Title: Modular Fluorescent Nanoparticle DNA Probes for Detection of Peptides and Proteins

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Abstract

Fluorescently labeled antibody and aptamer probes are used in biological studies to characterize binding interactions, measure concentrations of analytes, and sort cells. Fluorescent nanoparticle labels offer an excellent alternative to standard fluorescent labeling strategies due to their enhanced brightness, stability and multivalency; however, challenges in functionalization and characterization have impeded their use. This work introduces a straightforward approach for preparation of fluorescent nanoparticle probes using commercially available reagents and common laboratory equipment. Fluorescent polystyrene nanoparticles, Thermo Fisher FluoSpheres™, were used in proof-of-principle studies. Particle passivation was achieved by covalent attachment of amine-PEG-azide to carboxylated particles, neutralizing the surface charge from -47 to -17 mV. A conjugation-annealing handle and DNA aptamer probe was attached to the azide-PEG nanoparticle surface either through reaction of pre-annealed handle and probe or through a stepwise reaction of the nanoparticles with the handle followed by aptamer annealing. Nanoparticles functionalized with DNA aptamers targeting histidine tags and VEGF protein had high affinity (EC_{50}s ranging from 2-7 nM) and specificity, and were more stable than conventional labels. This protocol for preparation of nanoparticle probes relies solely on commercially available reagents and common equipment, breaking down the barriers to use of nanoparticles in biological experiments.

Introduction

Fluorescently labeled antibody and aptamer probes are used to visualize and quantify biological molecules in the fields of biology, chemistry, and biomedicine[1]. They are employed in applications to study receptor-ligand binding in plate-based assays[2], single-molecule fluorescent microscopy analyses[3], and flow cytometry[4] and in applications requiring cell-type specific targeting such as theranostics[5–7] and *in vivo* imaging[8,9]. Several characteristics are universally important for fluorescent probes: stability, affinity, brightness, and biocompatibility. Conventional fluorescent labels such as luminescent metal complexes, proteins, and organic dyes can suffer from insufficient brightness, poor stability, and photobleaching[10–12]. In addition, traditional labels often chemically interact with biomolecules to the detriment of both the fluorescent probe and the biological system[13].

Nanoparticles offer numerous advantages over conventional labels. First, fluorescent nanoparticles can be much brighter than conventional labels due to their high molar extinction coefficients and quantum yields[14,15]. Second, nanoparticles have high photostability due to protective encapsulation of fluorescent dyes or to the mechanism of their fluorescence[12,16]. Finally, nanoparticles can be coated with biologically inert chemicals like poly(ethylene-glycol) (PEG) to improve biocompatibility[17]. Several types of nanoparticles have been used as fluorescent labels[16,18,19], each with its own unique set of advantages and disadvantages. Dye-doped polymer particles are composed of polymer matrices such as polystyrene or polyacrylate and encapsulate the fluorescent dyes. Polymeric nanoparticles are inexpensive, bright, easy to functionalize, and biocompatible; however, they interact non-specifically if insufficiently passivated and are subject to photobleaching, albeit on far greater time scales than conventional labels[16]. Quantum dots, which are composed of semiconducting materials and are intrinsically

fluorescent, have high quantum yields and molar extinction coefficients, are very photostable, and can be modified with exterior organic capping to enhance biocompatibility[20]; however, quantum dots are cytotoxic, limiting their use *in vitro* and *in vivo*[21]. Furthermore, quantum bots blink, which can be a hinderance in single-molecule studies[12]. Evaluation of other nanoparticle types, such as gold, up-converting, carbon, and silica nanoparticles, as fluorescent labels is ongoing[18].

Although fluorescent nanoparticles may offer improvements to conventional labels, challenges in synthesis, functionalization, and passivation have limited their use. For example, incorporating dyes into silica nanoparticles or polymeric matrices requires a detailed understanding of fluorophore quenching behavior and expertise in emulsion and polymerization techniques[16,19,22]. The synthesis of quantum dots requires specialized equipment for techniques like e-beam lithography, formation of microemulsions, and sputtering for vapor-phase synthesis[23,24]. Once synthesized, nanoparticles require specialized equipment for characterization, such as electron microscopy, not available to every laboratory[16,25]. After synthesis, nanoparticles are functionalized by attaching probes to the surface, an often arduous and expensive process that requires specific expertise[26,27]. For example, in the case of silica nanoparticles, a reaction with (3-aminopropyl)triethoxysilane (APTES) is often used to introduce a biocompatible amine-functionality to the particle, but this reaction must be controlled to avoid APTES polymerization and can require additional chemical modification to allow compatibility with probe functional groups[28,29]. In addition to functionalizing the nanoparticle with an active chemistry, the affinity reagent probe must be functionalized with a compatible conjugation-annealing handle. Reagents linked to common chemical moieties like thiols and amines are readily available from commercial vendors, but specialized and often more desirable functional

groups require custom synthesis and characterization. Finally, achieving sufficient passivation on the nanoparticle surface can be technically challenging. For instance, quantum dots must be capped with hydrophilic materials, like amphiphilic polymers, to improve solubility and colloidal stability in aqueous solutions[23,30]. For polymeric particles, passivation prevents undesirable interactions with the biological environment. Moreover, the passivated layer provides functional groups for further attachment of probes[16]. Although individual solutions to nanoparticle challenges have been identified, no common set of best practices are available.

To simplify the preparation and use of nanoparticle-based probes, we set out to develop a protocol for functionalizing off-the-shelf reagents to generate fluorescent nanoparticle probes. For proof-of-principle experiments, commercially available dye-laden polystyrene nanoparticles (FluoSpheres™, ThermoFisher) and quantum dots (Qdot™ 655 ITK™ Carboxyl Quantum Dots, ThermoFisher) were chosen due to their stability, brightness, and biocompatibility. These nanoparticles were modified with PEG to improve affinity and colloidal stability. Finally, aptamer probes were attached to the particle by annealing disparate probes to a common oligonucleotide conjugated to a chemical moiety for covalent attachment to the nanoparticle; we call this oligonucleotide the conjugation-annealing handle. This modular design allows nanoparticle to be easily functionalized with a new probe. In our proof-of-concept studies, we demonstrated the utility of our method by functionalizing FluoSphere and Qdot fluorescent nanoparticle probes with previously described DNA aptamer probes[31,32]. Our studies show that our fluorescent nanoparticle probes meet the stability, affinity, brightness, biocompatibility, modularity, and reagent availability requirements necessary for a fluorescent label. We provide a supplemental protocol with easy-to-follow instructions so that other researchers can create their own fluorescent nanoparticle probes.

Results

Fluorescent nanoparticle probe fabrication

Our method consists of three steps: passivation with chemically active PEG, attachment of a conjugation-annealing handle to enhance modularity, and attachment of a DNA-based aptamer probe (**Figure 1**). Our method utilizes only commercially available materials (**Table 1**). For initial protocol optimization and characterization, 40-nm and 200-nm yellow-green FluoSpheres, which are nanoparticles encapsulating fluorescent dyes within a polymeric polystyrene matrix, were used as the nanoparticle label. To attach the passivating PEG-layer, the FluoSpheres were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/ *N*-hydroxysuccinimide (EDC/NHS) and reacted with PEG functionalized with an amine and an azide moiety for DNA conjugation. We attached the handle and DNA aptamer probe to the nanoparticle either through a single reaction between the nanoparticle and the pre-annealed handle and probe complex or through a stepwise reaction with conjugation of the handle to the nanoparticle followed by annealing of the aptamer (**Figure 1A**). The handle consists of a 3' dibenzocyclooctyne (DBCO) for conjugation to the PEG layer and either a 24-base-pair annealing sequence (Illumina P7 primer sequence[33]) or a poly-T sequence (21 thymidine bases; **Figure 1B**). The aptamer includes a complimentary sequence that anneals to the handle. The optimized protocol is included in the Supplementary Information.

Optimization of nanoparticle PEGylation

To demonstrate that methoxy-PEG (mPEG) was effectively conjugated to the nanoparticles, we first introduced either 1.6-kDa mPEG-amine (reactive), methoxy-PEG-OH (unreactive), or buffer only at a concentration of 10^8 PEG molecules/particle and examined the impact of PEG conjugation on nanoparticle zeta potential. Zeta potential measurements rely on scattering of

incident laser light to measure the velocity of particle motion under application of an electric field[34]. Changes in zeta potential of reacted particles indicate consumption of highly negatively charged carboxylate groups during conjugation and charge-shielding of any remaining negative charge by the PEG layer[35], which should only occur if chemical conjugation has occurred. Zeta potentials of -43 mV in the buffer-treated and mPEG-OH-treated samples indicated minimal non-specific interactions of PEG with the activated nanoparticle. The zeta potential was -15.4 mV when nanoparticles were incubated with 10^8 mPEG-amine molecules/particle (**Figure 2A**), indicating that PEGylation occurred via covalent linkage to carboxylate-modified nanoparticles. Next, we tested a range of PEG grafting densities by evaluating the zeta potential of nanoparticles reacted with 10^6 to 10^8 PEG molecules/particle. Increased PEG density reduced the magnitude of negative nanoparticle surface charge from -35 mV to -14 mV, suggesting a more highly passivated particle at higher PEG densities (**Figure 2B**).

