Video Article

Design and Development of Aptamer–Gold Nanoparticle Based Colorimetric Assays for In-the-field Applications

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Abstract

The design and development of an aptamer–gold nanoparticle (AuNP) colorimetric assay for the detection of small molecules for in-the-field applications was examined. Target selective AuNP based color assays have been developed in controlled proof-of-concept laboratory settings. However, these schemes have not been exerted to a point of failure to determine their practical use beyond laboratory settings. This work describes a generic approach to design, develop, and troubleshoot an aptamer-AuNP colorimetric assay for small molecule analytes and using the assay for in-the-field settings. The assay is advantageous because adsorbed aptamers passivate the nanoparticle surfaces and provide a means to reduce and eliminate false positive responses to non-target analytes. Transitioning this system to practical uses required defining not only the shelf-life of the aptamer-AuNP assay, but establishing methods and procedures for extending the long-term storage capabilities. Also, one of the recognized concerns with colorimetric readout is the burden placed on analysts to accurately identify often subtle changes in color. To lessen the responsibility on analysts in the field, a color analysis protocol was designed to perform the color identification duties without the need for performing this task on laboratory grade equipment. The method for creating and testing the data analysis protocol is described. However to understand and influence the design of adsorbed aptamer assays, the interactions associated with the aptamer, target, and AuNPs require further study. The knowledge gained could lead to tailoring aptamers for improved functionality.

Video Link

The video component of this article can be found at https://www.jove.com/video/54063/

Introduction

Colorimetry is one of the oldest techniques used in analytical chemistry. For this technique, a qualitative or quantitative determination of the analyte is made based on the production of a colored compound[1]. Typically, color assays use reagents that experience a color shift in the presence of the analyte species, which results in an observable or detectable color change in the visible light spectrum. Colorimetry has been used in the detection of targets ranging from atoms, ions, and small molecules to complex biological molecules such as deoxyribonucleic acids (DNA), peptides, and proteins[2-4]. For the past two decades, nanomaterials have revolutionized the field of detection assays, particularly with color based assays[5-6]. Combining the unique chemical and physical properties of nanomaterials with a target selective recognition element, such as antibodies, oligonucleotide aptamers or peptide aptamers, has led to the resurgence in the design and development of colorimetric detection assays[7].

Metal nanoparticles have a demonstrated size-dependent color change property, which has been exploited in the design of numerous colorimetric assays. Gold nanoparticles (AuNPs) are of particular interest due to a distinctive red-to-blue color shift, when the dispersed solution of particles is induced to aggregate[8], typically through the precise addition of salt. The ability to control the transition from the dispersed (red) to the aggregated (blue) states has led to the creation of colorimetric sensors for ionic, small molecular, peptide, protein, and cellular targets[2-4,9]. Many of these sensors employ aptamers as the target recognition motif.

Aptamers are DNA or ribonucleic acid (RNA) molecules selected from a random pool of 10^{12}-10^{15} different sequences[10-11]. The selection process identifies target recognition elements with binding affinities in the low nanomolar regime, and the systematic evolution of ligands by exponential enrichment (SELEX) is the most commonly known process[12-13]. Advantages of oligonucleotide based aptamers for sensing applications include ease of synthesis, controllable chemical modification, and chemical stability[14-15].

One approach to creating a colorimetric assay combines nanomaterials with recognition elements, consists of combining these two species through the physical adsorption of DNA-aptamer molecules to AuNP surfaces. Through target-aptamer binding, the aptamer experiences a structural change[16-18] that alters the interaction of the aptamer with the AuNP surface, which leads to an inducible red-to-blue color response[19]

with the addition of salt. This astonishing feature of AuNPs provides an observable colorimetric response mechanism for aptamer-based devices that can be used to design colorimetric assays for different analytes.

Color assays designed using non-covalent, physically adsorbed DNA aptamers on AuNP surfaces have the stigma of being a weak sensor platform due to issues with robustness, a propensity for failure outside of controlled laboratory settings, and the lack of information available for use in practical settings. However, the aptamer-AuNP based colorimetric assay was of interest because of the simplicity of operation and observable color response. The goal of this work is to provide a protocol for the design, development, operation, reduction of surface related false positive response, and long-term storage of DNA-AuNP based colorimetric assays using cocaine as the representative analyte. Furthermore, we proposed this adsorbed aptamer assay approach (**Figure 1**) as being advantageous due to simplicity and ease of use that resulted in fewer steps than the conventional approach for these aptamer-AuNP assays. For this assay, the aptamer was first added to the AuNPs, which were allowed to adsorb to the surface for an extended period of time. An additional advantage to this approach was the reduction of response to non-target analyte molecules related to AuNP surface interactions. However, the reduction in false positive response was at the expense of assay sensitivity. Therefore a balance between surface protection and analyte accessibility is necessary to maintain proper assay function. Moreover, a major defect of analyzing color assays through means other than with instrumentation is that the results are often subjective and open to interpretation from analyst-to-analyst, particularly when trying to differentiate subtle differences in color. Conversely, there are a number of issues with making laboratory based instrumentation usable outside the lab, such as availability of power, practicality with portability, *etc.* In this work, a color analysis protocol was developed for more portability and to eliminate some of the guesswork commonly associated with color based assay interpretation[20-21]. Compared to previous approaches, this effort strived to push these assays to their limits for applications beyond laboratory settings.

