Aptamer-Conjugated Nanoparticles for Cancer Cell Detection

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Abstract

The material contained in this section includes the detailed nanoparticle synthesis procedures for both the fluorescent and magnetic nanoparticles, and the various nanoparticle surface modifications used in the primary research manuscript as well as the bioconjugations used to attach proteins and aptamers to the nanoparticle surfaces. Detailed descriptions of the study of the optimized fluorophore encapsulated in the nanoparticles (Table S-1), nanoparticle conjugation and the effect that the conjugation method used has on the overall signal output from the assay as a result (Figure S-1), and the description of an alternative multiple aptamer approach investigated for this assay.

Methods

Fluorescent Nanoparticle Synthesis

FITC-, TMR- and Cy5-doped NPs were synthesized according to the following method: TMR- and Cy5-NHS were each dissolved in DMSO at a concentration of 5 mg/mL, and APTS was added at a molar ratio of 1.2:1 APTS:dye. The APTS was allowed to conjugate to the amine-reactive dye for 24 h in the dark with shaking prior to synthesis of the particles. Glass reaction vessels and Teflon-coated magnetic stir rods were washed with 1 M NaOH solution for 30 min, rinsed with DI water and ethanol, and allowed to dry. This wash step was performed to clean the glass vessel and stir rods and smooth the inside surface of the glass vessel, which prevents unwanted seeding and NP formation. After conjugation, 4.19 mL of ethanol was mixed with 239 µL of ammonium hydroxide solution in the reaction vessel. A 36 µL volume of TMR-APTS conjugate or 54 µL of Cy5-APTS conjugate was added to the reaction vessels. A 177 µL volume of TEOS was added rapidly to the reaction mixture, and the vessels were sealed. The reaction was allowed to proceed for 48 h in the dark before the particles were recovered by centrifugation at 14 000 rpm. The particles were washed three times with phosphate buffer to remove any weakly bound dye molecules.

Tris(2,2'-bipyridyl)dichlororuthenium (II) hexahydrate (RuBpy) dye-doped nanoparticles (NPs) were synthesized through reverse microemulsion. First, 1.77 mL Triton x-100, 7.5 mL cyclohexane and 1.6 mL n-hexanol were added to a 20 mL glass vial with constant magnetic stirring. Then, 400 µL of H_2O and 80 µL of 0.1M RuBpy dye (MW=748.63) were added, followed by the addition of 100 µL tetraethyl orthosilicate (TEOS). After thirty minutes of stirring, 60µL NH_4OH was added to initiate

silica polymerization. After 18 hours, carboxyl-modified silica post-coating was initiated by adding 50 μL TEOS, 40 μL monocarboxyl-silane, and 10 μL phosphonate-silane. Polymerization proceeded for 18 hours, and particles were centrifuged, sonicated, and vortexed four times with 95% ethanol, followed by one wash with H_2O.

Fluorescent Nanoparticle Bioconjugation

Tricarboxyl-Modified NPs (3-COOH): A 40 μL aliquot of tricarboxyl-silane (3-COOH) was added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. DNA modification was performed by EDC/NHS chemistry as described below.

Tricarboxyl-Modified NPs Variant Preparation (3-COOHV): After the third wash, the NPs were suspended in 500 μL of 30 mM hydroxylamine with 1% BSA in 10 mM PBS (pH 7.4) buffer and incubated for 30 minutes. Finally, the NPs were washed three times and suspended in 500 μL aliquots of 10 mM PBS with 0.05% Tween 20 and 0.1% BSA at pH 7.4. The final concentration of the NPs was 2 mg/mL, and the samples were stored at 4 ˚C until used. DNA modification was performed by EDC/NHS chemistry as described below.

Streptavidin-Coated NPs (SA-COOH): A 250 μL aliquot of the carboxyl-modified NPs was washed three times with 250 μL aliquots of a 0.5 mM MES (pH 5.0) buffer. Modification with streptavidin was achieved by adding 2.0 mg of EDC and 5.0 mg of NHS to the washed particles and incubating for fifteen minutes. To the activated NPs, 100 μg of streptavidin was added. This solution was allowed to react for two hours with

constant mixing. The NPs were washed three times with 1 mL aliquots of 10 mM PBS buffer. The sample was stored at a concentration of 2 mg/mL at 4 ˚C until used.

