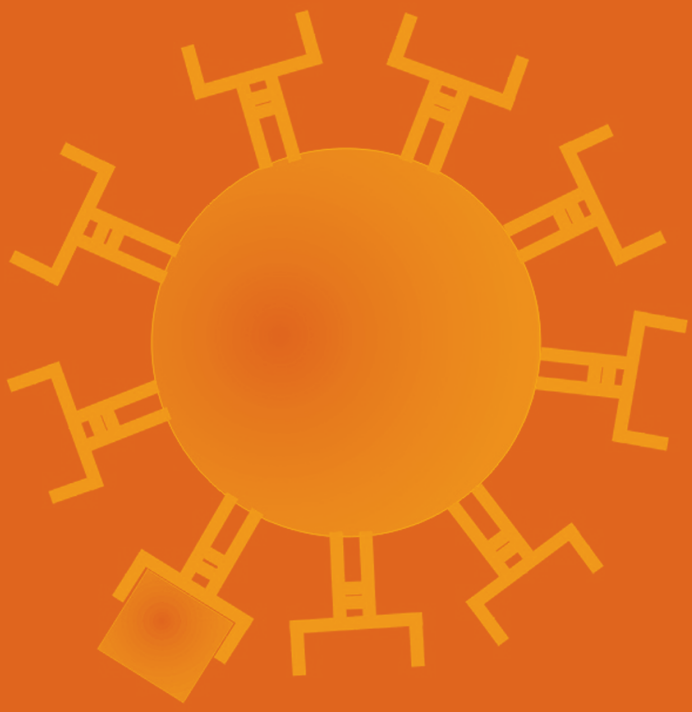
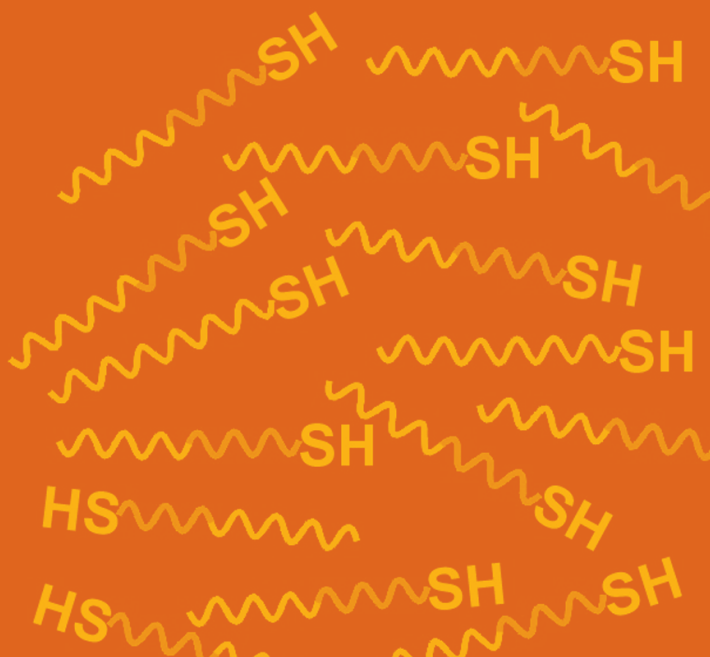
PSA



Barcode DNA
Detection



DNA-Functionalized Gold Nanoparticles as Probes in Biomolecule Detection Assays

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Abstract

Oligonucleotide-functionalized gold nanoparticles (DNA-Au NPs) find ubiquitous use in various biomolecular detection schemes due to their unique chemical and physical properties. This study investigates the effects of nanoparticle size and concentration of salt in solution on the DNA loading of the Au NP surface. The DNA loading studies are performed by functionalization of the Au NPs with fluorophore-labeled DNA. The Au NPs are further utilized for the detection of prostate specific antigen (PSA) using a highly sensitive bio-barcode assay. The amplification of the signal in the bio-barcode assay is proportional to the number of barcode strands per particle. Therefore, the determination of the barcode oligonucleotide loading on the Au NPs at different conditions is a crucial step in the optimization of the bio-barcode assay.