We also evaluated the impact of PEG molecular weight on nanoparticle surface charge and colloidal stability. Colloidal stability was inferred by dynamic light scattering (DLS). In DLS, particle velocity is measured by subjecting the nanoparticle suspension to an incident laser and measuring the constructive and destructive interference patterns produced by scattered light over time, which can then be used to calculate the hydrodynamic radius of the nanoparticles[34,36]. In general, PEGs with molecular weights of about 2 kDa (PEG-45) or larger are used for nanoparticle passivation[37], so we tested PEGs ranging from PEG-12 to PEG-112. As the molecular weight of PEG increased, nanoparticle surface charge neutralization increased, indicative of more complete passivation. PEG-112, the longest polymer tested, had the greatest decrease from an activated zeta potential of -46 mV to a final zeta potential of -7.75 mV (**Figure 2C**). PEG-112-conjugated particles had a polydispersity index (PDI) of 0.36 and hydrodynamic

diameter of 628 nm; particles conjugated with lower molecular weight PEGs had PDIs of 0.04-0.15 and diameters of 332-394 nm (**Figure 2D and E**). As a PDI greater than 0.1 suggests particle aggregation[34], PEG-112 was eliminated from further study. Particles conjugated to PEG-45 had a zeta potential of -17 mV, a PDI of 0.14, and diameter of 369 nm, indicating that passivation was achieved and colloidal stability was maintained. Thus, PEG-45 was used for all subsequent studies at a concentration of $3.5x10^7$ PEG molecules per 200-nm particle and $1.4x10^5$ PEG molecules per particle for 40-nm nanoparticles (**Figure S1**).

We hypothesized that a higher percentage of azide-PEG-amine would allow conjugation of more probes per particle, which would result in higher affinity of the nanoparticle probe to its target. We compared mPEG-amine:azide-PEG-amine ratios of 95:5, 99.5:0.5, 99.95:0.05, and 99.995:0.005 in protein binding studies. The optimal on-target binding was observed at the ratio of 95:5 (**Figure S2**). To achieve the highest binding possible while conserving reagents, we selected a ratio of 95:5 mPEG-amine:mPEG-azide for the remainder of these studies.

Quantification of DNA aptamer probe-to-nanoparticle ratio

Next, we evaluated changes in particle charge and size with addition of the conjugation-annealing handle and of the DNA aptamer probe. The addition of the handle to the nanoparticle surface increased the overall negative charge on the nanoparticle from -13 mV to -19 mV, and subsequent annealing of the DNA aptamer probe further increased negative charge to -21 mV (**Figure 3A**). Nanoparticle diameter increased as each component was added from 335 nm for the PEGylated particle to 367 nm for fully functionalized fluorescent nanoparticle probes (**Table 2**). The PDI remained close to 0.1, indicating that colloidal stability was maintained. The

decrease in surface charge and slight increase in size indicated successful attachment of both the conjugation-annealing handle and the probe to the nanoparticle.

Next, we quantified aptamer attachment to the nanoparticles using qPCR, a technique commonly used to measure the quantity of DNA on a nanoparticle[38]. Amplification was performed of oligonucleotides attached to particles (on-particle) and of oligonucleotides removed from fabricated nanoparticles by heat (off-particle). The number of DNA molecules was determined by comparing cycle threshold (Ct) values of the experimental group to a standard curve of the DNA aptamer. In both on- and off-particle amplification methods, about 430 DNA aptamer probes were detected per particle (**Figure 3B**). For particles treated with a negative-control aptamer, which did not have sequence complimentary to the conjugation-annealing handle, 4-7 aptamers were detected per particle. These data indicate successful aptamer conjugation and negligible non-specific binding between aptamer and nanoparticle.

Assessment of fluorescent nanoparticle probe binding

We used a plate-based assay binding assay to determine whether the addition of the label to the probe altered the binding affinity of the probe and to interrogate the differences between several probe attachment methods. In these proof-of-principle studies, we used the B1 aptamer, which binds histidine tags[31], as our probe. A biotinylated B1 probe detected with traditional detection methods (amplification of signal by streptavidin HRP or conjugation to a fluorescently labeled streptavidin conjugated) bound histidine-histidine-histidine (HHH) with an observed equilibrium dissociation constant (K_d) of 55 nM (**Figure S3**), comparable to the reported K_d of 120 nM[31].

When we evaluated binding of the nanoparticle probes to immobilized HHH peptide, we observed a 19.5-fold higher binding by the B1 probe than by the nanoparticles functionalized

with a negative control aptamer (**Figure 4A**). To evaluate non-specific binding, we conducted the assay with HHH and with proline-asparagine-glycine (PNG), a peptide that the B1 aptamer does not recognize. Streptavidin AlexaFluor™ 647 Conjugate, a conventional label, was used as a control to ensure that specific and non-specific binding were not due to the label type. Nanoparticles were functionalized with the B1 (on-target) and Her2[39] (off-target) aptamers to differentiate non-specific binding by the aptamer versus non-specific binding due to the functionalized nanoparticle. Both the Streptavidin AlexaFluor™ 647 Conjugate and fluorescent nanoparticle B1 probe bound to HHH, but we observed minimal non-specific binding to PNG (**Figure 4B**). Nanoparticles functionalized with the Her2 aptamer did not bind detectably to either HHH or PNG. Taken together, these results provide evidence that both particle passivation and probe attachment were successful.

We next evaluated on- and off-target binding of the nanoparticle B1 probe in complex protein mixtures. The nanoparticle B1 probes bound his-tagged Her2 protein with 2-fold signal over background lysozyme binding (**Figure 4C**). In the presence of *E. coli* cell lysate or myoglobin protein, background levels of binding were similar to the no-protein control (**Figure 4D**). Finally, we evaluated a concentration titration of fluorescent nanoparticle B1 probes against the his-tagged Her2 protein. The half maximal response concentration, EC_{50}, was 6.5 nM (**Figure 4E**). This EC_{50} value is lower than what was previously reported (120 nM)[31] and what we found experimentally with other conventional labels (55 nM; **Figure S3**). We hypothesize that this apparent increase in affinity could result from avidity effects, as described in the Discussion Section.

Attachment of alternate aptamers to fluorescent nanoparticles

To demonstrate modularity of the fluorescent nanoparticle probe system, nanoparticles were functionalized with H3T, a truncated version of the B1 aptamer[31], and a VEGF aptamer[32]. These aptamers bind their targets when they are detected with more traditional methods (**Figure S3**). We tested the binding of the HHH peptide by the nanoparticle H3T probe using a plate-based assay. The nanoparticles bound to the peptide target with an EC_{50} of 3.4 nM (**Figure 5A**), stronger affinity than the EC_{50} of 30 nM found using a more traditional detection approach (**Figure S3**) and that reported in literature (120 nM)[31]. Nanoparticle probes functionalized with the VEGF aptamer bound VEGF protein and not an off-target protein (**Figure 5B**). This demonstrates the modularity of our probe attachment approach.

Alternative attachment strategies

Finally, we evaluated the on-target binding of fluorescent nanoparticle probes fabricated using pre-annealing or post-annealing of the aptamer to the nanoparticle (**Figure 1A**). Pre-annealed and post-annealed fluorescent nanoparticle B1 probes were used to detect an on-target his-tagged protein in a plate-based binding assay. Both pre- and post-annealed particles detected the his-tagged protein with stronger affinity than the off-target myoglobin protein, but at the highest probe concentrations tested, the pre-annealed particles showed 2-fold higher binding to his-tagged protein than did the post-annealed particles (**Figure S4**). To explain these differences, further experimentation is required, but we hypothesize that the concentration of probe mixed with the particles in the post-annealing protocol was not high enough to fully saturate available conjugation-annealing handles.

In addition to annealing the probe to the particle, we evaluated a more traditional attachment approach by directly conjugating a DBCO-modified aptamer to the PEG-azide layer. We evaluated pre-annealed particles and the directly conjugated particles in plate-based binding

assays against his-tagged protein. Although both approaches resulted in higher on-target protein binding than off-target binding, the probe prepared using the pre-annealing approach had on-target binding that was about 2-fold greater than the probe prepared using direct conjugation approach (**Figure S5**). Additional studies will be required to determine the reason for this effect. These results indicate that both pre- and post-annealing of aptamers to nanoparticles can be used to achieve specific binding and that annealing approaches are as good as or better than traditional conjugation approaches. The pre-annealing fabrication was used in subsequent experiments.