DNA was attached to the particles by washing them three times with 20 mM Tris-HCl and 5 mM $MgCl_2$ at pH 8.0 and dispersing the NPs at 0.2 mg/mL in this buffer. The DNA was attached by adding biotin-labeled DNA (3 pmol) to 500 μL of streptavidin-coated NPs. The reaction was incubated at 4 °C for 12 hours, and three final washes of the particles were performed using 20 mM Tris-HCl and 5 mM $MgCl_2$ at pH 8.0. The NPs were stored at a final concentration of 0.2 mg/mL at 4 °C before use.

Microemulsion Carboxyl NPs (ME-COOH): The reverse micelle microemulsion was prepared by mixing 1.77 mL of Triton X-100, 7.5 mL of cyclohexane, and 1.6 mL of n-hexanol in a 20 mL screw- top glass vial. The solution was mixed continuously for fifteen minutes. A 408 μL aliquot of water was added along with a 72 μL volume of TMR-APTS conjugate followed directly by the addition of 100 μL of tetraethyl orthosilicate (TEOS) with 30 minutes of stirring. A 60 μL aliquot of ammonium hydroxide was added, and the reaction was mixed continuously for 24 hours. The carboxyl modification was achieved by adding an additional 50 μL aliquot of TEOS followed immediately by the addition of 40 μL of tricarboxyl-silane. The reaction was continuously mixed for an additional 24 hours. The NPs were centrifuged at 14,000 RPM for 15 minutes, and the sample was washed three times with fresh aliquots for 95 % ethanol and once with water. The samples were sonicated and centrifuged between each wash. DNA modification was performed by EDC/NHS as described below.

Monocarboxylic Acid- and Phosphonic Acid-modified NPs: The monocarboxyl-silane (1-COOH)- modified NPs were prepared using the same procedure as described for the tricarboxyl-modified NPs, differing only in that the silane used in the preparation of this sample set was the monocarboxyl-silane (1-COOH). For these NPs, five distinct silane compositions used in the modification procedure were prepared. The first reaction used a 40 µL aliquot of 1-COOH added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. This NP sample was referred to as 1-COOH . DNA modification was achieved by the method described below for carboxyl-modified NPs for all monocarboxylic acid-modified NPs.

75% 1-COOH and 25% phosphonate-silane samples were prepared using a 30 µL aliquot of 1-COOH followed directly by 10 µL of phosphonate-silane added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. This NP sample was referred to as 1-COOH 25%.

50% 1-COOH and 50% phosphonate-silane samples were prepared using a 20 µL aliquot of 1-COOH followed directly by 20 µL of phosphonate-silane added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. This NP sample was referred to as 1-COOH 50%.

25% 1-COOH and 75% phosphonate-silane samples were prepared using a 10 μL aliquot of 1-COOH followed directly by 30 μL of phosphonate-silane added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. This NP sample was referred to as 1-COOH 75%.

Finally, 5% 1-COOH and 95% phosphonate-silane samples were prepared using a 2 μL aliquot of 1-COOH followed directly by 38 μL of phosphonate-silane added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. This NP sample was referred to as 1-COOH 95%.

Carboxyl-Amine Conjugation: A 250 μL aliquot of the carboxyl-modified NPs was washed three times with 250 μL aliquots of a 10 mM PBS (pH 7.4) buffer. The DNA modification was achieved by adding 2.0 mg of EDC and 5.0 mg of NHS to the washed particles and incubating for fifteen minutes. A 10 μL aliquot of a 100 μM solution of amine-modified DNA aptamer was added to the NP sample. The sample was allowed to react for two hours with constant mixing at room temperature, and three final washes were performed using the 20 mM Tris-HCl, 5 mM $MgCl_2$ at pH 8.0. The NPs were stored at a final concentration of 0.2 mg/mL at 4 °C before use.