Introduction

Gold was the first metal transformed into a colloidal state, as reported by Michael Faraday in 1857.[1] As described by Faraday in his famous report,[1] "a beautiful ruby fluid" formed upon reduction of aqueous gold chloride with phosphorous dissolved in carbon disulfide. Today, there are a variety of methods to synthesize aqueous gold nanoparticles, and Au NPs have been the focus of extensive research.[2,3] DNA-Au NPs represent a two-component system comprised of oligonucleotide strands, with nanoparticles as their scaffolds. DNA-Au NPs are often used as probes in various biodetection assays[2] and as key building blocks in several assembly schemes that take advantage of the inherent recognition properties of DNA and the chemically programmable, sequence-specific hybridization properties of nucleic acids.[4] DNA-Au NPs have unique characteristics including high stability, low toxicity, cooperative binding properties, sharp melting profiles, unique optical properties, catalytic properties, chemical tailorability, and the ability to support multiple chemical functionalities on their surfaces.[5,6,7] Each of these properties gives the DNA-Au NP a demonstrated advantage in selectivity and sensitivity over conventional probes (e.g., fluorophore). However, the specific properties of oligonucleotide-modified nanoparticles will depend on several factors, such as the size of the particle used as a scaffold, the salt concentration of the solution, and the specific sequence design of the oligonucleotides.[5,6]

The goals of this research are to (1) investigate the optimal conditions for functionalization of Au NPs with oligonucleotides; and (2) utilize the Au NPs as probes to detect low concentrations of prostate specific antigen (PSA) in a highly sensitive biomolecule detection assay,[8,9] PSA was chosen as the target because of its importance in the detection of prostate cancer. Prostate cancer is the second leading cause of cancer death among American men, and early detection and treatment of prostate cancer could lead to improved survival rates.[10]

Background

The surface coverage of DNA strands on Au NPs can be determined by a fluorescence-based method.[11] Fluorescence is a technique used to determine the concentration of fluorophore-labeled oligonucleotides using a standard calibration curve. Fluorophore-labeled oligonucleotides can be synthesized using a standard phosphoramidite chemistry and fluorophore-labeled phosporamidites.[5] A commonly utilized fluorophore label for DNA is 6-carboxyfluorescein (6-FAM), which is compatible with most fluorescence detection equipment and can be attached to the oligonucleotide at the 5' or 3' end.[6] The concentration of the DNA-Au NPs can be calculated by using UV-vis spectroscopy. The extinction of the solutions at the surface plasmon frequency is measured and compared with the known values for the extinction coefficients at each particle size.[5] The fluorophore-labeled DNA strands are usually released from the Au NPs by a ligand exchange process induced by dithiothreitol (DTT), a disulfide

reducing agent.[6] Mercaptoethanol can also be used to release oligonucleotides from surfaces.[11] In the present study, DTT is employed for the ligand exchange reaction.

Protein targets are commonly detected using the heterogeneous enzyme-linked immunosorbent assays (ELISAs) coupled with optical probes (chemiluminescent or fluorophore).[12] However, ELISAs have slow antibody-antigen binding kinetics, which result in long assay times at low target levels. Additionally, in the case of multiplexed analysis, ELISAs suffer from overlapping spectral features, nonuniform photobleaching rates, false positive signals, and the need for multiple laser excitation sources and complex instrumentation for assay readout.[9,12] Currently PSA can be detected at low-picomolar concentrations using conventional assays.[8]

The bio-barcode assay is based on Au NPs functionalized both with oligonucleotides and a target recognition element (an antibody for proteins and a portion of the barcode for nucleic acids). This makes the bio-barcode assay a powerful amplification and detection system for proteins 8 and nucleic acids.[13] Indeed, it allows one to detect nucleic acid targets with sensitivity of polymerase chain reaction (PCR) and proteins targets at limits of detection that are four to six orders of magnitude lower than ELISAs (depending on capture antibody and background signal). In the assay, the barcode strands are used as a surrogate target and as a means of amplification. For each target binding event, a multitude of barcode DNA strands is released, which amplifies the signal. This makes the determination of the barcode oligonucleotide loading on the Au NPs under different conditions a crucial step in optimization.