Comparison to alternative commercial labeling options

To understand how our nanoparticle labels compare to commercially available labels, we conducted a series of studies to compare affinity, stability, and brightness of the fluorescent nanoparticle probe to off-the-shelf labels including Streptavidin AlexaFluor™ 647 Conjugate, Streptavidin APC Conjugate, and Streptavidin SureLight™ APC. In a plate-based binding assay, we compared the EC_{50} of our fluorescent nanoparticle B1 probe to commercially available labels attached to biotinylated B1 aptamer (**Figure 6A**). The fluorescent nanoparticle probe had binding affinity approximately equivalent to that of Streptavidin SureLight™ APC, with EC_{50}s of 2.2 and 2.3 nM, respectively. Streptavidin AlexaFluor™ 647 and Streptavidin APC Conjugates had EC_{50} values of 28 nM and 30 nM, respectively. The total number of binding sites per label may impact observed binding affinity due to avidity effects. Information from the vendor indicates that Streptavidin AlexaFluor 647 Conjugate and the Streptavidin APC Conjugate consist of one streptavidin per label, resulting in approximately three biotinylated probe binding sites per label. The Streptavidin SureLight APC consists of multiple streptavidin and APC molecules per conjugate, and conversations with the vendor indicated that the total number of binding sites can vary batch-to-batch. Although more studies will be required to fully understand the observed

results, we conclude that the affinity of the fluorescent nanoparticle B1 probe is equal to or greater than commercially available labels.

To compare stabilities of fluorescent labels, we evaluated on-target and off-target binding over 16 weeks. The fluorescent nanoparticle B1 probe showed consistent binding affinity for 12 weeks, whereas the Streptavidin APC and Streptavidin AlexaFluor 647 conjugates showed diminished probe affinity at 3 weeks (**Figure 6B**). Fluorescence intensity over 2 weeks was consistent for all labels, although a slight decrease was observed for Streptavidin SureLight APC Conjugate (**Figure S6**). This suggests that the alternative labels have reduced affinity for their targets over time, whereas affinity of the fluorescent nanoparticle probe is maintained for several months.

Finally, we evaluated the relative brightness of the fluorescent nanoparticle probe and alternative labels. The fluorescent nanoparticles were 4- to 5-fold brighter than Streptavidin SureLight™ APC and Streptavidin AlexaFluor™ 647 Conjugate labels on a molar basis (**Figure 6C**). In summary, the fluorescent nanoparticle probes had superior stability and brightness as compared to alternative labels.

Use of an alternative nanoparticle core

Finally, we assessed if target specificity was similar when the same functionalization method was applied to a different nanoparticle core, the commercially available Qdot™ 655 ITK™ Carboxyl Quantum Dots. In a plate-based assay, binding affinity for his-tagged Her2-his protein was about 2.4-fold higher than background, and the measured EC_{50} was 60 nM (**Figure 7**). We observed little off-target binding. As noted above, observed affinity can be impacted by both avidity and the detection method, and further studies will be needed to determine why the

affinity was lower for the nanoparticle probes with a Qdot core than a FluoSphere core. Overall, this experiment demonstrates that our protocol is robust to swap-in of an alternative core.

Discussion

In these studies, we describe a straightforward method for fabrication of fluorescent nanoparticle probes with the aim of enabling broader use of nanoparticle-based labels. Our protocol utilizes commercially available reagents to create nanoparticle probes with high affinity and brightness, good biocompatibility and colloidal stability, modularity, and long-lasting activity. The first step of our method is nanoparticle passivation. As nanoparticle surfaces tend to non-specifically adsorb proteins[15,16,40–43], dense PEG layers are commonly used to passivate particles due to favorable interactions with water molecules that energetically disfavor interactions with other biomolecules [35,42,44]. For the fluorescent nanoparticle probes presented here, nanoparticles were PEGylated using industry-standard techniques that reduce protein binding[37,43]. We optimized PEG density and molecular weight to maximize particle passivation while maintaining colloidal stability. As PEG density and molecular weight increased, particle passivation increased. This was expected as short, low-density and low-molecular-weight PEG grafting results in a "mushroom" conformation, ineffective at passivating surfaces, whereas high-density and high-molecular-weight PEG grafting results in "brush" conformations, leading to highly effective passivation[35,44,45] (Supplementary Information, ***PEG Density Calculations***). With optimized PEGylation conditions, off-target nanoparticle probes showed low non-specific binding to proteins. The zeta potential of our nanoparticles was never neutral, however, suggesting that passivation of the particle surface was not complete. We may be able to improve PEGylation of 200-nm particles by increasing the PEG concentration until we see a plateau in

zeta potential as we observed for 40 nm particles. Additionally, mixing high and low molecular weight PEGs has been used to improve surface passivation in single molecule detection experiments[46], and could also improve passivation of nanoparticle surfaces. We could also evaluate other passivating polymer and surfactant combinations[47].

The next step of our method is the attachment of the conjugation-annealing handle and the DNA aptamer probe. In many probe-labeling protocols, each new probe requires optimization, which can be time-consuming and expensive. Our strategy largely avoids this by achieving modularity via a conjugation-annealing handle, a short DNA sequence designed to anneal aptamer probes on the 5' end and to covalently attach to the PEG layer through a DBCO moiety on the 3' end. By utilizing the conjugation-annealing handle, a multitude of probes can be prepared without needing to functionalize each probe with a conjugation handle. Here, we demonstrate this modularity by demonstrating activity of fluorescent nanoparticles functionalized with B1 and its truncated version H3T, both designed to bind his-tags, and an aptamer that binds the VEGF protein[32]. The probe can be attached to the conjugation-annealing handle before or after conjugation to the particle so fluorescent nanoparticles conjugated to the handle can be prepared in large batches. This method could also be used to prepare antibody probes functionalized with a short DNA sequence complementary to the conjugation-annealing handle. Our strategy eliminates problems arising from incompatible chemical modifications of commonly available functionalized nanoparticles and antibodies. One limitation of this approach is that the probes are not covalently attached to the nanoparticles. Although in our hands, nanoparticle probes were stable for months, stronger attachment could be achieved by optimizing the annealing sequences or employing non-natural nucleotides, such as locked nucleic acid[48].

We used the B1 aptamer in proof-of-principle experiments due to its high affinity and potential utility in other applications. B1 binds his-tags that are used for the isolation of recombinant proteins via immobilized metal affinity chromatography[49]. The fluorescent nanoparticle B1 probe bound both peptides and proteins specifically. Both the B1 aptamer and its truncated version, H3T, bound targets with EC_{50} values ranging from 3.4 to 6.5 nM, lower than the 120 nM affinities previously reported for these aptamers[31]. Although this suggests that attachment to the nanoparticle increases aptamer affinity, it is important to note that we report concentration on a nanoparticle probe basis, but local concentration of probe may be much higher due to the multivalent nature of the nanoparticle probe[20]. Furthermore, the presence of multiple probes per nanoparticle could help stabilize the interaction with the target, decreasing the off-rate and thus reducing the EC_{50}[50,51]. This avidity is a major advantage over traditional labels, which only have one probe per fluorescent label. When we compared fluorescent nanoparticle labels to other off-the-shelf labels, we found that the affinity increased as the ratio of probe to label increased. In addition, when we varied the percentage of PEG with a conjugation handle for probe binding, we noted that higher percentages results in increased probe binding to the target. We hypothesize that both observed effects are due to avidity. We note that EC_{50} measurements are highly dependent on the experimental conditions and do not provide the kinetic information. Studies that utilize approaches like surface plasmon resonance will improve our understanding of how the fluorescent nanoparticle label impacts probe binding kinetics.

The type of nanoparticle used in the creation of fluorescent nanoparticle probes will depend upon the specific research application. The protocol we describe can be used with different nanoparticle types as demonstrated by our validation of Qdot-based probes. B1 probes with a Qdot core showed specific binding to a his-tagged protein and an HHH peptide with EC_{50} values

of about 60 nM. This EC_{50} is higher than that measured with the FluorSphere-based probe, possibly due to lower affinity, inadequate detection of Qdot fluorescence, or fewer probes per nanoparticle. Thus, this strategy is highly versatile but will likely require some optimization studies when utilizing a new nanoparticle core.

The fluorescent nanoparticle probes described in this work have brightness profiles and stability on par or better than other commercially available labeling techniques, have high affinity for targets, minimal non-specific binding, and are biocompatible. Our nanoparticle probes take inspiration from nanotechnologies used in other applications: Polystyrene nanoparticles have been used *in vitro* to study targeted cellular interactions and uptake by conjugating cell membrane-targeting moieties to the nanoparticle surface[52,53] and quantum dots have been functionalized with aptamers for use as theranostic agents in targeted cancer drug delivery applications[54–57] and as biomolecule sensors[58]. Previous applications have involved use of custom probes, nanoparticles, or conjugation chemistries, making broad adoption challenging. In contrast, each component of the fluorescent nanoparticle probes presented here is commercially available. In addition, our approach is modular, enabling the same core to be used for multiple probes. We successfully generated nanoparticle probes against his-tags and the VEGF protein with little specialized equipment and reagents that were all commercially available. In the future, clinically relevant DNA, RNA, protein, or small-molecule based probes could be conjugated to the particles using a similar technique.