Protocol

1. Synthesis via Citrate Reduction of Gold Nanoparticles (AuNP) and Characterization

1. Clean an Erlenmeyer flask (500 ml) and large stir bar with 5 ml concentrated nitric acid and 15 ml concentrated hydrochloric acid in chemical safety hood.
    1. Wet the entire surface of the flask with the acid wash, rinse the flask with nuclease free water, and allow the flask to dry.
2. Add 100 ml of 1 mM gold(III) chloride; use a sheet of aluminum foil to cover the top of the acid cleaned Erlenmeyer flask and heat with continuous stirring on a hot plate until boiling.
3. Add 10 ml of 38.8 mM sodium citrate. The color will change from clear/gray, to dark blue/black, and finally dark red over several minutes. Continue stirring with the heat off for 10 min.
4. Allow the AuNP suspension to cool to room temperature and add 110 µl of diethylpyrocarbonate (DEPC) with continuous stirring.
5. Cover the entire flask with aluminum foil and allow the DEPC treatment to incubate overnight. Store all AuNPs in the dark, in amber storage containers or covered with aluminum foil.
6. Autoclave the AuNP suspension, cool to room temperature, and filter through a 0.22 µm pore cellulose acetate membrane. Store the filtered, autoclaved AuNP stock solution in the dark at 4 °C.
   NOTE: Treatment with DEPC, sterilization via autoclave, and storage at 4 °C will improve the shelf-life of the aptamer-AuNP assay. Storage in this manner will allow for the assay to remain functional for more than 2 months.
7. Calculate the AuNP concentration by obtaining Ultra Violet-Visible absorption at 520 nm, and use the extinction coefficient (Ɛ) 2.4 x 10^8 L mol^{-1} cm^{-1} with Beer's Law by calculating concentration (c). The concentration was determined to be 10 nM with a size of 15 nm determined by dynamic light scattering.
   NOTE: Concentrations will vary from batch-to-batch. Dilute the AuNP stocks with nuclease free water as necessary to maintain the desired 10 nM AuNP suspension.

2. DNA-aptamer, Buffer, Solution, and Assay Preparation

1. Purchase or synthesize the following cocaine binding aptamer sequences using standard phosphoramidite chemistry[22]:
   MN4[19]: 5'-GGC GAC AAG GAA AAT CCT TCA ACG AAG TGG GTC GCC-3'
   MN6[19]: 5'-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-3'
2. Purify the aptamers using standard desalting[23]. Reconstitute oligonucleotides in nuclease-free water at either 100 µM or 1 mM stock solutions. Aliquot and store at -20 °C for several months.
3. Purchase or prepare stocks of sterile 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 100 mM magnesium chloride ($MgCl_2$), and 1 M sodium chloride (NaCl).
4. Prepare 50 ml of buffer in nuclease-free water with concentrations of 20 mM HEPES, 2 mM $MgCl_2$, pH 7.4 and store at room temperature for months.
5. Incubate the DNA with the stock AuNP solution (10 nM) for 3-4 hr at room temperature and protect from the light. Vary the volume of AuNPs as desired to provide enough sample for the tests to be performed (2.5-7.5 ml).
    1. Here, use loading densities of 90, 120, 150, and 180 DNA molecules/AuNP in this work. Vary the volume and concentrations of DNA accordingly. Tune the DNA coverage to reduce unintended AuNP surface related color responses of non-target analytes.
       NOTE: Increasing the DNA coverage will reduce the assay sensitivity. The loading densities are calculated from knowing the concentration of the AuNP stock, and calculating the total number of AuNPs present in the volume desired for use in experiments. If the actual coverage densities are desired, protocols exist to obtain those values[7]. The DNA coverage was determined by using a 50 kDa molecular weight cutoff spin column to separate the AuNP bound DNA from the free DNA. The AuNPs are too large to pass through the spin column, while the free DNA will pass through easily. The next step is to quantify the free DNA collected using absorbance measurements or a single stranded DNA fluorescent dye.

6. Add an equal volume of 20 mM HEPES, 2 mM $MgCl_2$, pH 7.4 buffer and place the sample at 4 °C in the dark overnight. The aptamer-AuNP assay was in a 10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 (assay buffer).

