% Tween 20. Bound particles were released by adding 100 µl 50 mM DTT and incubated for 2 h at 25ºC (as per manufacturer's instructions). 10 µl sample from each well was mixed with 10 µL 2x qPCR Master Mix (containing 1 µM primers) and DNA was then amplified using qPCR (1 cycle at 95 °C for 10 min, then 50 cycles of 95 °C for 15 s and 60 °C for 30 s).

RESULTS AND DISCUSSION

Design and preparation of multi-template PCR-amplifiable DNA reporter

Using the NCBI Nucleotide Blast (megablast algorithm, optimized for highly similar sequences), no significant similarity was found between the NCBI nucleotide collection database and our 79-bp synthetic DNA template, indicating that our *de novo* designed PCR amplifiable DNA reporter is not present in any sequenced biological specimen. We then constructed plasmid DNAs containing 1 to 7 repeats of this reporter and tested the hypothesis that including more repeats of the amplifiable reporter template in the plasmid DNA would increase detectability. As shown in Figure 3, C_t decreased substantially with each additional repeat from 1 to 4, with diminishing returns for additional repeats up to seven. The C_t value of 5×10^5 DNA copies of

plasmid with 1 repeat and 4 repeats of template corresponded were 18.22 and 13.26 respectively. Based on these results we chose the plasmids that contain 1 and 4 repeats of the template for construction of the DNA-avidin reporter nanoparticles.

We confirmed the cloning of the repeats of the reporter sequence in the pBC plasmid by gel electrophoresis of the pBC plasmids containing 1 to 7 repeats of the reporter sequence, digested with restriction enzymes KpnI and BpmI (supplementary information Figure SI 1). We also confirmed the insertion of the repetitive sequences by Sanger sequencing of the various plasmids containing the repeats with primers M13R and M13F. Figure SI 2 in the supplementary information shows the Sanger sequencing result for pBC plasmid containing 7 repeats.