We have highlighted the utility of the nanoparticle probes in studying probe-target interactions in plate-based assays, but this could be extended to single-molecule studies and flow cytometry. The brightness and resistance to photobleaching of single nanoparticle labels make these probes particularly suitable for both applications. In addition, the nanoparticle probes

developed in this work could be used for targeted cellular uptake studies, and the surface functionalization techniques could be extended to create nanoparticles capable of controlled release for intracellular biomolecule delivery. Finally, the modularity of the probe attachment approach allows extension of this method to include other types of biological probes such as proteins and small molecules. In sum, the method for fabricating fluorescent nanoparticle probes described could have vast applications with only slight modifications and will enable researchers to achieve fluorescent sensitivity that is unattainable with traditional labeling approaches.

Materials and Methods

Materials for nanoparticle functionalization and passivation

Table 1 lists the vendors and catalog numbers for materials used in these studies. Carboxylate-modified FluoSpheres™ and QDot™ nanoparticles were purchased from ThermoFisher. mPEG-amine (molecular weight (MW): 2,000 g/mol) and azide-PEG-amine (MW: 2048 g/mol) were from Creative PEGWorks. DNA conjugation annealing handles and probes were purchased from Integrated DNA Technologies (IDT) or Eurofins Scientific; sequences are listed in Table 3.

Nanoparticle dispersion and washing

To ensure that nanoparticles are well-distributed in an aqueous solution, tubes of particles were dispersed by pipetting up and down while partially immersed in a sonication bath (Branson Bransonic Ultrasonic Cleaner 8510R-DTH). Nanoparticle washes were performed as follows: 1) nanoparticles were centrifuged at 31,000 x*g* for 30 minutes (pre-PEGylation) or 60 minutes (post-PEGylation) in a 1.5-mL tube; 2) supernatant was removed, taking care not to disturb the pellet; 3) appropriate volume of buffer was added; and 4) the pellet was redispersed by pipetting up and down while sonicating until no large aggregates of nanoparticles were visible.

Nanoparticle activation and PEGylation

Carboxylate-modified microspheres were activated by reacting $5.3x10^{13}$ or $1.7x10^{11}$ nanoparticles/mL for 40-nm and 200-nm nanoparticles, respectively, with 50 mM (N-3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Sigma) and 100 mM N-hydroxysuccinimide sodium salt (NHS; Sigma) in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES; Sigma), pH 6, 500 mM NaCl at 24 °C for 1 hour, shaking at 800 revolutions per minute (RPM) on a ThermoMixer dry block. The protocol outlined here was generally performed on $5.3x10^{13}$ 40-nm nanoparticles or $2.13x10^{10}$ 200-nm nanoparticles (see Supplementary Information, ***Fluorescent Nanoparticle Probe Protocol***). Particles were washed, resuspended in 1 mL phosphate buffer saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and washed again. Particles were resuspended in a 100 mg/mL 95:5 mPEG-amine:azide-PEG-amine solution (Table 1) in PBS such that the PEG:nanoparticle molar ratio was $3.5x10^{7}$ and $1.4x10^{5}$ for 200- and 40-nm nanoparticles, respectively. The reaction was incubated at 24 °C for 1 hour shaking at 800 RPM. Then, 250 µl/180 PBS was added to the sample and samples were washed twice with resuspension in 500/125 µl PBS for 40-nm/200-nm nanoparticles, respectively.

Quantum dots were functionalized using the same procedure with the following modifications: the nanoparticles were activated by reacting $7.23x10^{14}$ nanoparticles/mL with 5 mM EDC and 10 NHS. For PEGylation, nanoparticles were resuspended in 100 mg/mL of a 75:25 mPEG-amine:azide-PEG-amine in PBS to a PEG:nanoparticle molar ratio of $2x10^{4}$.

DNA aptamer probe attachment

For both pre-annealing and post-annealing processes, 125 µM of the DNA aptamer probe-complex or the conjugation annealing handle was reacted with particles at a 30,000:1, 125:1, or

25:1 DNA:nanoparticle molar ratio for 200-nm FluoSpheres, 40-nm FluoSpheres, or Qdots, respectively. For pre-annealing, the probe and the handle were combined at concentrations of 125 µM each and incubated at 95 °C for 5 minutes and at room temperature for 10 minutes, and 10x PBS was added to the complex to a final concentration of 1x PBS. The complex was then mixed with PEGylated nanoparticles and reacted at 24 °C overnight with shaking at 800 RPM to allow the DBCO/azide click reaction to proceed. Following incubation, 250/180 µL PBS was added to the sample of 40-nm/200-nM particles, respectively, which was washed twice and resuspended in 500/125 µL PBS for-40 nm/200-nM particles, respectively. The final sample was stored at 4 °C.

For post-annealing, the annealing handle was reacted with 200 nm nanoparticles at 24 °C overnight with shaking at 800 RPM. Nanoparticle were washed twice and resuspended in 180 µL PBS. The DNA aptamer probe was then added to the dry nanoparticle pellet at a concentration of 125 µM and the same molar ratios as the annealing handle, and incubated at 95 °C for 5 minutes and at 24 °C for 10 minutes while shaking at 800 RPM. Nanoparticles were washed twice, resuspended in 125 µl PBS, and stored at 4 °C.

Preparation of fluorescent probes with alternative off-the-shelf labels

Biotinylated aptamer probes were attached to the following streptavidin conjugates: Streptavidin AlexaFluor™ 647 Conjugate (Thermo Fisher Scientific, S32357), Streptavidin APC Conjugate (ThermoFisher Scientific, S32362), and Streptavidin SureLight™ APC (allophycocyanin, Columbia Biosciences, D3-2212). The conjugation reaction was performed in a buffer containing 10 mM HEPES, 1.2 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl pH 7.4 (HEPES buffer). Streptavidin conjugates at a 1 uM concentration were combined with biotinylated aptamers at a 1:4 ratio and incubated for 30 minutes while shaking at 600 RPM in HEPES buffer. Unoccupied biotin

binding sites were then blocked by addition of 100 mM D-biotin (Thermo Fisher) in dimethyl sulfoxide (Thermo Fisher) at a 40-molar excess of D-biotin to streptavidin and incubation for 30 minutes while shaking at 600 RPM.

Fluorescent nanoparticle probe characterization

Dynamic light scattering (DLS) measurements for nanoparticle hydrodynamic diameter, polydispersity index (PDI), and zeta potential were obtained using a Malvern Zetasizer ZSP Zen5600. All reported measurements were carried out in 2% PBS with a 60-second delay between measurements. Aliquots of 2-5 µl of sample were added to 700 µl of 2% PBS and loaded into a cuvette (BrandTech Scientific, 759150). Zeta potential was measured in Folded Capillary Cell cuvettes (Malvern, DTS1070).

qPCR was used to measure the number of probes/particle and was carried out using the "on-particle" and "off-particle" methods. In the on-particle measurement, the total number of probes/particle was assessed while probes were attached to the particle, and the standard curve included nanoparticles at the same concentration as in the samples. In the off-particle method, the total number of probes/particle was assessed by conducting qPCR once the probes were removed from the particles. Removal was achieved by heating the sample to 95 °C for 10 minutes, centrifuging at 31,000 x*g* for 16 minutes, and collecting supernatant for assessment. For both methods, qPCR was performed on an Applied Biosystems QuantStudio 3 Real-Time PCR Instrument. A 10-point standard curve with concentrations ranging from 1000 pM to 0.1 pM of the aptamer probe was made in DNase/RNase free water; a no-template control was included. A PCR reaction master mix was prepared with 12.5 µl of 2x PowerUp SYBR Green Master Mix (Applied Biosystems, 100029283), 1.25 µl of 25 µM forward primer, and 1.25 µl of 25 µM reverse primer per sample (see **Table 4** for primer sequences). A 15-µl aliquot of the master mix

was combined with 10 µl of sample in a 96-well PCR plate (VWR, 83007-374). The plate was mixed on a Qiagen TissueLyser II for two 25-second intervals at 13 Hz then centrifuged for 2 minutes at 1000 x*g*. The qPCR program was as follows: 1) 50 °C for 2 minutes, 2) 95 °C for 2 minutes, 3) 95 °C for 15 seconds, 4) 50 °C for 1 minute, 5) cycle steps 3 and 4 repeated 40 times. The concentration of DNA/nanoparticle was calculated by comparing the Ct values of the experimental sample to the standard curve. Two replicates were run per sample.

Binding assessment via plate-based assay

The HHH peptide (Cys-biotin-PEG2-Gly-His-His-His-Gly(COOH)) was purchased from Thermo Fisher. It was 84% pure as determined by mass spectrometry. The PNG peptide was synthesized in-house using an Intavis Multipep RSi synthesizer. Recombinant Her2-his (Sino Biological, 1004H08H) and VEGF (Sino Biological, 11066-HNAH) were resuspended according to the manufacturer's protocol, aliquoted, and stored at -80 °C. Lysozyme (Sigma, L4919), myoglobin (Sigma-Aldrich, M1882), and *E. coli* lysate (MCLab, ECCL-100) were prepared in 15 mM Na_2CO_3, 35 mM $NaHCO_3$ (referred to as 50 mM carbonate-bicarbonate buffer), pH 9.6 and stored at 4 °C.