Approach

DNA Loading Study

Functionalization of Au NPs with DNA
For the investigation of the particle size effect on the DNA loading, Au NPs with average diameters of 15, 60, and 100 nm were studied (Au colloids were purchased from Ted Pella, Inc., and 5'-thiol-modified and 3'-fluorophore-labeled oligonucleotides were obtained from Integrated DNA Technologies.) The DNA functionalization of the Au NPs was performed following a previously published procedure with a few modifications.[5] DNA, freshly deprotected by dithiothreitol (DTT, Pierce) and purified by a NAP-5 column (GE Healthcare), was added to each Au colloid (final oligonucleotide concentration is ~ 3 μM), and the solution was brought to desired concentrations of NaCl (0.1, 0.3 and 0.5 M) in phosphate buffer (10 mM phosphate, 0.01% SDS, pH 7.4) through a stepwise process over a 24-hour period. The excess DNA was removed by repeated centrifugation and washing with a buffer solution (the same concentration of NaCl, 10 mM phosphate, 0.005% Tween 20, pH 7.4).

Fluorophore-Labeled DNA Coverage Study
The fluorophore-labeled DNA strands were released from the Au NPs by a ligand exchange process induced by DTT (final DTT concentration was 0.5 M). After DTT addition, the solutions were incubated at 50° C for 5 min and held at room temperature for 1 hr. The solutions were centrifuged at 10,000 rpm for 15 min to isolate the Au NPs, and the supernatants containing the released fluorophore-labeled DNA strands were collected for the fluorescence analyses.

A series of dilutions (0, 1, 5, 10, 25, 50, 75 and 100 nM) of the fluorophore-labeled DNA were prepared in 0.5 M DTT solution in phosphate buffer (0.5 M NaCl, 10 mM phosphate, 0.005% Tween 20, pH 7.4) to obtain a standard calibration curve. The fluorescence of the DNA samples was measured in a 96-well plate format using Gemini EM fluorescence/Chemiluminescence Plate Reader (Molecular Devices). The number of DNA strands per particle was calculated from the measured fluorescence intensity and the standard calibration curve.

The Bio-barcode Assay
The bio-barcode assay utilizes two sets of probes – magnetic microparticle (MMP) probes and 30 nm Au NP probes cofunctionalized with an antibody (monoclonal or polyclonal) that recognizes the target protein and barcode oligonucleotides as surrogates for the target antigen. The target antigen is captured in solution by the MMP probes conjugated with a monoclonal antibody specific for an epitope of the target antigen different from the one recognized by the Au NP probe. The target MMP complexes are then sandwiched by an Au NP probe with an antibody that can bind to the target in a different region than the MMP. These complexes are isolated with a magnetic field and washed, and the barcode strands are released by a ligand exchange process induced by the addition of DTT (Figure 1). The barcode strands are then identified by the chip-based scanometric method.[7]