3. Salt Titration and Assay Setup

1. Determine the initial salt concentration needed to induce the assay color response by salt titration with the assay blank. Add 20 µl of methanol (blank) to 180 µl aliquots of aptamer-AuNP assay in a 96-well plate. Titrate the samples with increasing volumes of stock NaCl solution (1 M or 2 M) and determine the equivalence point (**Figure 2**).
   NOTE: The assay can be scaled to smaller volumes by keeping the ratio of methanol blank (or dissolved analyte) to aptamer-AuNP assay the same.
   1. Here, determine the NaCl volume needed to cause the slightest color change by visual observation. The starting concentration for the assay was 75 mM and 130 mM for MN4 and MN6, respectively at a 60 DNA molecule/AuNP coverage density.
      NOTE: For a quantitative determination for the initial salt concentration, the midpoint of the titration curve serves as a good starting point. Also, concentrations used will vary based on the aptamer, DNA coverage densities, from day-to-day performance, and batch-to-batch.
2. Optimizing the assay response, add 20 µl of analyte molecules diluted in methanol to 180 µl aliquots of aptamer-AuNP assay in a 96-well plate at room temperature. Immediately add the NaCl concentration determined in the previous step to initiate the assay color response.
   NOTE: Utilize a multichannel pipette to perform multiple experiments simultaneously.
3. Obtain the largest color change possible by increasing or decreasing the NaCl concentration, and comparing the target response to the blank response. Use the NaCl concentration that provides the largest response difference.
4. Observe or measure the assay response 150 sec following NaCl addition. Analyze the absorbance at 650 nm and 530 nm using a spectrometer or obtain a digital camera photo of the assay response (see section 4 for the photo analysis protocol).
   NOTE: A microplate reader was used in obtaining the measurements for this work.
5. Plot the results as the ratio of absorbance obtained at 650 nm and 530 nm (E_{650}/E_{530}) as a function of analyte concentration. Normalize the assay response to the blank signal as was done in this work.

4. Photo and Digital Image Color Analysis Protocol Analysis

1. Prepare the assay samples as described (sections 3.2-3.3). Place the 96-well plate on a transilluminator.
   NOTE: A standard laboratory transilluminator is typically too bright to obtain usable digital images for this analysis. These transilluminators cause regularly spaced "dark lines" to appear in the digital image due to the intensity of the light source. Making a transilluminator from a light emitting diode (LED) based light box and a piece of opaque plastic works well.
2. Obtain photos of the 96-well plate at 150 sec after NaCl addition, import the images into image analysis software, and calculate the average red, green and blue (RGB) values, using an incremental averaging technique, as shown in equation 1[24]:

   (1) $$AVE_N = AVE_{N-1} + \left(\frac{Pixel_N - AVE_{N-1}}{N}\right)$$
3. Convert the RGB values from standard RGB (sRGB) color space to the chromaticity diagram (CIExyY) color space, using the following equations[24]:

   (2) $$C_{linear} = \left(\frac{C_{srgb} + 0.055}{1.055}\right)^{2.4}$$

   (3) $$\begin{vmatrix} X \\ Y \\ Z \end{vmatrix} = \begin{vmatrix} 0.4124 & 0.3576 & 0.1805 \\ 0.2126 & 0.7152 & 0.0722 \\ 0.0193 & 0.1192 & 0.9505 \end{vmatrix} \begin{vmatrix} R_{linear} \\ G_{linear} \\ B_{linear} \end{vmatrix}$$

   (4) $$x = \frac{X}{X+Y+Z} \quad y = \frac{Y}{X+Y+Z}$$
4. Convert the exponential RGB values to linear RGB values using equation 2. The matrix specified in equation 3 is used to calculate the X, Y and Z values of the CIE color space[24].
5. Calculate the x and y chromaticity values using equation 4 representing the average color of the pixels in the area selected for analysis[24].
6. Perform the analysis in every well and plot the chromaticity values to generate a calibration curve (**Figure 4**). Obtain the standard error by analyzing the color of different areas of the same well.

5. Freezing Aptamer-AuNP Assay for Long-term Storage

1. Prepare the aptamer-AuNP assay components as described in sections 2.4 and 2.5. Make separate solutions containing 1 g/ml trehalose and 1 g/ml sucrose in nuclease-free water to make the Cryogen Solution.
   NOTE: High concentrations of trehalose and sucrose were used to reduce the dilution factor when preparing the assay for freezing. Heat the sugar solutions on a hot plate in a beaker of water to thoroughly dissolve the sugars before use.
2. Make a solution that contains 19.2 mg/ml trehalose and 4.8 mg/ml sucrose with the 60 MN4-DNA/AuNP assay at a final volume of 200 µl in 1.5 ml microcentrifuge tubes. Final cryogen solution concentrations will vary with DNA coverage.
   NOTE: Samples to be frozen should not exceed 300 µl. Larger volumes may not freeze properly.
3. Flash freeze the samples using a -146 °C freezer or in liquid nitrogen. Store the samples frozen until use. Storage can be in a -80 °C or -20 °C once flash freezing is complete.
4. For this work, leave the samples in the -146 °C freezer overnight and then transfer to a -20 °C freezer for long-term storage.
   NOTE: Flash freezing can cause the aptamer-AuNPs to aggregate. Test the integrity of the freezing process by monitoring the absorbance profile and comparing it to an unfrozen sample. If aggregation is observed, increase the amount of cryogen solution to compensate for this issue.