Peptide ELONA

Wells of streptavidin-coated 384-well plates (Pierce, 15506) were washed twice with 100 µL of wash buffer (0.1% Tween-20, 10 mM HEPES, 1.2 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, pH 7.4) then incubated with 50 µL of 1 µM biotinylated peptide in peptide binding buffer (1% sheared salmon sperm DNA (Invitrogen, AM9680), 10 mM HEPES, 1.2 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, pH 7.4) and were shaken at 500 rpm for 30 minutes. Wells were then washed six times with 100 µL wash buffer and blocked with blocking solution (100 µL QBlock (Grace Biolabs, 105106) containing 0.12% Span-80, 0.1% Tween-20, 1% sheared salmon sperm DNA, and 100

µM D-biotin) while shaking at 500 RPM for 30 minutes. A 25-µL aliquot of fluorescently labeled aptamer probe in binding buffer (wash buffer containing 1% sheared salmon sperm DNA and 300 µM dextran sulfate sodium salt (40 kDa, Sigma-Aldrich, 42867)) was added to each well and allowed to incubate for 60 minutes while shaking at 500 RPM. Wells were then washed 6-8 times with 100 µL of wash buffer followed by one wash with 100 µL wash buffer without Tween-20 to remove residual bubbles. Finally, 50 µL of wash buffer without Tween-20 was added to each well, and the plate was read using a Tecan Infinite 200 microplate spectrophotometer.

Protein ELONAs: Fluorescent nanoparticle probes and Quanta Red HRP Substrate

High-binding, 384-well plates (Corning 3577) were coated with 50 µL of 50 nM protein overnight at 4 °C in 50 mM carbonate-bicarbonate buffer, pH 9.6. The protein solution was washed out with 100 µL of wash buffer per well and blocked with 100 µL of blocking solution for 90 minutes. The plates were washed six times with 100 µL of wash buffer per well and then 25 µL biotinylated aptamer probes or 25 µL of fluorescent nanoparticle probes were added in binding buffer and incubated for 1 hour. For biotinylated probes, wells were then washed three times with wash buffer, and 50 µL of binding buffer containing 5 µg/mL Streptavidin Poly-HRP (ThermoFisher Scientific 21140) was added to each well and incubated for 30 minutes with shaking at 500 RPM. A solution of QuantaRed HRP substrate (ThermoFisher Scientific, 15159) was prepared according to manufacturer's protocol, and 50 µL of the substrate was added to each well and incubated for 5 minutes followed by addition of 5 µL stop solution. For fluorescent nanoparticles probes, wells were washed six times with wash buffer after the 1 hour incubation with probes. Fluorescence was measured on a Tecan Infinite 200 microplate spectrophotometer.

Determination of EC_{50} values

After obtaining raw fluorescence intensities from ELONAs, the concentration of nanoparticles used was converted to logarithmic scale. GraphPad Prism was used to perform the half maximal effective concentration (EC50) analysis. The figures were analyzed using the "Sigmoidal, 4PL, X is log(concentration)" option. This fits a four-parameter logistic curve (4PL) and the Hill Slope from the data. Then, EC50 was obtained from the interpolated "Best-fit values" post analysis.

Comparison to other fluorescent labels

Biotinylated aptamer probes conjugated to alternative off-the-shelf labels were stored at concentrations of 1 µM in 10 mM HEPES, 1.2 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, pH 7.4 at 4 °C. Stability of these alternative fluorescent probes was determined by conducting a peptide ELONA immediately and 1-3 weeks post-conjugation. Fluorescent nanoparticle B1 probes were stored in 500 µl PBS at 4 °C. Stability of the fluorescent nanoparticle probes was assessed by performing a peptide ELONA immediately and 8, 12, and 16 weeks post-conjugation using a 3-fold serial dilution of probe from 100 nM to 7 fM in 100 µl 1X NV buffer using a 384-well black microplate (Corning 3601).

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

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Author Contributions

TER, PM, and CMS wrote the main manuscript text, and CMS, MB, and TER prepared figures. TER, CMS, MB, SST, RPG, JKR, and PM contributed to the planning, experimental design, and data analysis. CMS, MB, TER, DA, and JKR contributed to experimental execution and data compilation. All authors reviewed the manuscript.

Additional Information

The authors declare no competing interests.

Figures and Figure Legends

Figure 1

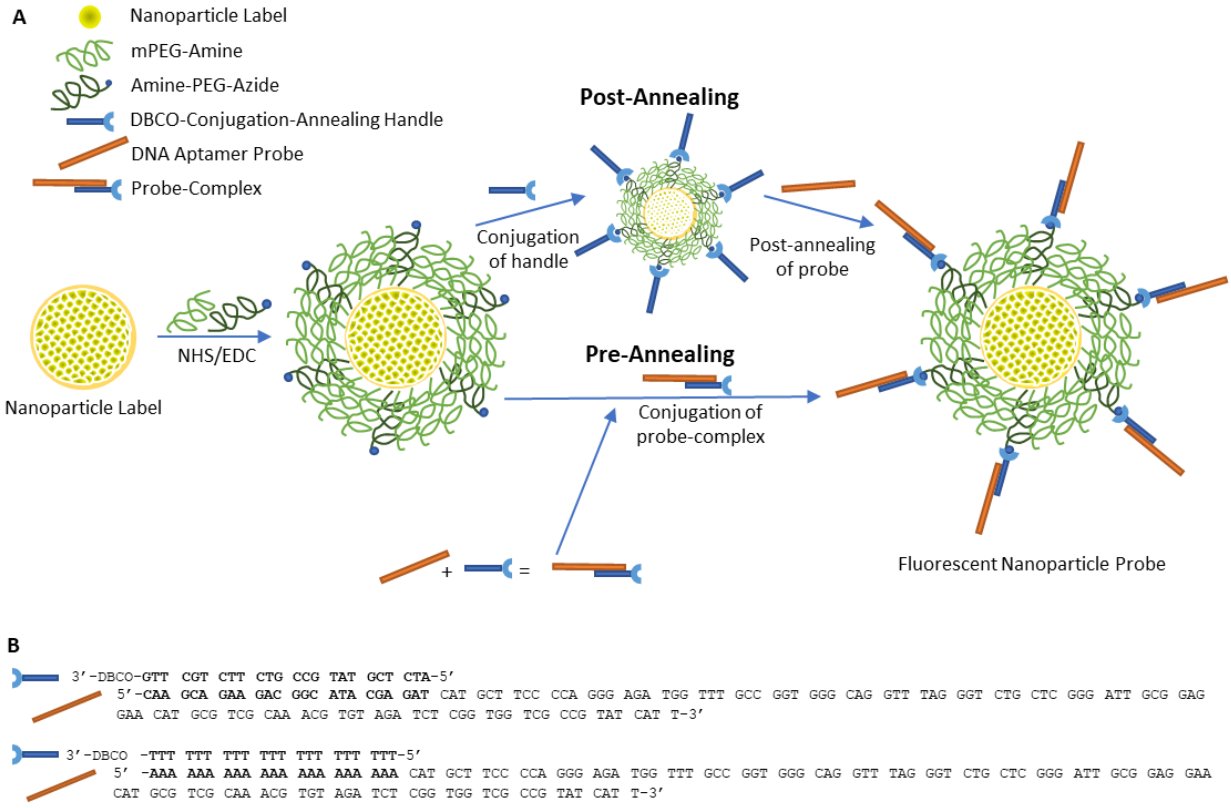
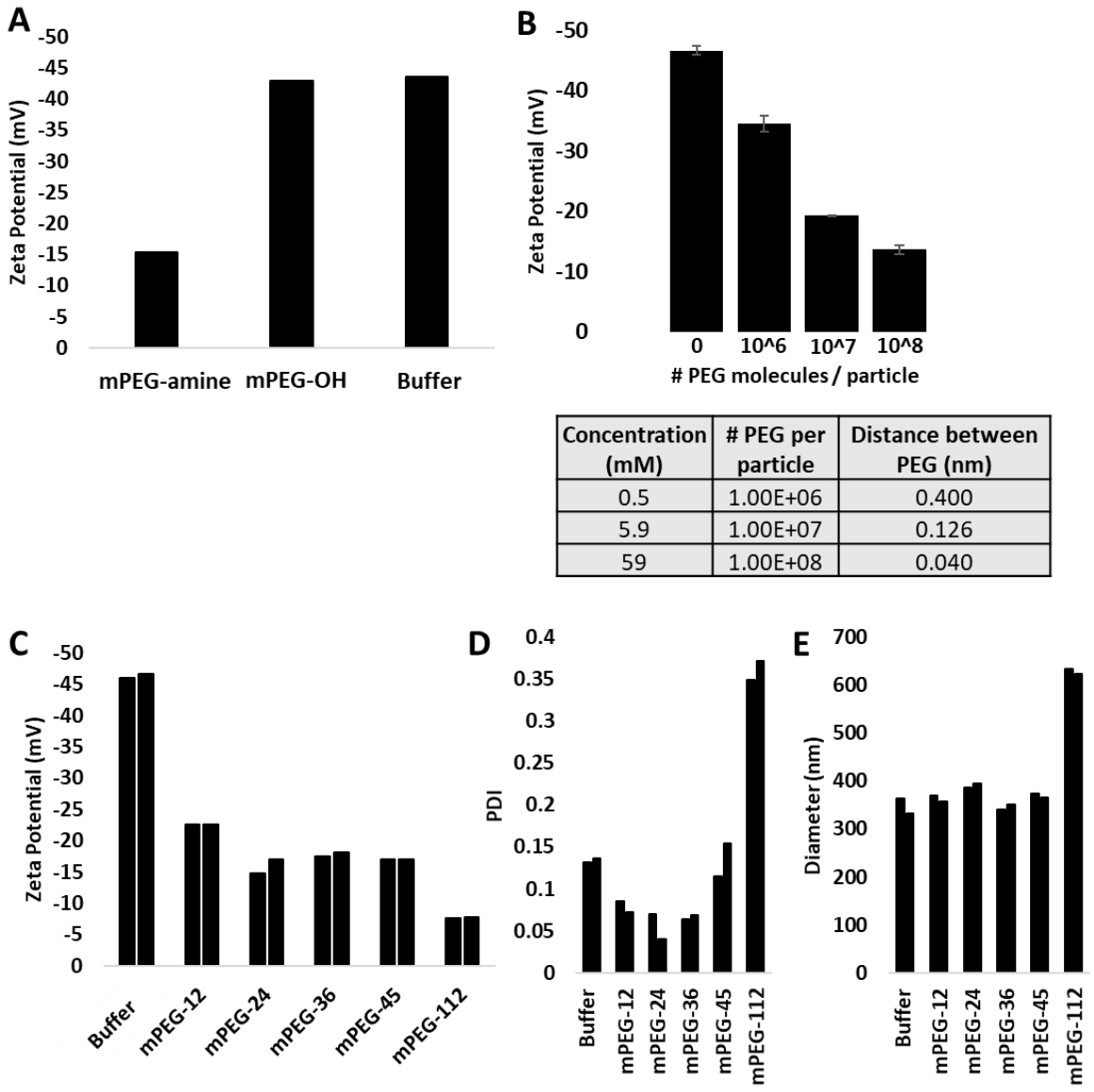