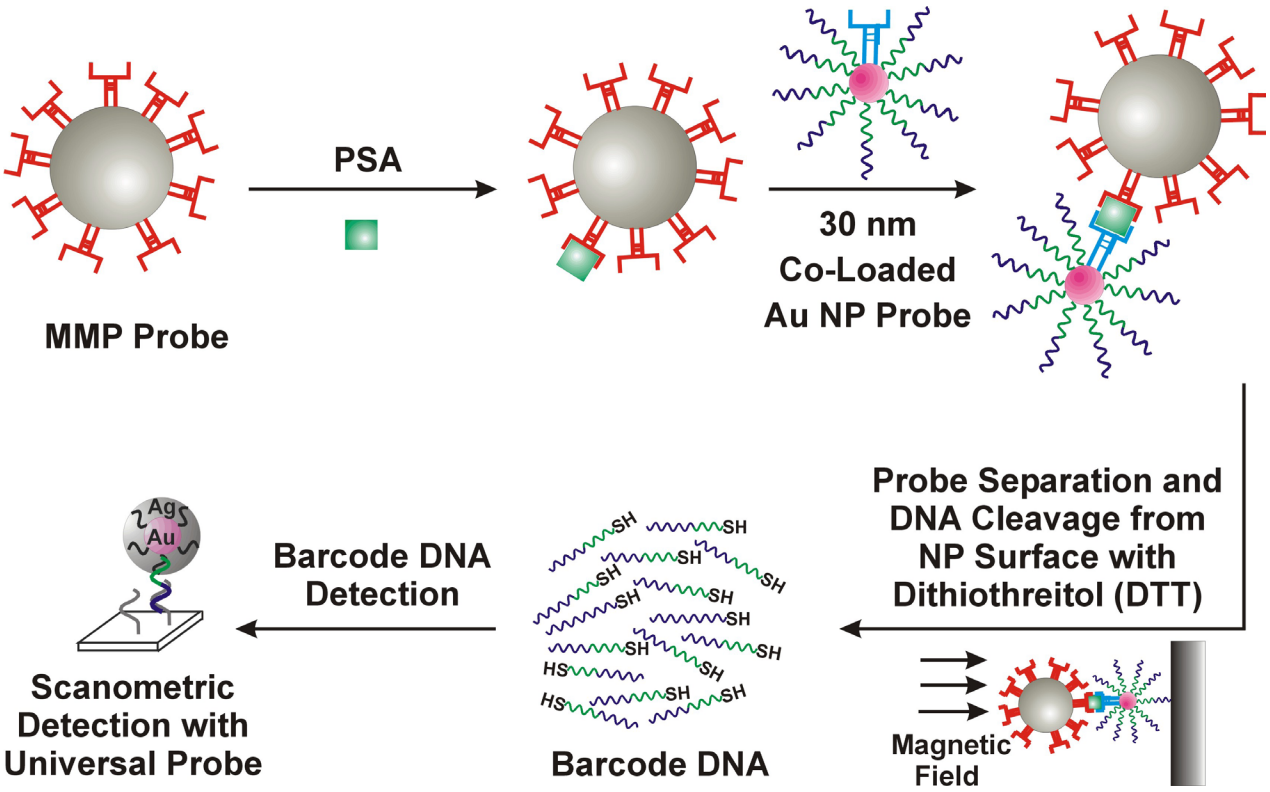


Figure 1: Schematic representation of the bio-barcode assay used for PSA detection.

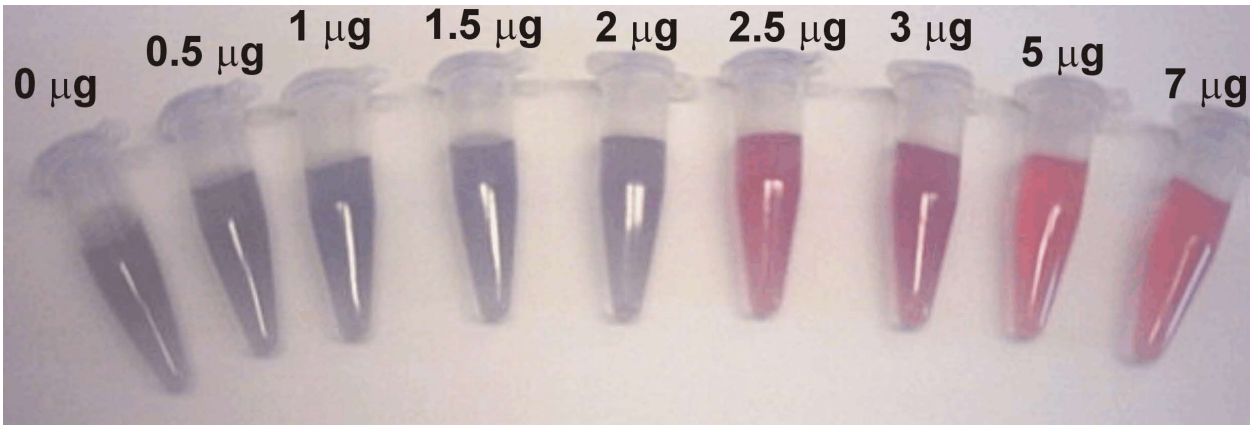


Figure 2: 30 nm Au colloids with variable amounts of polyclonal anti-PSA antibody. The color of the colloids changes from red to blue upon NaCl addition as the amount of Ab is decreased.