5. Thaw the samples at room temperature and use only enough samples as necessary for experimentation. Obtain absorbance spectra of thawed samples and compare to the baseline spectra of an unfrozen cryogen solution treated sample. Measure the absorbance from 400 nm to 700 nm.
6. Perform the salt titration (section 3.1), test the assay (section 3.2), and plot the results (sections 3.3 and 3.4) as described previously.

Representative Results

The primary objective of this work was to develop and investigate the stability and robustness of aptamer based AuNP colorimetric assays for use in the field. As highlighted in a previous publication, two distinct strategies for creating the assay were investigated[7]. The assays were termed the Free Aptamer Assay and the Adsorbed Aptamer Assay. The Adsorbed Aptamer Assay was more appealing for the purposes of a fieldable detection assay (**Figure 1**).

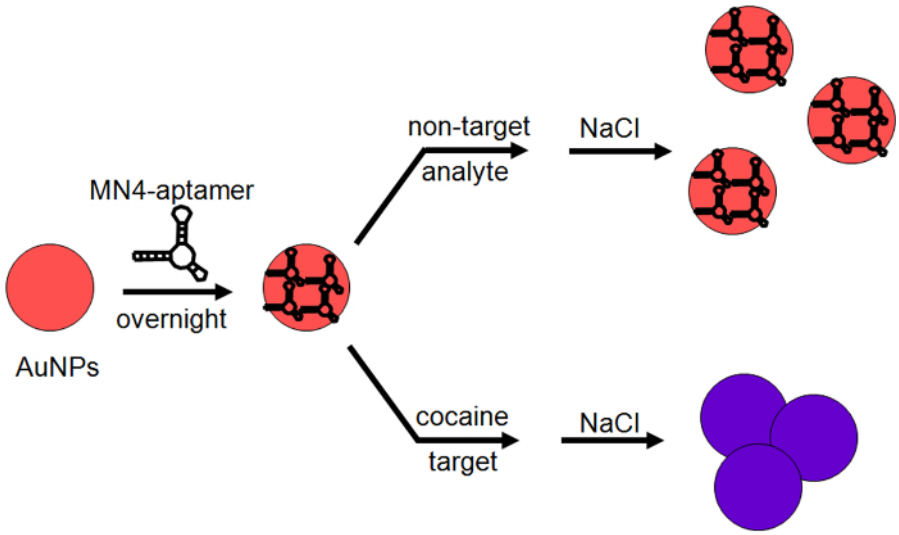

Figure 1. Schematic representation of the Adsorbed Aptamer Assay. The aptamer was mixed with AuNPs and incubated overnight; analyte molecules dissolved in methanol were added to the Absorbed Aptamer Assay, immediately followed by the addition of sodium chloride (NaCl) to induce AuNP aggregation when the target was added. Please click here to view a larger version of this figure.

This was due to the reduction in false positive rates to nontarget analytes, relative simplicity, and ease of use of the Adsorbed Aptamer Assay. To prepare the Adsorbed Aptamer Assay, the DNA-aptamer was mixed with AuNPs and incubated overnight in the assay buffer. This allowed the aptamer to readily adsorb on the AuNP surface, which reduced the susceptibility of the assay for false positive responses to nontarget analyte molecules such as masking or cutting agents. However, only the preformed structure of the MN4 cocaine aptamer was active in the presence of cocaine (target) in this assay design. The assay was employed by adding analyte solutions followed by the immediate addition of the appropriate concentration of NaCl. Salt solutions were used to initiate the observable and measurable color change of the assay. The assay was executed within 2-3 min. after the addition of the analyte solution.

A critical action in the execution of these physically adsorbed DNA based colorimetric assays is the induced color response, through the addition of the appropriate concentration of salt. The addition of salts are known to destabilize AuNP suspensions resulting in aggregation of the particles by masking the negative charges of the citrate layer that stabilize the AuNPs, and then diminishes the interparticle electrostatic interactions. The result is the observable red-to-blue color change. The same effect is observed with DNA treated AuNPs. In the case of DNA-aptamers, analyst determine the concentration of salt necessary to cause minimal observable AuNP destabilization (blue coloration). With the addition of target, the stabilization of the AuNPs by the DNA-aptamer is significantly reduced resulting in an observable, measurable target dose response color change. This color change can only be perceived with the addition of the appropriate predetermined amount of salt.