Figure 1. Fluorescent nanoparticle probe fabrication and DNA aptamer probe attachment. (A) Carboxylate-modified nanoparticle labels are PEGylated for passivation and click chemistry modification using a combination of mPEG-amine and amine-PEG-azide and NHS/EDC chemistry. DNA aptamer probes are attached to the particles via annealing to the conjugation-annealing handle either before or after it is conjugated to the PEG layer. Figure is not to scale. (B) The conjugation-annealing handle is used to conjugate a DNA aptamer probe to the nanoparticle. The same handle can be used to conjugate different aptamer probes. The handle consists of a DNA sequence complementary to a short region on the 5' end of the DNA aptamer probe and a DBCO functional group on the 3' end for covalent attachment to the particle. The

DNA aptamer probe consists of a variable probe region that can bind to a target of interest and an annealing region that allows attachment to the conjugation-annealing handle.

Figure 2

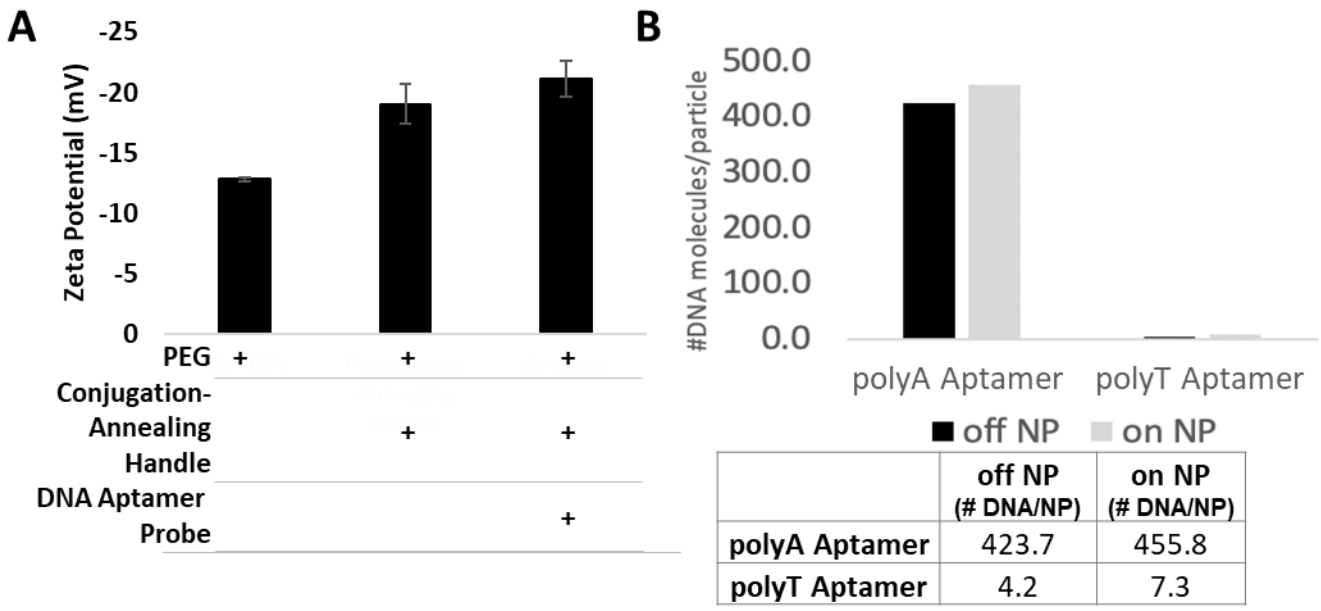


Concentration (mM)	# PEG per particle	Distance between PEG (nm)
0.5	1.00E+06	0.400
5.9	1.00E+07	0.126
59	1.00E+08	0.040

Figure 2. PEGylation of carboxylate-modified FluoSpheres™. (A) Zeta potential measurements for carboxylated FluoSpheres™ activated with NHS/EDC and reacted with mPEG-amine, mPEG-methoxy, or buffer only. Data are means of duplicates from one of two experiments. (B) Zeta potential measurements for carboxylated FluoSpheres™ activated with NHS/EDC and reacted with increasing concentrations of mPEG-amine. Data are means (±

standard deviation) of one of two independent experiments, analyzed in triplicate. Table lists distances between PEG molecules at each concentration. (C-E) FluoSpheres™ were conjugated with PEGs of indicated molecular weights, and C) zeta potentials, D) PDIs, and E) hydrodynamic diameters were determined. Shown are data from both replicates of one of two experiments.

Figure 3



	off NP (# DNA/NP)	on NP (# DNA/NP)
polyA Aptamer	423.7	455.8
polyT Aptamer	4.2	7.3

Figure 3. Characterization of DNA aptamer probe attachment to PEGylated nanoparticle. (A) Zeta potential measurements after PEGylation, conjugation of conjugation-annealing handle, and annealing of DNA aptamer probe. Data are means (± standard deviation) of one of two independent experiments, analyzed in triplicate. (B) qPCR quantification of aptamer numbers determined on particle and off particle for particles functionalized with complimentary polyA aptamer or non-complimentary (negative control) polyT aptamer. Data shown are means of one of two experiments performed in duplicate.