MMP Probe Preparation
Tosyl-functionalized MMPs (1 mm diameter, Invitrogen) were covalently linked to the monoclonal anti-PSA antibody at 37° C in borate buffer solution at pH=9.5. The MMPs were passivated with bovine serum albumin (BSA) by the addition of blocking buffer (0.15 M NaCl, 10 mM phosphate, 0.025% Tween 20, 0.5% BSA, pH=7.4). The passivation step proceeded for 24 hrs under vortex at 1,400 rpm at 37° C. The MMP probes were washed three times with storage buffer (0.15 M NaCl, 10 mM phosphate, 0.025% Tween 20, 0.1% BSA, pH=7.4), resuspended in the storage buffer at 10 mg/mL final concentration, and stored at 4° C.

Determination of the Optimal Amount of Anti-PSA Polyclonal Antibody for the Functionalization of 30 nm Coloaded Au NP Probes
To determine the amount of polyclonal anti-PSA antibody for the functionalization of the 30 nm Au NPs, several Au colloidal solutions were incubated with different amounts of antibody. Each solution was then salted to 0.2 M NaCl concentration. Figure 2 illustrates the color change that occurs after the addition of NaCl, which reflects the amount of polyclonal anti-PSA antibody in the system. This color change was used to qualitatively determine the optimal amount of antibody. The NP stability increases as the amount of protein is increased. Simultaneously, the Au NPs have to be further loaded with barcode DNA sequences. To estimate the DNA loading on the 30 nm Au NP probes at different antibody amounts, the Au NPs were functionalized with fluorophore-labeled barcode DNA strands (Figure 3). Based on the results shown in Figure 2 and Figure 3, the 30 nm Au NPs were functionalized with polyclonal anti-PSA antibody at a concentration of 2 mg/mL Au colloid.

30 nm Coloaded Au NP Probe Preparation
Gold nanoparticles were purchased from Ted Pella, Inc., with an average diameter of 30 nm. Prior to functionalization, the pH of the Au colloid was adjusted to 9.2 using 0.2 M NaOH. First, the 30 nm Au particles were functionalized with antibodies by adding the antibody at 0.1 mg/mL starting concentration to the Au colloid and incubating the solution for 30 min at 10° C under slow vortex. The final concentration of the antibody in the Au colloid was 2 mg/mL. Next, freshly deprotected thiol-functionalized barcode DNA (from Integrated DNA Technologies, Inc.) was added to the Au colloid (final concentration ~ 3 μM), and the solution were shaken gently for 5 min

at 10° C. The colloid was buffered to 10 mM phosphate concentration, pH=7.2 including 0.02% Tween 20 (Sigma-Aldrich), and the NaCl concentration was brought to 0.15 M in one step under vortex. The solution was incubated for 30 min at 10° C, purified from excess DNA by repeated centrifugation at 10,000 rpm at 10° C for 10 min, and washed with 0.15 M NaCl, 0.025% Tween 20, 1% BSA, 10 mM phosphate buffer, pH=7.2 (assay buffer). Finally, the Au NP probe was redispersed in assay buffer at a concentration of ~ 6 nM. The exact Au nanoparticle concentration was determined by reading the extinction of the colloid at 530 nm. The molar extinction coefficient for 30 nm particles at 530 nm is 3 x 10^9 $M^{-1}cm^{-1}$ (calculated from the measured UV-Vis extinction of a colloid with a known particle concentration). The Au NP probes were stored at 4° C prior to use.

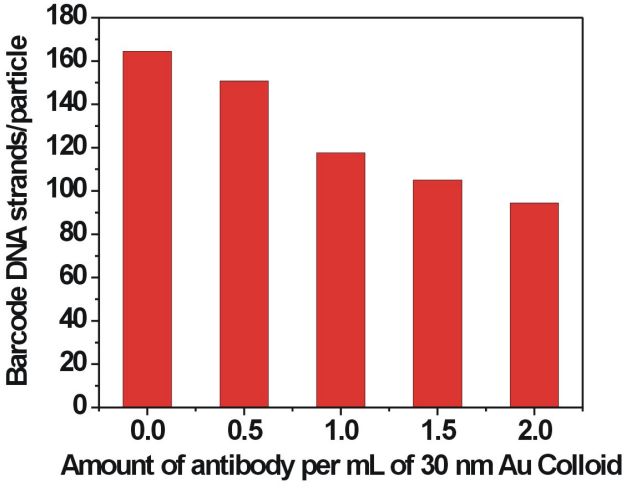


Figure 3: DNA loading depending on the amount of polyclonal anti-PSA antibody per mL of 30 nm Au colloid.