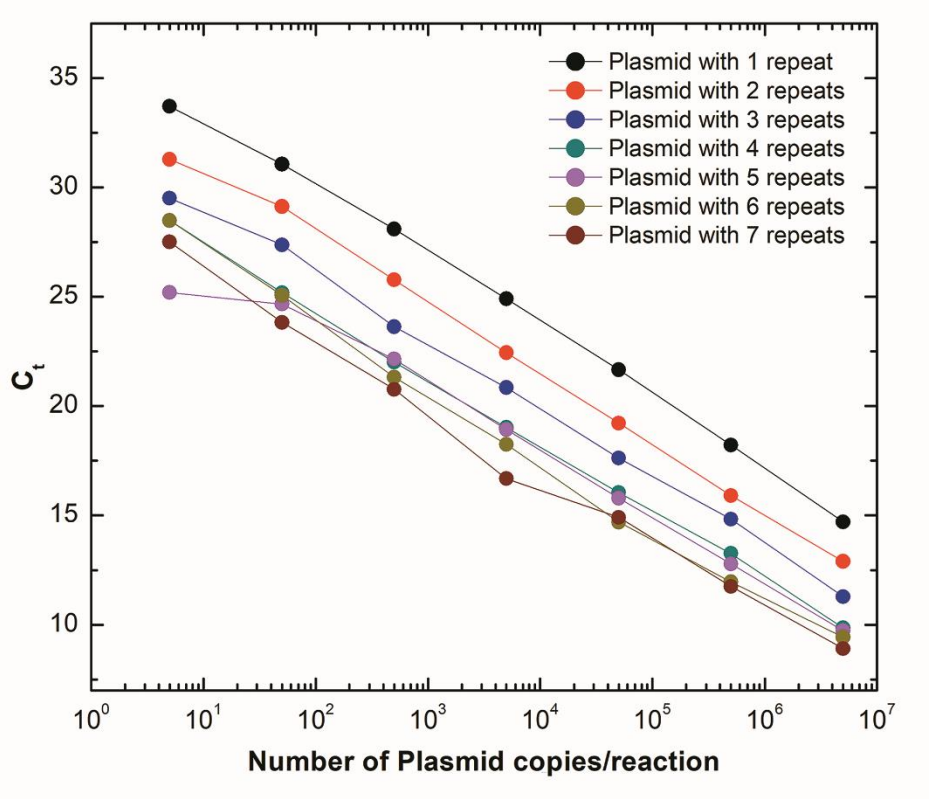

Figure 3. qPCR standard curves of plasmid DNA constructs containing 1 to 7 repeats of target template. The plasmid with no template showed C_t over 35.

Construction and Characterization of DNA-avidin nanoparticles

Polycationic compaction agents, (e.g., spermidine[29]) bind the major or minor grooves of dsDNA, neutralizing its charge and reducing its volume by four to six orders of magnitude.[30] In vivo, they function to package genomic DNA, e.g., into sperm.[31] We have previously reported the use of compaction agents such as spermine and spermidine for the condensation and selective purification of DNA.[32,33] Avidin, a 68-kDa, very cationic biotin-binding glycoprotein found in chicken egg white,[34] has been shown to condense DNA through high-affinity interactions with DNA without impairing avidin's biotin-binding activity, leading to the formation of 120 nm nanoparticles of toroidal shape.[23] Selective PEGylation through the available biotin-binding sites in avidin further stabilizes the nanoparticles, allowing their use as ELISA-based immunodetection reporters with low non-specific binding.[24,25]

Our DNA-avidin nanoparticles were constructed through condensation and neutralization of plasmid DNA containing 1 ("Particle 1") or 4 ("Particle 4") repeats of target template with avidin, and coating with PEG-biotin. We mixed the plasmid DNA containing one or four repeats of template with avidin such that there was one avidin molecule present for every 4 bp of DNA. This was followed by addition of a hydrophilic 2-arm, 10 kDa biotin-PEG polymer such that the PEG-biotin occupied 30% of avidin biotin-binding sites. These DNA-avidin nanoparticles were found to be stable for 6 months when stored in water at 4 ^{o}C. As shown in Figure 4, the C_t values of 5×10^5 copies of Particles 1 and 4 were respectively 20.79 and 14.64. Samples with 5×10^5 DNA copies of plasmid DNA with 4 repeats of template or of Particle 4 derived from that plasmid gave similar C_t values (13.26 and14.64, respectively), suggesting that one nanoparticle contained one condensed plasmid DNA molecule.