Figure 4

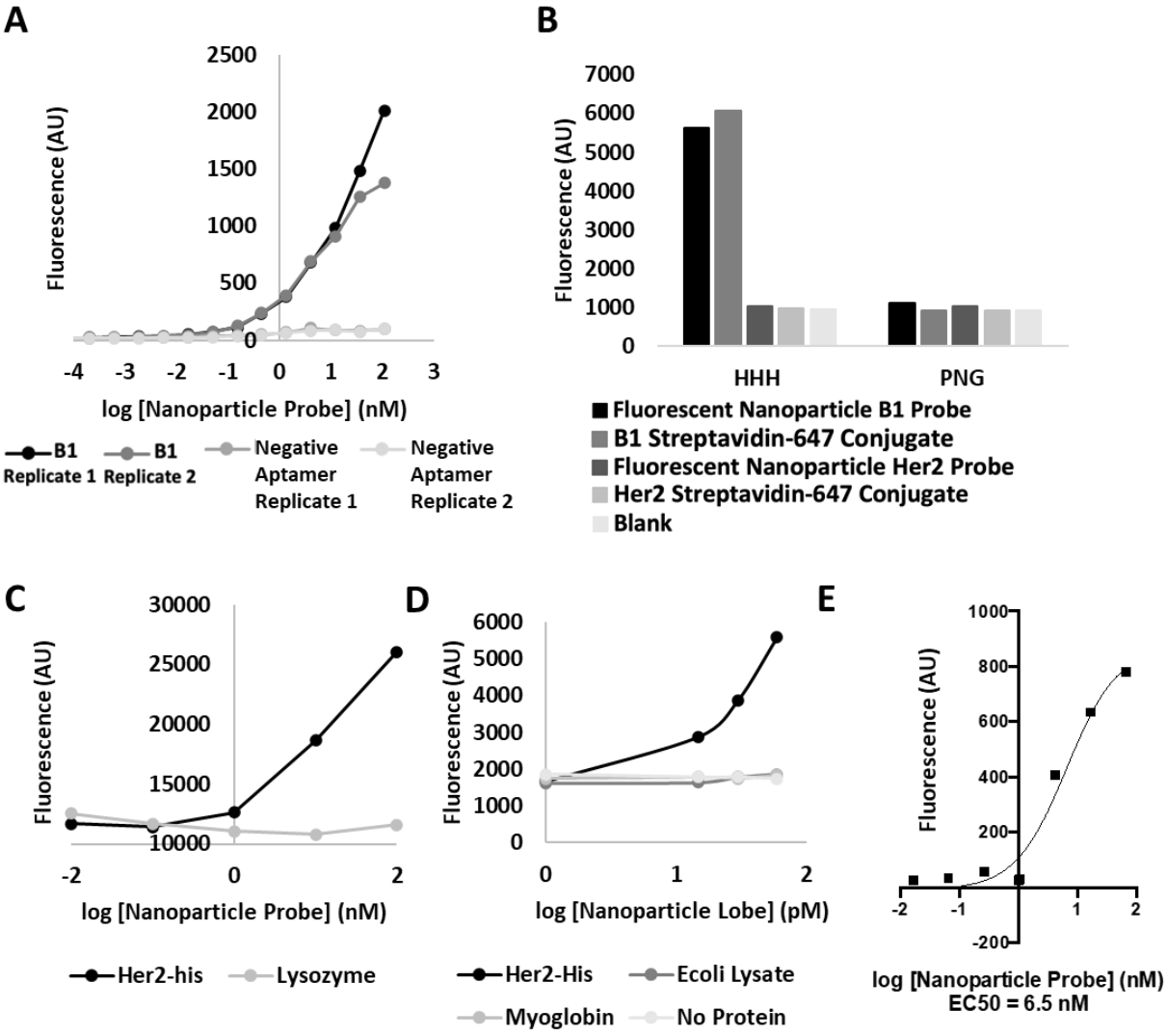


Figure 4. Fluorescent nanoparticle B1 probe binds specifically to HHH peptide and his-tagged protein. (A) Fluorescence signals as a function of concentration of nanoparticle B1 probe and negative control probe upon binding to an HHH peptide. Shown are data from two replicates of a representative experiment that was repeated at least five times with one or two replicates per experiment. (B) Fluorescence signals from fluorescent nanoparticle B1 and Her2 probes and B1 and Her2 Streptavidin 647 conjugates against HHH (on-target) or PNG (off-target) peptides. Data is from a single experiment. (C) Signal from nanoparticle B1 probe against his-tagged Her2

(on-target) and lysozyme (off-target). Data are representative at least five experiments. (D) Signal from nanoparticle B1 probe against his-tagged protein (on-target), E. coli lysate (off-target), myoglobin (off-target), and no protein. Data are representative of at least three experiments. (E) Binding curve of the B1 probe against his-tagged Her2. Data are representative of at least five experiments.

Figure 5

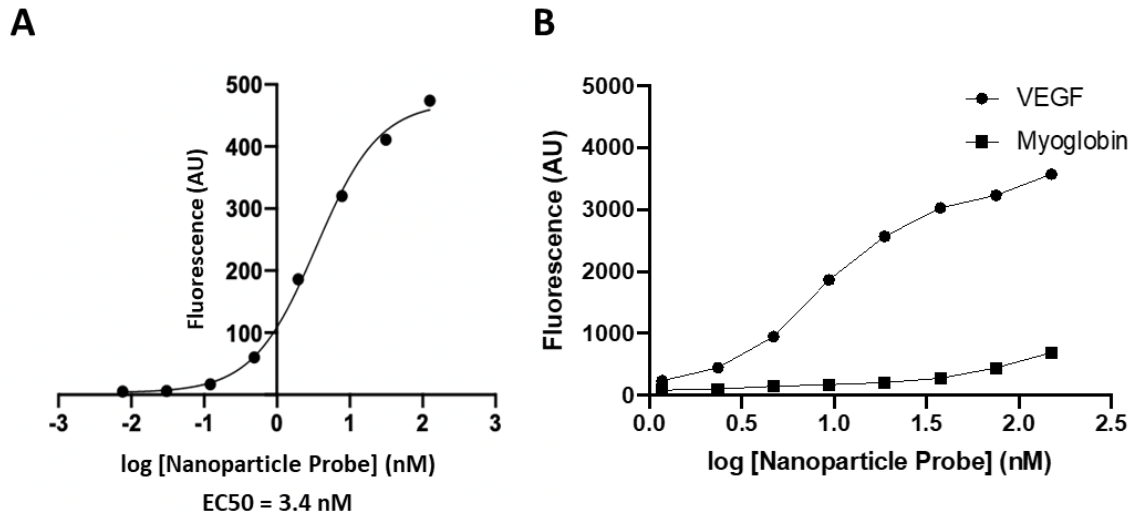


Figure 5. Fluorescent nanoparticles functionalized with various aptamers bind specifically. (A) Binding curve for fluorescent nanoparticle H3T probe against HHH peptide. Data shown are means from one of two experiments performed in duplicate. (B) Binding curve for fluorescent nanoparticle VEGF probe against VEGF (on-target) and myoglobin (off-target). Data shown are means from one of two experiments performed in duplicate.

Figure 6

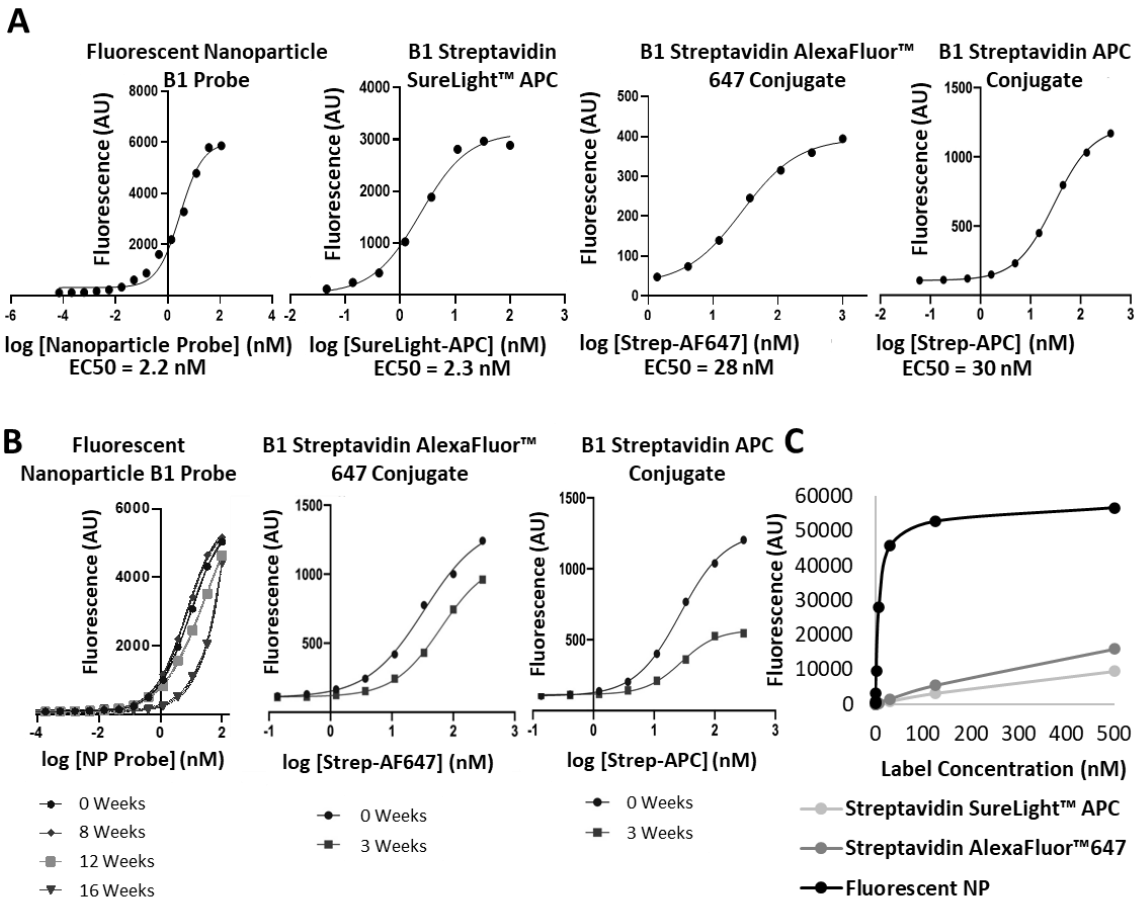


Figure 6. Fluorescent nanoparticle probes compare favorably to commercially available labels. (A) Binding curves and EC50 values for fluorescent nanoparticle B1 probe, B1 Streptavidin SureLight™ APC, B1 Streptavidin AlexaFluor™ 647 Conjugate, and B1 Streptavidin APC Conjugate against HHH targets. Data shown are means from one of at least three experiments performed with one to three replicates per experiment. (B) Fluorescence intensities from binding curves of fluorescent nanoparticle B1 probe, B1 Streptavidin AlexaFluor™ 647 Conjugate, and B1 Streptavidin APC Conjugate against HHH targets at noted timepoints post fabrication. Data shown are from one of two experiments. (C) Signal intensity versus concentration of the fluorescent nanoparticle B1 probe, B1 Streptavidin APC Conjugate,

and B1 Streptavidin AlexaFluor™ 647 Conjugate. Data are from one of two experiments for the nanoparticle probe, three experiments for the Streptavidin AlexaFluor™ 647 Conjugate, and three experiments for the Streptavidin APC Conjugate.