Equally crucial is the process in which the appropriate concentration of salt was determined. This was accomplished by performing a titration curve by adding increasing concentrations of sodium chloride solution to a series of assay blanks (**Figure 2**).

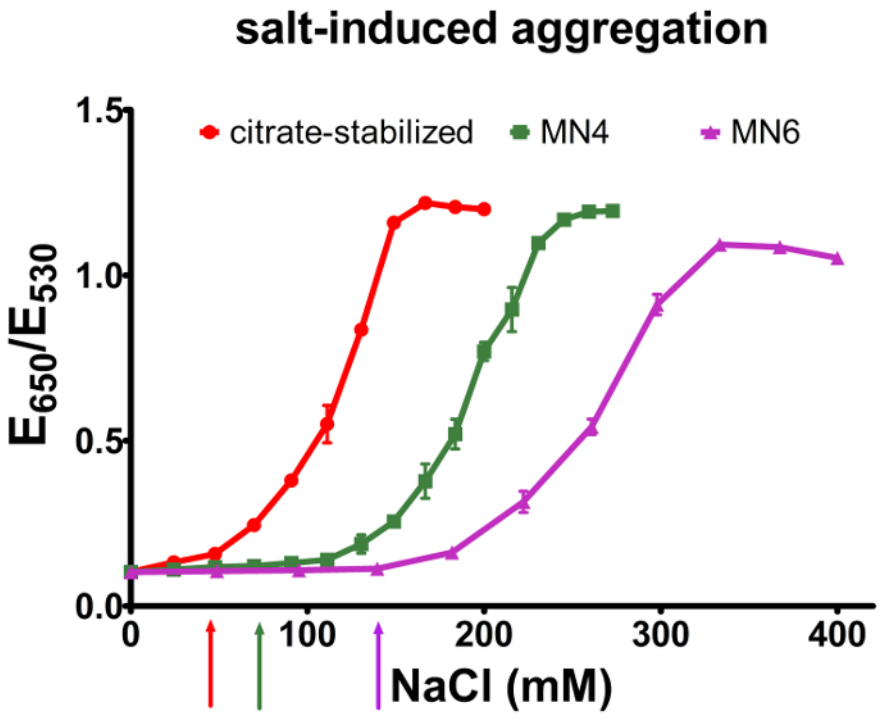

Figure 2. Salt-induced titration curve. Citrate-stabilized (red), MN4 cocaine binding aptamer (green), and MN6 cocaine binding aptamer (purple) treated AuNP samples were titrated with sodium chloride (NaCl). The initial salt concentrations obtained visually were indicated with corresponding colored arrows for each curve. Error bars are defined by the standard deviation in triplicate measurements. Please click here to view a larger version of this figure.

Here, the initial salt concentration used was determined by visual inspection, and was taken to be the salt concentration that caused the slightest blue coloration observable with the assay blank. However for a more quantitative approach, researchers new to this area of research can use the midpoint of the titration equivalence point as a starting salt concentration. Beyond this point the color response begins to increase rapidly. Furthermore, the salt concentration used in the execution of the assay was adjusted to maximize the color difference between the assay blank and cocaine responses. This was accomplished by increasing and decreasing salt concentration from the amount determined by titration. The salt titration procedure was performed daily to account for day-to-day variability with the assay. The batch variability in salt concentration needed for the assay was no more than 20%. **Figure 2** has three distinct AuNP samples, citrate-stabilized (no DNA), MN4 (DNA-aptamer stabilized), and MN6 (DNA-aptamer stabilized). The DNA coverage for the MN4 and MN6 samples were 60 DNA molecules/AuNP, and each titration curve has a different titration equivalence point and midpoint based on the level of stability provided by the surface treatment. Citrate provided little stability, MN4 (double stranded like structure) provided more stability and MN6 (single stranded like structure) provided the most stability to the AuNPs. The initial salt concentrations used in this work were determined to be ~50 mM, ~90 mM, and ~130 mM visually. The trend agrees with conventional understanding of DNA structure and its stabilizing effect on AuNPs[24-25]. When performing this test visually, the assay blank showed a slight blue coloration with the salt concentrations as identified in **Figure 2** with arrows, which are close to the midpoints as discussed. The salt concentrations prior to theses points provide little or no observable color change, and beyond these points the color increases rapidly. For this work, the initial salt concentration was determined visually and then fine-tuned using microplate reader measurement comparisons of assay blank and cocaine samples.