Amine-Modified NPs: The amine-modified NPs were prepared using the same procedure as described for the carboxyl coating, as described previously, except that the silane used in the preparation of this sample set was an amino-silane (NH2). For these NPs, four distinct silane compositions were used in the modification procedure. The first reaction used a 40 μL aliquot of NH2 added to 1 mL of 10 mg/mL silica-coated NPs in 10 mM PBS (pH 7.4) and continuously mixed for four hours. The sample was washed three times with 10 mM PBS and stored at a concentration of 10 mg/mL at room temperature until used. This NP sample was referred to as 100% NH2. Thus, NH2 25%, 75%, and 95% NPs were prepared by changing the reaction conditions to include 30 μL NH2 and 10 μL phosphonate-silane (NH2 25%), 10 μL NH2 and 30 μL phosphonate-silane (NH2 75%), and 2 μL NH2 and 38 μL phosphonate-silane (95% NH2), respectively.

DNA modification was achieved by adding GMBS to activate the amine-modified NPs. To 250 μL of the NH2-modified NPs, 2.0 mg of GMBS was added, and the samples were incubated for fifteen minutes. A 10 μL aliquot of a 100 μM solution of thiol-modified DNA aptamer was added to the NP sample. The sample was allowed to react for two hours with constant mixing at room temperature, and three final washes were performed using the 20 mM Tris-HCl, 5 mM $MgCl_2$ at pH 8.0. The NPs were stored at a final concentration of 0.2 mg/mL and stored at 4 °C before use.

Magnetic Nanoparticle Synthesis

The iron oxide core magnetic nanoparticles (MNPs) were prepared by means of precipitating iron oxide by mixing ammonia hydroxide (2.5%) and iron chloride at 350

RPM using a mechanical stirrer (10 minutes). The iron chloride solution contained ferric chloride hexahydrate (0.5 M), ferrous chloride tetrahydrate (0.25 M), and HCl (0.33 M). After three washes with water and once with ethanol, an ethanol solution containing ~1.2 % ammonium hydroxide was added to the iron oxide NPs, yielding a final concentration of ~7.5 mg/mL. To create the silica coating for the magnetite core particles, TEOS (200 µL) was added, and the mixture was sonicated for 90 minutes to complete the hydrolysis process. For post-coating, an additional aliquot of TEOS (10 µL) was added, and additional sonication was performed for 90 minutes. The resulting NPs were washed three times with ethanol to remove excess reactants.

Magnetic Nanoparticle Bioconjugation

For avidin coating, a 0.1 mg/mL silica-coated MNP solution and a 5 mg/mL avidin solution were mixed and then sonicated for 5-10 minutes. The mixture was incubated at 4° C for 12-14 hours. The particles were then washed three times with 10 mM phosphate buffered saline (PBS) (pH 7.4) and dispersed at 1.2 mg/mL in 10 mM PBS, and the avidin coating was stabilized by cross-linking the coated NPs with 1% glutaraldehyde (1 hour at 25° C). After another separation, the particles were washed three times with 1M Tris-HCl buffer. Then, the particles were dispersed and incubated in the 1M Tris-HCl buffer (3 hours at 4° C), followed by three washes in 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0. DNA was attached to the particles by dispersing the particles at 0.2 mg/mL in a buffer of 20 mM Tris-HCl and 5 mM $MgCl_2$ at pH 8.0. The DNA was attached by adding biotin-labeled DNA (3 pmol) to 500 µL of avidin-coated NPs. The reaction was incubated at 4 °C for 12 hours, and three final washes of the particles were performed

using 20 mM Tris-HCl, 5 mM $MgCl_2$ at pH 8.0. The NPs were stored at a final concentration of 0.2 mg/mL at 4 °C before use.

Optimization of the Fluorophore in the Nanoparticles

Because of the different excitation and emission wavelengths, the variability of the instrument at different wavelengths, and the possible variations in the NPs, the fluorescent nanoparticles (FNPs) were compared using their signal-to-background ratios. For these experiments, the signal value was determined using 100,000 target cells after magnetic extraction, while the background value was determined using 100,000 control cells after magnetic extraction, and each measurement was repeated 5 times. This method enabled evaluation of the NPs based on their signal strength, and accounted for interactions between the FNPs and either the MNPs or the inside of the container, as both conditions could influence the final result. The FNP with the highest signal-to-background ratio was used for subsequent experiments to evaluate the performance of the assay in regards to the optimum dye for detection. The results of these experiments are shown in Table S-1.