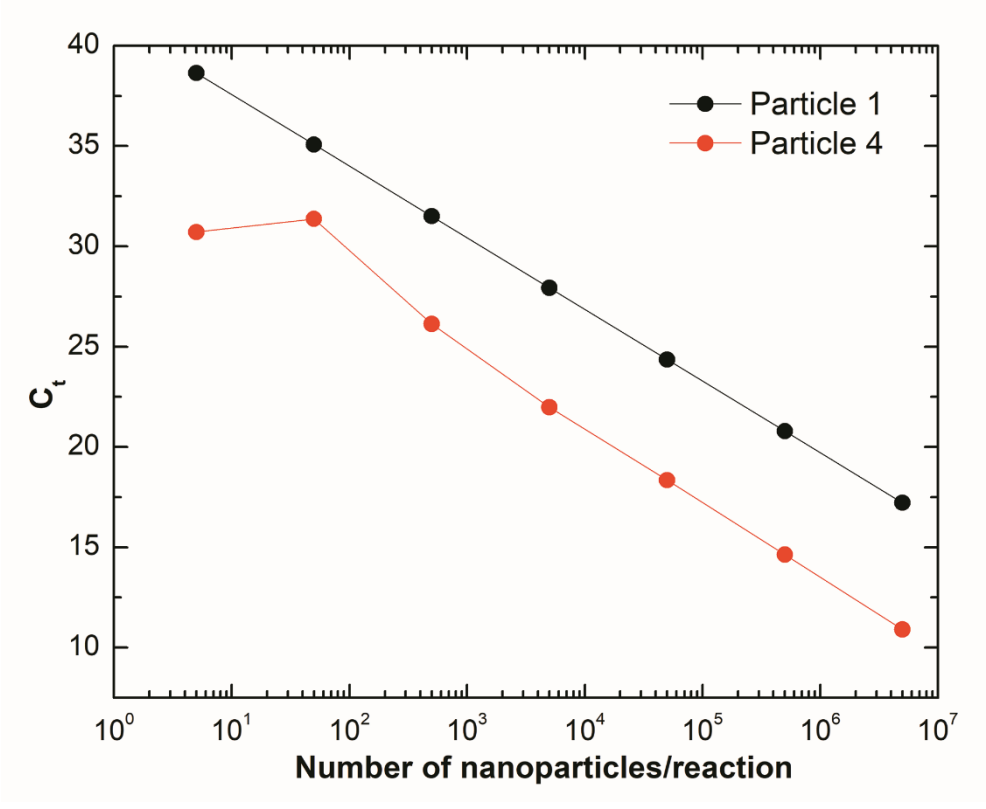

Figure 4. qPCR standard curves of DNA-avidin nanoparticles (n=1). Particle 1 (solid black) and Particle 4 (solid red)

Characterization of DNA-avidin nanoparticles

A. Nanoparticle tracking analysis (NTA)

Nanoparticle sizes and concentration were estimated from the collected nanoparticle tracking analysis movies for Particle 1 and Particle 4. As shown in Figure 5, the average sizes of Particle 1 and Particle 4 were found to be 109 ± 3.8 nm, and 95 ± 3.7 nm respectively. The approximate undiluted stock concentrations of Particles 1 and 4 were found to be 6.7×10^{10} particles/ml and 7.2×10^{10} particles/ml.