With the Free Aptamer Assay approach, false positive responses were an issue. Surface interactions of analyte molecules are one source for this issue, as described in a previous publication[7]. To prevent the unintentional color responses due to nonspecific AuNP surface interactions, the DNA coverage densities of the target binding aptamer was controlled (**Figure 3**).

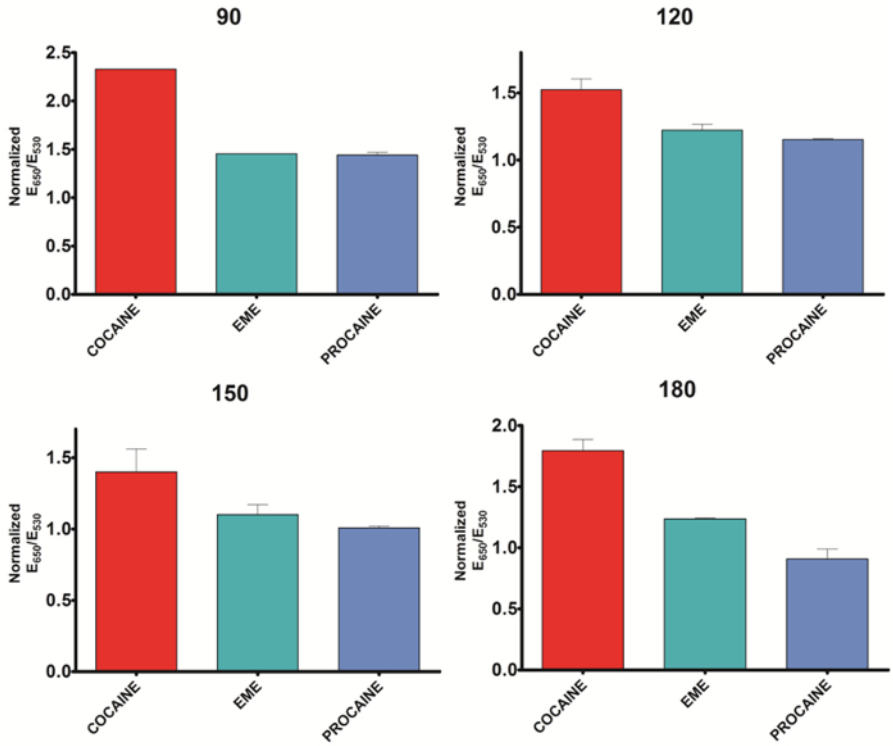

Figure 3. Adsorbed aptamer assay response with varying DNA coverage density. Aggregation response to cocaine (red, target), EME (green, control), and procaine (blue), a AuNP surface active molecule, for the 90, 120, 150, and 180 DNA/AuNP coverage densities were displayed. All the samples analyzed were dissolved in methanol at 1 mg/ml. Error bars are defined by the standard deviation in triplicate measurements. Please click here to view a larger version of this figure.

In general, the false positive response rate of nontarget analyte molecules due to AuNP interactions was reduced with increasing DNA coverage densities. However, the assay sensitivity was reduced as a consequence of increased DNA coverage. In this work, DNA densities of 60, 90, 120, 150, 180, and 300 DNA/AuNP were investigated. Densities of 60 and 300 were extensively described in previous work[7]. **Figure 3** represents additional DNA densities studied. Procaine was one of the more highly surface active nontarget analytes surveyed. For each of the individual DNA coverages, the assay response was maximized by adjusting the salt concentration as described. In general as the DNA coverage increases, the response difference between control (EME) and cocaine response decreases. Similarly, the assay response in the presence of procaine decreases to background levels with increasing coverages. The 180 DNA/AuNP density eliminated surface related false positive response for procaine, while retaining a high target response. This assessment describes a process for tuning these color assays for reducing surface related false positive responses, while preserving target response as much as possible. Improved sensitivities can be achieved through the reduction of DNA coverage. However, false positive responses might become an issue depending on the application.

Colorimetric assays are commonly used for quick, simple often presumptive qualitative and even quantitative tests. Common issues with colorimetric determinations are the subjective nature of the color discernment, particularly with subtle and borderline color differences. The judgement calls which must be made by the analyst can result in misinterpretation of the data. Measurements on laboratory equipment reduce the uncertainty and indecision associated with assessing the assay results. However in this work, the intent was to provide a field ready assay that provided immediate outcomes to the analyst. As such, a photo image analysis technique was established that provided a more decisive color result (**Figure 4**).