Table S-1: Comparison of extracted Target and Control Cells with different dye doped nanoparticles

Dye	Target Cell Signal	Control Cell Signal	Signal/Background
Cy5	7,800 (±2%)	650 (±4%)	12
TMR	49,100 (±4%)	1,200 (±4%)	40
RuBpy	37,000 (±4%)	1,300 (±4%)	29
FITC	16,000 (±4%)	5,500 (±4%)	2.9

Based on the signal-to-noise ratios of the extractions, the TMR-doped particles possessed the best signal-to-background ratio based on the instrumentation that was used. The RuBpy NPs had an excellent signal-to-background ratio, but not as good as the TMR-based particles. The Cy5 particles had a lower fluorescent intensity and the lowest background. In a previous publication, various dyes were used for multiple detection capabilities.[17] However in this investigation, the focus of the study was on determining which dye provided the greatest signal-to-background difference. This likely resulted from the instrument limitations with the far red dye Cy5. The FITC NPs had a much lower fluorescent intensity and appeared to have had a much higher background as a consequence of using a higher gain on the instrument. The lack of fluorescence signal could have arisen from the poor incorporation of the dye inside of the particle or self-quenching of the dye. Several preparations of these particles failed to produce a highly fluorescent batch. Each of the FNPs, except for the FITC NPs, had a relatively low %RSD, ranging from 4% for the Cy5 NPs to 5% for the RuBpy NPs. Based on these results, subsequent experiments utilized the TMR-based FNPs. For use as a clinical diagnostics tool for detection of exfoliated disease cells, the need for greater signal differentiation over background is crucial.

Nanoparticle Conjugation Fluorescence Intensity

While high selectivity indicated a lower background signal and lower signal from a negative sample, the signal associated with a positive result also needs to be as high as possible. Since exfoliated tumor cells are present at extremely low levels in the blood stream, positive responses must generate as much fluorescence intensity (i.e., sensitivity) as possible to be effective. In order to evaluate the conjugation methods for the highest

fluorescence intensity, six of the conjugation chemistries were compared including 1) avidin-coated NPs with biotinylated aptamer (A/Biotin, Fig. 3A), 2) streptavidin-coated NPs with a carboxyl group attached through EDC chemistry to an amine-labeled aptamer (SA-COOH Fig. 3B), 3) NPs prepared through the microemulsion method with carboxyl surface modification conjugated with EDC and an amine-labeled aptamer (ME-COOH), 4) and tricarboxyl-modified Stöber NPs conjugated with EDC and an amine-labeled aptamer (3-COOH, Fig. 3C) and 5) monocarboxyl modified Stöber NPs conjugated with EDC and an amine-labeled aptamer (1-COOH Fig. 3D), and 6) amine-modified NPs conjugated through a dithiol-labeled aptamer (NH2, Fig. 3E). Fluorescence intensities were measured and then normalized against the highest fluorescence intensity generated, which belonged to the 3-COOH NPs, and finally plotted in Figure S-1. The results generally followed a trend where the least selective NPs generated the highest fluorescence signal. This can be explained by the tendency of FNPs to nonspecifically bind to the cells, whereas the more selective NPs were limited to binding to their targets. There were a few exceptions in that the A/Biotin NPs had higher fluorescence intensity than the SA-COOH and NH2 NPs, despite having the highest selectivity. This could be a result of the coupling between the nanoparticle and aptamer having a much lower yield, thereby resulting in fewer aptamer sequences on the nanoparticle surface, as these chemistries tend not to be as efficient as the direct amine-to-carboxyl coupling utilizing EDC. The next step in the comparison investigated the effects of fluorescence intensity and selectivity on the limit of detection, and this will be explored in a later section. The results of the conjugation chemistry evaluation showed that sensitivity and selectivity tend to work against each other as indicated by the highest fluorescence intensity

belonging to the least selective NPs, while the most selective NPs exhibited much lower fluorescence intensity than that of the tricarboxyl-modified NPs. For clinical applications, the tendency would be to use a more selective NP modification for treatment to scavenge for disease cells versus using a more sensitive modification for diagnostics.