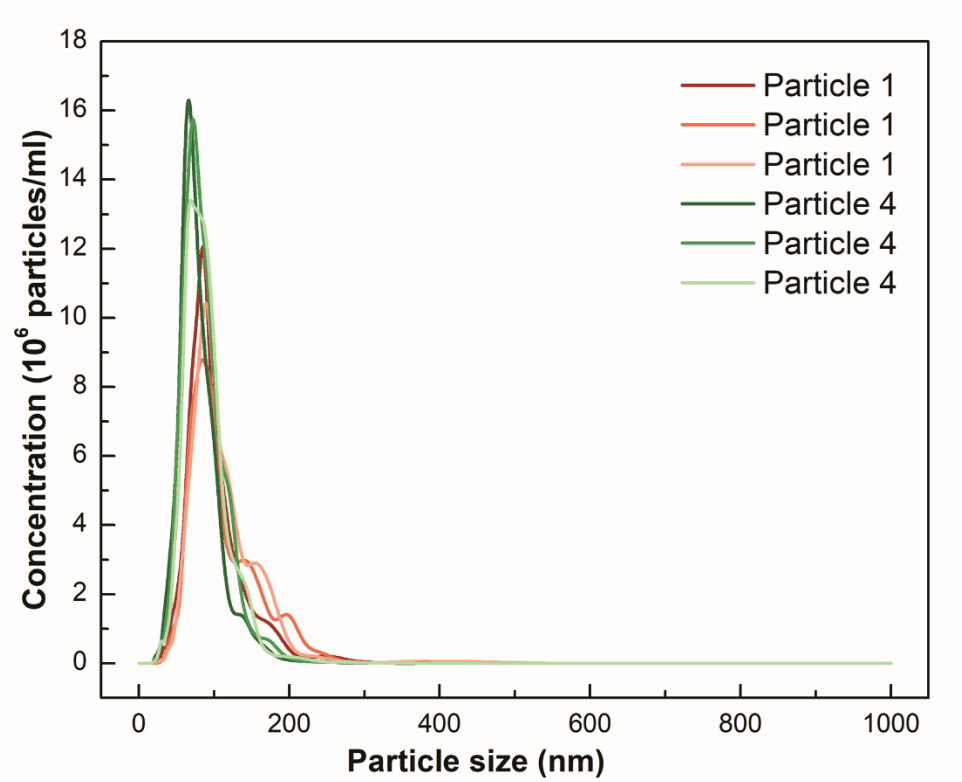

Figure 5. Nanoparticle tracking analysis of DNA-avidin nanoparticles. Three distinct fields of view were observed for calculating size and concentration of both Particle 1 and Particle 4. Curves shown in shades of red correspond to Particle 1 stock solution diluted 100-fold with water. The average size and undiluted concentration of Particle 1 were found to be 109 ± 3.8 nm and 6.7×10^{10} particles/ml respectively. Curves shown in shades of green correspond to Particle 4 stock solution diluted 100-fold with water. The average size and undiluted concentration of Particle 4 were found to be 95 ± 3.7 nm and 7.2×10^{10} particles/ml respectively.

B. Zeta Potential

The zeta potential of Particle 4 was found to be 1.17 ± 1.4 mV in contrast to plasmid DNA with 4 repeats of template (-5.2 ± 2.4 mV) indicating avidin largely neutralized the negatively charged phosphate groups of DNA.

DNA-avidin nanoparticle-based iPCR assay

We investigated the feasibility of nanoparticle-based iPCR by detecting human chorionic gonadotropin (hCG). hCG is an ideal model protein, commonly used to validate novel assay technologies, as it is extensively studied and many anti-hCG antibodies are commercially available.

To demonstrate detection of hCG, we immobilized monoclonal antibodies recognizing the hCG beta-chain in the wells of a microplate. Biotinylated (DTT-cleavable biotin) detection antibodies (anti-hCG alpha chain mAb) were added followed by the addition of Particle 4 DNA-avidin nanoparticles. As shown in Figure 6, -Delta C_t increased monotonically with the concentration of hCG; we estimated the limit of detection (LOD) at 25 pg/ml (signal higher than the no-hCG control plus 3 times the standard deviation of the no-hCG control; 660 fM; 100 µl sample volume). The data from iPCR assay of hCG using Particle 4 was then analyzed using a nonlinear regression (logistic regression), five-parameter (5PL) fit to determine the dynamic range of the assay. The dynamic range of protein quantification for the assay was found to be 25 pg/ml to 10,000 pg/ml. The LOD of our iPCR platform (6×10^{-17} moles in 100 µl sample volume or 25 pg/ml) is close to that of a previously published study[35] having LOD of 10^{-17} moles in a 50 µl sample volume for hCG.

We then tested the assay with a complex matrix like 25% human serum. We spiked different concentrations of hCG ranging from 10 pg/ml to 1000 pg/ml in 25% human serum (100% serum diluted to 25% in PBS containing 1% IgG-free BSA). We found the limit of detection to be 50 pg/ml for hCG spiked in 25% human serum, as shown in Figure SI 3. We have also compared the reproducibility of our iPCR platform with different batches of Particle 4 as immuno-reporter for

detection of hCG spiked in PBS+1% BSA, which showed similar sensitivity (Figure SI 3 of supplementary information).