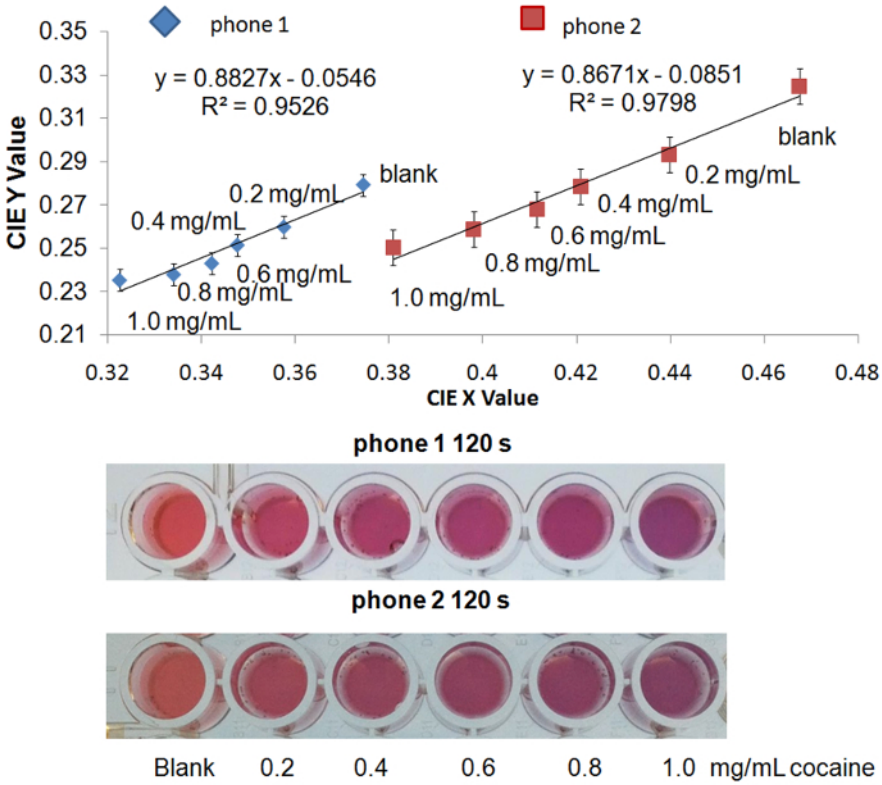

Figure 4. Calibration curve plotted from digital photo image analysis. Digital images of the Adsorbed Aptamer Assay with increasing concentrations of cocaine were obtained using smartphones (bottom), Phone 1 (upper) and Phone 2 (lower) images. These images were used to generate the chromaticity values that were plotted as calibration curves (top), Phone 1 (blue diamonds) and Phone 2 (red squares). Error bars are defined by the standard deviation in triplicate measurements. Please click here to view a larger version of this figure.

With increasing concentrations of cocaine, the assay color resulted in an increasing blue color. Digital photos of calibration curves were obtained using two different smartphones, and imported to a standard laptop computer for analysis using ImageJ software. The chromaticity values were used to plot the calibration curves as shown in **Figure 4**. The image analysis provided a linear response to increasing cocaine concentration with both sets of smartphone images as indicated by the R^2 values. This method was transitioned to a Smart-device app to aid the analyst in the field. A detailed evaluation of the app was performed in a previous publication[7]. The app eliminated much of the uncertainty and indecision associated with subtle color change results of the lowest cocaine concentrations.

Long-term storage of physically adsorbed DNA assays of this type have not been studied in great detail and one of the goals of this work was to find conditions to extend the assay shelf-life. Treatment of the assay components for storage at 4 °C was detailed in a previous publication[6]. For this work, lyophilization of the prepared assay components was considered for long-term use and storage of the assay. Lyophilizing the assay components has the distinct advantage of keeping and storing the samples at ambient temperature, which would eliminate the need for a refrigerator or freezer. The primary step in the lyophilizing process is to first freeze the sample. To check the AuNP samples survive the freezing process, perform a comparison of the absorbance spectra of the assay prior to freezing to that of the thawed sample. The scans should match exactly. If the samples do not survive the freezing process, the thawed sample spectrum will have increased absorbance in the region above 525 nm. This indicates that the AuNPs aggregated during freezing and the thawed sample is compromised. The viability of the thawed assay was tested with cocaine and EME (**Figure 5**).