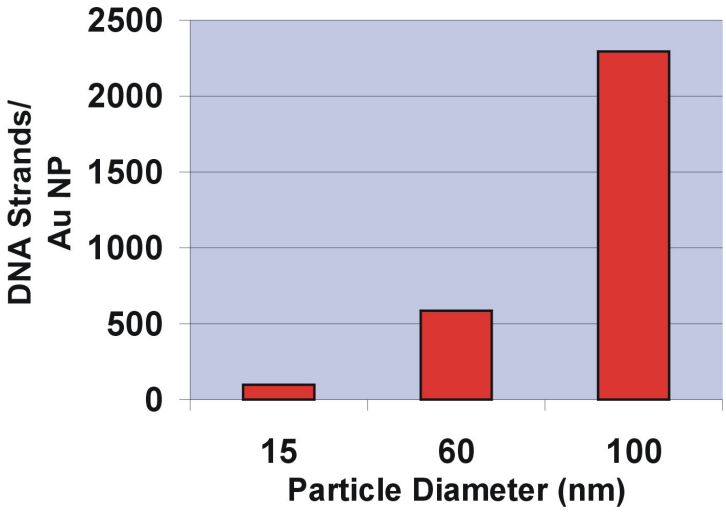


Figure 4: The number of DNA strands per particle with Au NPs of different sizes.

Results and Discussion

DNA Loading Study

Au NP Size Effect

It has been suggested that Au NPs functionalized with DNA strands, have almost consistent surface coverage.[6] This research project began with the hypothesis that if the particle size increased, the number of DNA strands on the particle surface would increase proportionally. To test this hypothesis, the number of DNA strands per particle was studied using a series of particles of different sizes (15, 60, and 100 nm) and fluorophore-labeled DNA. As shown in Figure 4, the number of DNA strands calculated by fluorescence measurement increased significantly as the particle size

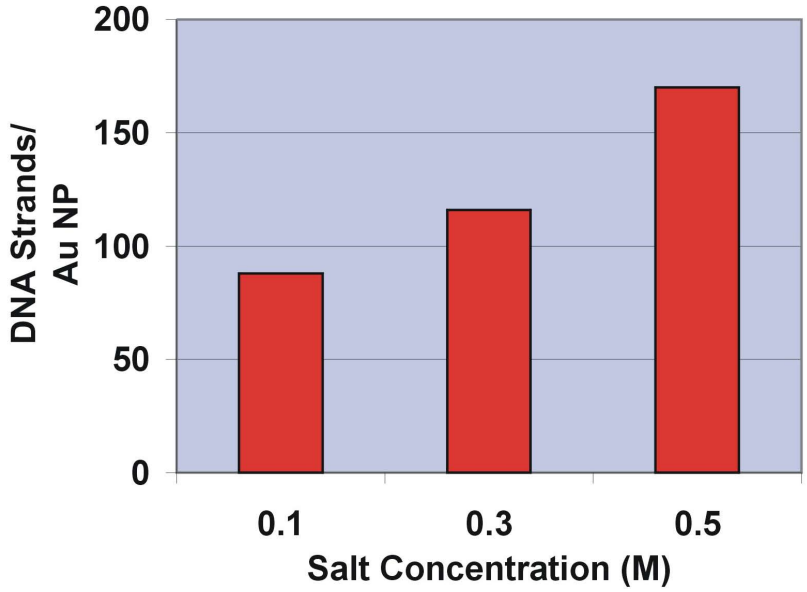
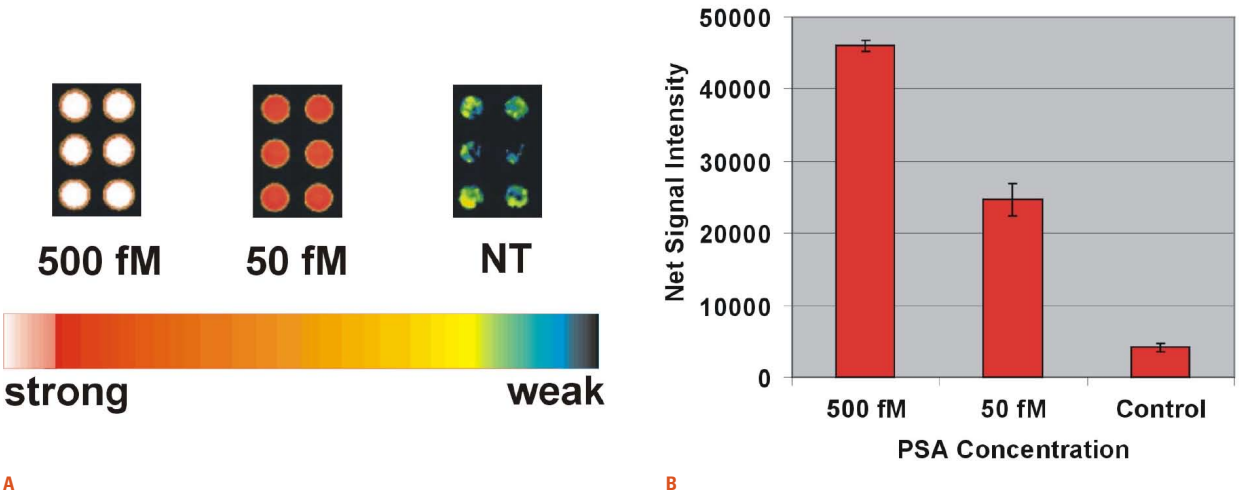