Figure 7

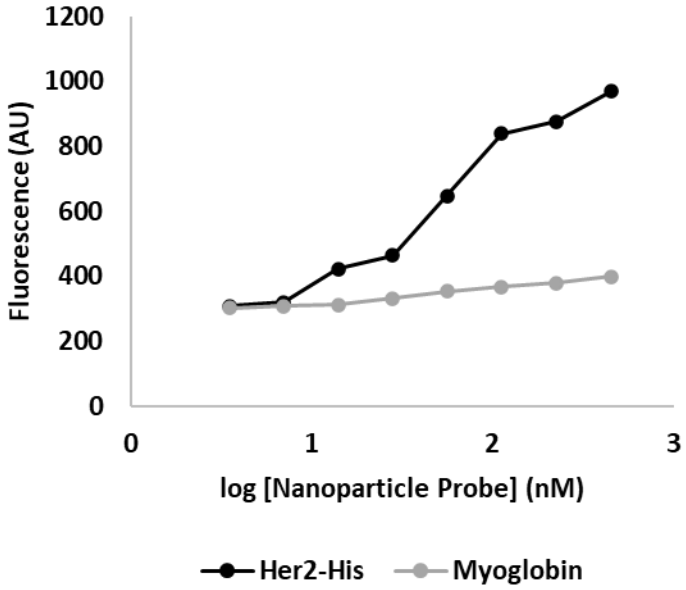


Figure 7. Fluorescent nanoparticle probes can be prepared with a quantum dot core. (A) Binding curves of Qdot fluorescent nanoparticle B1 probe to his-tagged Her2-his (on-target) and myoglobin (off-target). Data are from one of two experiments.

Tables

Table 1. Commercially Available Reagents for Fluorescent Nanoparticle Probes

Component of Fluorescent Nanoparticle Probe	Vendor	Catalog No.	Product Name
Nanoparticle label	Thermo Fisher: Molecular Probes	F8795	FluoSpheres™ Carboxylate-Modified Microspheres, 0.04 µm, yellow-green fluorescent (505/515), 5% solids, azide free
	Thermo Fisher: Molecular Probes	F8811	FluoSpheres™ Carboxylate-Modified Microspheres, 0.2 µm, yellow-green fluorescent (505/515), 5% solids
	Thermo Fisher: Molecular Probes	F8807	FluoSpheres™ Carboxylate-Modified Microspheres, 0.2 µm, dark red fluorescent (660/680), 5% solids
	Thermo Fisher: Molecular Probes	Q21321MP	Qdot™ 655 ITK™ Carboxyl Quantum Dots
Passivation and Functionalization	Creative PEGWorks	PHB-1882	Amine-PEG-azide, MW 2 kDa
	Creative PEGWorks	PLS-269	mPEG-Amine, MW 2 kDa
Conjugation Annealing Handle	Integrated DNA Technologies	Custom sequence; Table 3	<custom_sequence>/iSp9//3DBCON/, HPLC purification
DNA Aptamer Probe	Integrated DNA Technologies	Custom sequence; Table 3	20 nmole Ultramer

Table 2. Summary of particle size, PDI, and zeta potential of EDC/NHS activated carboxylate-modified FluoSpheres™, PEGylated FluoSpheres™ and fluorescent nanoparticle probes of a 200-nm diameter. Representative data from more than five experiments are shown as means (± standard deviation).

Stage in Nanoparticle Fabrication	Particle size (nm)	Polydispersity index (PDI)	Zeta Potential (mV)
Activated Nanoparticle	335 ± 3.0	0.13	-46.9 ± 1.0
PEGylated Nanoparticle	367 ± 6.0	0.12	-17.3 ± 0.3
Fluorescent Nanoparticle Probe	367 ± 0.1	0.12	-21.1 ± 1.5

Table 3. Oligonucleotides for the generation of fluorescent nanoparticle probes

Component	Name	Annealing Region	Oligonucleotide Sequence (annealing region in bold)
DNA Aptamer Probe	P7-B1 or B1	P7	5'-**CAA GCA GAA GAC GGC ATA CGA GAT** CAT GCT TCC CCA GGG AGA TGG TTT GCC GGT GGG CAG GTT TAG GGT CTG CTC GGG ATT GCG GAG GAA CAT GCG TCG CAA ACG TGT AGA TCT CGG TGG TCG CCG TAT CAT T-3'
	polyA-B1	polyA	5'-**AAA AAA AAA AAA AAA AAA AAA** CAT GCT TCC CCA GGG AGA TGG TTT GCC GGT GGG CAG GTT TAG GGT CTG CTC GGG ATT GCG GAG GAA CAT GCG TCG CAA ACG TGT AGA TCT CGG TGG TCG CCG TAT CAT T-3'
	H3T	P7	5'- **CAA GCA GAA GAC GGC ATA CGA GAT** GTT TGC CGG TGG GCA GGT TTA GGG TCT GCT CGG GAT TGC GGA GGA ACA TGC GTC GCA AAC GTG TAG ATC TCG GTG GTC GCC GTA TCA TT -3'
	Her2	P7	5'- **CAA GCA GAA GAC GGC ATA CGA GAT** TCT AAA AGG ATT CTT CCC AAG GGG ATC CAA TTC AAA CAG CGT GTA GAT CTC GGT GGT CGC CGT ATC ATT -3'
	polyA-Aptamer2	polyA	5'-AGC GTC TCT CGA TCT CAT TCT CAG GTG GGG GGT ATA TTT AGA GAC GGA AGA TCG AGA GCA AGC GTT GTT TTG ATG GCC C**AA AAA AAA AAA AAA AAA AAA**-3'
	polyT-Aptamer2	polyT	5'-AGC GTC TCT CGA TCT CAT TCT CAG GTG GGG GGT ATA TTT AGA GAC GGA AGA TCG AGA GCA AGC GTT GTT TTG ATG GCC C**TT TTT TTT TTT TTT TTT TTT**-3'
	Negative-control Aptamer (Figure 4)	P7	5'-**CAA GCA GAA GAC GGC ATA CGA GAT** CGC TTG AAG TAG ATG ACT TTG CTA GTA TGT GGT TCT TTC CAA ATT ATT AGA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GTG TGT AGA TCT CGG TGG TCG CCG TAT CAT T-3'
	VEGF	P7	5'- **CAA GCA GAA GAC GGC ATA CGA GAT** CCC GTC TTC CAG ACA AGA GTG CAG GGG TGT AGA TCT CGG TGG TCG CCG TAT CAT T -3'
Conjugation-Annealing Handle	DBCO-polyT	polyT	5'-**TTT TTT TTT TTT TTT TTT TTT**-DBCO-3'
	DBCO-P7 (revcom)	P7 reverse complement	5'-**ATC TCG TAT GCC GTC TTC TGC TTG**-DBCO-3'
Direct attachment	DBCO-B1	NA	5'-[DBCO-C7]-CAA GCA GAA GAC GGC ATA CGA GAT CAT GCT TCC CCA GGG AGA TGG TTT GCC GGT GGG CAG GTT TAG GGT CTG CTC GGG ATT GCG GAG GAA CAT GCG TCG CAA ACG TGT AGA TCT CGG TGG TCG CCG TAT CAT T-3'
	Biotin-B1		5'-/5Biosg/CA TGC TTC CCC AGG GAG ATG GTT TGC CGG TGG GCA GGT TTA GGG TCT GCT CGG GAT TGC GGA GGA ACA TGC GTC GCA AAC-3'
	Biotin-Her2		5'-/5Biosg/TC TAA AAG GAT TCT TCC CAA GGG GAT CCA ATT CAA ACA GC-3'
	Biotin-H3T		5'-/5Biosg/GTT TGC CGG TGG GCA GGT TTA GGG TCT GCT CGG GAT TGC GGA GGA ACA TGC GTC GCA AAC-3'

Table 4. Primers

Target	Forward	Reverse
B1	5'-AATGATACGGCGACCACCGAGATCTACAC-3'	5'-CAAGCAGAAGACGGCATACGAGAT-3'
Aptamer 2	5'- AGCGTCTCTCGATCTCATTCTC-3'	5'-GGGCCATCAAAACAACGCTTG-3'