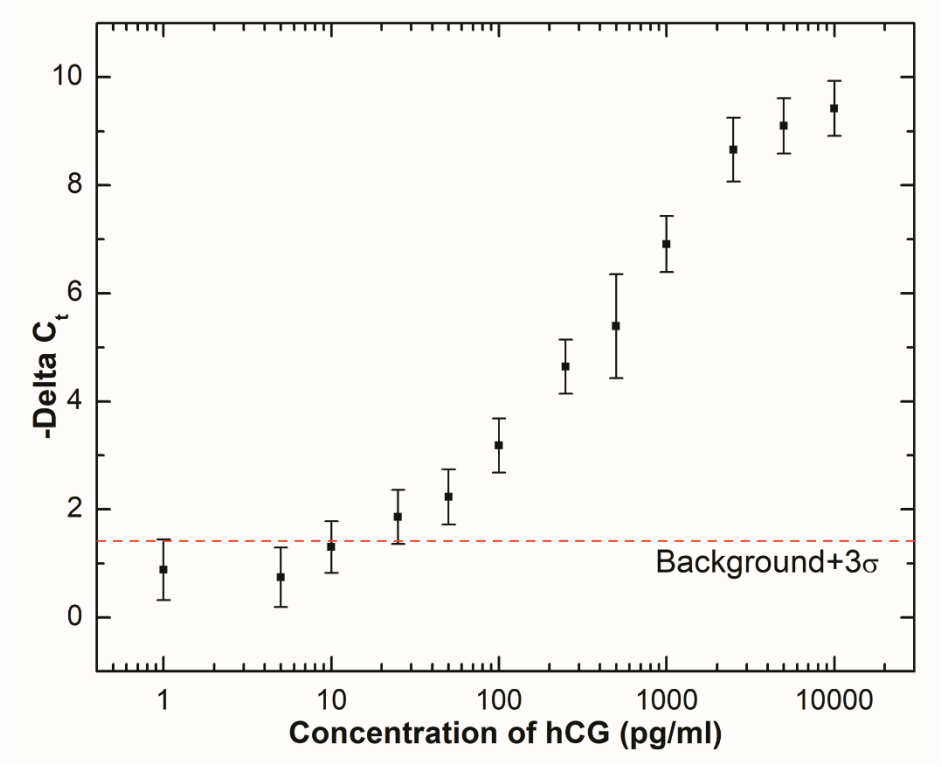

Figure 6. Quantification of hCG spiked in PBS +1% BSA using DNA-avidin nanoparticle (with four repeats of template)-based iPCR (n=3, error bars ±1 SD; non-template control gave no C_t). The dashed red line is the detection threshold of the assay, which is defined as the average -delta C_t value of the no-hCG control plus 3 times the standard deviation of the no-hCG control. A standard approach was used to estimate the Limit of Detection as the lowest analyte concentration that gave a signal clearly distinguishable from the detection threshold. The Limit of Detection was estimated at 25 pg/ml hCG (660 fM).

CONCLUSIONS

In summary, we have demonstrated an ultra-sensitive iPCR platform using novel ultra-detectable, reduced-nonspecific binding DNA-avidin nanoparticles. The nanoparticles carry

multiple repeats of a *de novo* designed synthetic PCR amplifiable DNA sequence for enhanced detectability and are modified with hydrophilic PEG for reduced non-specific binding, one of the major problems in traditional iPCR formats with naked DNA. Traditional iPCR universal platforms require prior preparation of antibody-DNA oligo conjugates using either thiol-maleimide or biotin-streptavidin chemistry.[36,37] However, covalent conjugation of DNA to antibodies can affect the affinity of the antibody.[21] Additionally, the tetrameric structure of the avidin and streptavidin results in the generation of a heterogeneous pool of DNA-antibody conjugates, thereby affecting the robustness of iPCR assay.[38] An additional potential advantage of our iPCR platform based on DNA-avidin nanoparticles is the homogeneity of the reporter nanoparticle, which have a single copy of condensed plasmid DNA with four repeats of the reporter DNA template. Our nanoparticle reporters are relatively easily prepared, and provide a generic, readily-customizable platform for the detection of proteins for which high-affinity antibodies exist. This technology could readily be applied to other protein targets, including microbial antigens,[39] cytokines, tumor markers,[40] and anti-drug antibodies induced by biologic therapeutics[41] to precisely quantitate target analytes at ultra-low levels.

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The authors declare the following competing financial interest(s): Several of the authors of this manuscript are named inventors on pending IP which overlaps the topics of this manuscript.

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