Figure 5: The number of DNA strands per particle with different salt concentrations.



A B

Figure 6: PSA detection with the bio-barcode assay. (A) Scanometric identification of the released barcodes from the 30 nm Au NP probes. (B) Quantitative analysis of the net signal intensity. The gray scale images from the Verigene ID system are converted into a color scale using GenePix Pro 6 software (Molecular Devices).

increased (96 strands for 15 nm Au NP, 586 strands for 60 nm Au NP, and 2,294 strands for 100 nm).

Salt Concentration Effect
Addition of salt during the functionalization of the Au NPs with oligonucleotides minimizes the electrostatic repulsion between the negatively charged oligonucleotide strands on the nanoparticle surface and allows more oligonucleotides to approach the particle surface.[5] This effect increases as the salt concentration increases. The number of DNA strands from the DNA-Au NPs (15 nm in diameter) functionalized at three different salt concentrations (0.1, 0.3, and 0.5 M) was investigated, and it was observed that as the salt concentration increased, so did the DNA loading. The number of DNA strands per particle is 88 for 0.1 M NaCl, 116 for 0.3 M NaCl, and 170 for 0.5 M NaCl (Figure 5).

PSA Detection Using 30 nm Coloaded Au NP Probes and the Bio-barcode Assay
To evaluate the sensitivity of the system, tests were run at different concentrations of PSA and a control sample that contained no PSA. The barcode strands were released with DTT and detected with the chip-based scanometric method. The signal is identified with a Verigene ID system (Nanosphere, Inc.) that measures the scattered light from the developed spots after silver amplification. The scanometric identification of the barcode oligonucleotides is shown in Figure 6A. The quantification of the net signal intensities is given in Figure 6B. The gray scale images from the Verigene ID system are converted into a color scale using GenePix Pro 6 software (Molecular Devices). White/red color indicates a positive signal, and yellow/black color indicates negative signal. The net signal intensity at 50 fM PSA is clearly distinguishable from the control signal intensity (Figure 6B), indicating that PSA can be detected at 50 fM concentration under the bio-barcode assay conditions used in the present work.

Conclusions

This research studied the effect of particle size and salt concentration on the amount of DNA loading of DNA-Au NPs. Larger particles have more DNA strands on them due to the larger surface area. The addition of salt during the functionalization process induces higher loading of DNA strands due to reduced electrostatic repulsion. Au NPs particles cofunctionalized with both antibody and DNA (coloaded Au NP probes) were investigated to determine the appropriate amount of antibody to use with the bio-barcode assay. The detection of PSA using bio-barcode assay was demonstrated with a detection limit of 50 fM. Further studies including using the bio-barcode assay with larger Au NP probes to increase the sensitivity are currently under investigation.

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