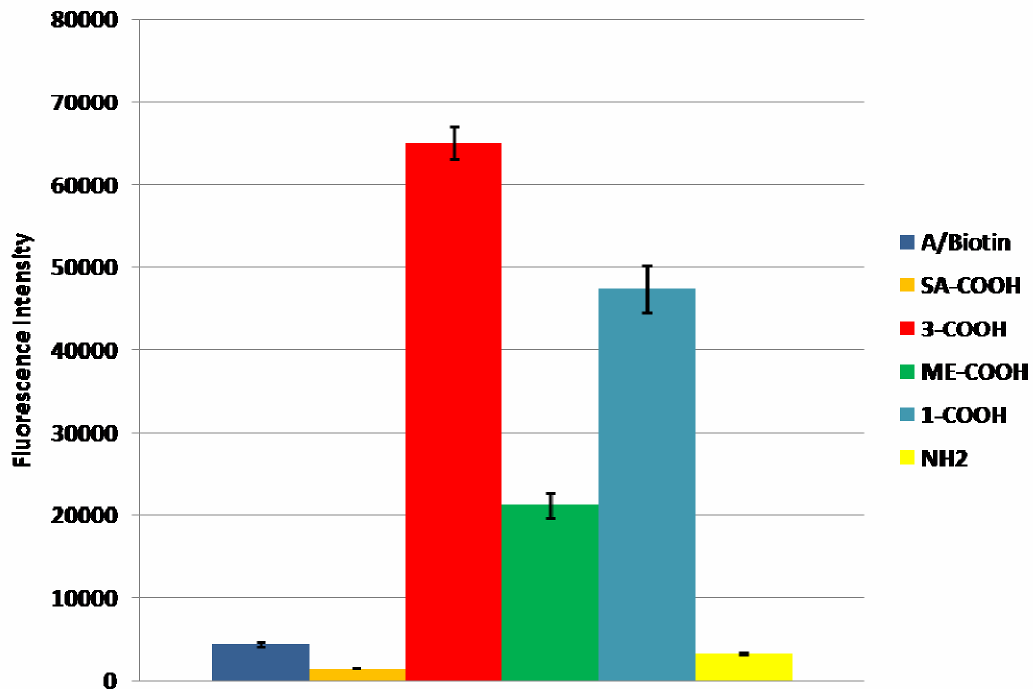

Figure S-1: Each conjugation chemistry was evaluated for its overall fluorescence intensity to determine which conjugation scheme would produce the most sensitive assay. Chemistries evaluated include 1) avidin-coated NPs with biotinylated aptamers, 2) streptavidin-coated NPs with biotin- labeled aptamers, 3) several varieties of carboxyl-modified NPs with amine-labeled aptamers, and 4) amine-coated NPs linked to thiol-modified aptamers.

Alternative Strategy

The first approached used multiple NPS each with a different aptamer sequence immobilized to target multiple sites on every cancer cell. The NPs with different sequences were then mixed together in equal amounts. Both the MNPs and FNPs utilized

the same aptamer sequences and combinations with the same total nanoparticle concentration as in the previously mentioned protocols. Then, mixtures of two, three, and four different ACNPs were evaluated versus the single ACNPs. For these experiments, 20,000 target and control cells were utilized (data not shown). However, the signal generated from the target cells decreased with each additional aptamer sequence. Based on these results, it is theorized that while the cells expressed all of the proteins targeted, only some of the proteins were highly expressed. Therefore, those ACNPS that were selective for a protein with low expression were not as effective at extracting the cells as the ACNPs with a highly expressed target. Consequently, using the same total amount of NPs resulted in decreasing the amount of ACNPs targeting the highly expressed proteins, thereby negatively affecting the absolute sensitivity of the assay. Since it is unlikely that every target for the aptamers would be highly expressed, a certain percentage of the ACNPs would not extract cells. This limitation could be addressed by using the full amount of NPs for each aptamer sequence. However, this would result in using four times as much of the total particles, thus increasing both the material costs and the possibility of nonspecific interactions, resulting from the excessive amount of NPs in the sample. Therefore, a different methodology was tested to increase the number of available aptamer sequences without changing the total amount of NPs in the sample.