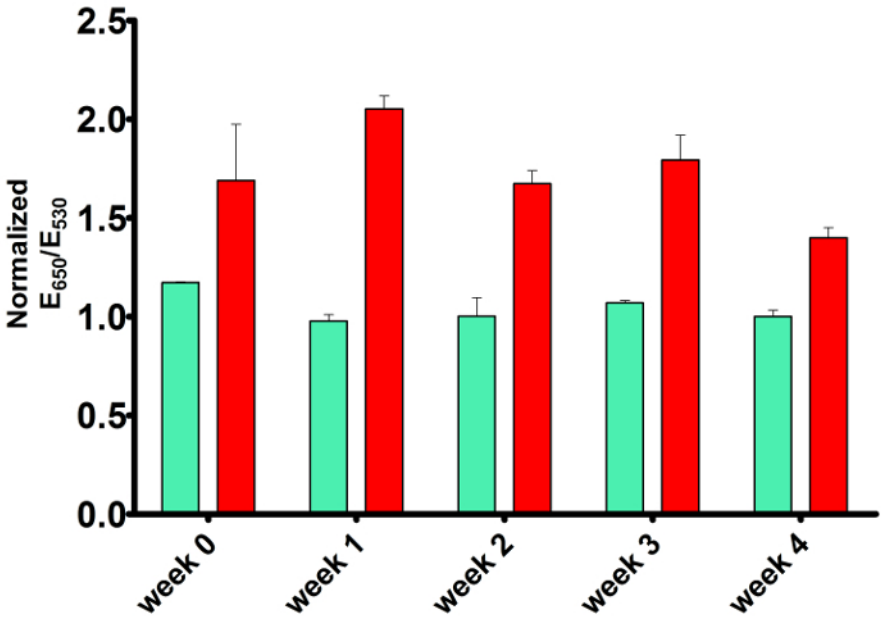

Figure 5. Adsorbed Aptamer Assay response shelf-life study. Quantification of the assay response to EME (green, control), and cocaine (red, target) performed with frozen and then thawed Adsorbed Aptamer Assay samples during a period of four weeks. Error bars are defined by the standard deviation in triplicate measurements. Please click here to view a larger version of this figure.

The thawed assay blank samples remained at the same value over the study period as samples that were made and used immediately (no storage). The responses of the cocaine and EME samples were consistent with typical responses observed with samples made and used immediately (no storage). The frozen samples were monitored for the period of 4 weeks with no change to the assay performance as compared to samples stored at 4 °C over the same period of time[7] or to assay samples made and used immediately. Cocaine responses were relatively constant during the 4 week period, which was also observed for the 4 °C samples[7]. The decrease in EME signal from week 1 to week 2 is often observed with room temperature and 4 °C storage as well. A phenomenon we attributed to maturation of the assay during the first week. This approach augmented the options available for long-term storage of the adsorbed DNA colorimetric assays, and provided a gateway to additional long-term storage options, namely lyophilization.

Discussion

Over the past decade, nanoparticle based colorimetric assays have been developed for the detection of targets include small molecules, DNA, proteins, and cells[2-4]. Assays that use DNA-aptamers with nanoparticles have been gaining interest. Typically, these colorimetric assays are performed by mixing the DNA-aptamer with analyte molecules followed by addition to AuNPs[9-10]. However, these assays have been utilized in proof-of-concept demonstrations with controlled laboratory settings and with limited, selected controls. Recent advancements to transition this technology into the field have been made[7]. In this approach, the DNA-aptamer was adsorbed to AuNP surfaces prior to addition of the analyte molecules to be tested (**Figure 1**). The development of either aptamer–gold nanoparticle based colorimetric assays requires optimization at various stages of the fabrication/analysis process. Thus, those new to the discipline must be aware of the nuances associated with refining and troubleshooting these assays to be successful.

The adsorbed aptamer–gold nanoparticle assays require careful optimization for each aptamer/target pair. However, following the steps explained here offers a consistent protocol to performing optimization of these colorimetric assays. The determination and adjustment of the salt concentration that results in the maximum observable or measurable color change between the target and assay blank is one of the more critical optimization steps (**Figures 2** and **3**). For some aptamer/target pairs, we have observed that using high salt concentrations near the end point of the titration curve have resulted in a blue-to-red color shift[18]. This indicates a stabilization of the AuNPs by the aptamer upon the addition of the target and salt.

Other factors that might influence the assay performance include buffer components and concentrations, amount of DNA-aptamer coverage, solvents used, temperature, aptamer sequence and structure, target-assay incubation time (time when target is added to the assay, prior to salt addition), and color development time (time needed for the color to develop, after salt addition). A 10 mM HEPES, 1 mM $MgCl_2$ pH 7.4 buffer was the assay buffer of choice in many of the target/aptamer pairs used in our work. However, this buffer may not be ideal for all aptamers. To obtain proper folding of the aptamer, the assay buffer components may need to be tailored, and kept as close as possible to the composition used in the aptamer selection buffer. When utilizing a buffer with AuNPs, the buffer components and concentrations must be considered, particularly with ionic substances. High concentrations of ionic compounds could cause premature aggregation of the AuNPs. DNA/AuNP coverage was demonstrated in **Figure 3**. As the DNA coverage increases from 90-180 DNA/AuNP, the target analyte response decreased causing reduced assay sensitivity. Therefore, a trade-off is needed that depends on each aptamer, due to its particular folding and degree of interactions with the AuNP surface.

Additionally, solvents needed to dissolve analyte molecules may result in unintended aggregation of the AuNPs. Water, assay buffer, and methanol have all been used without issue. Used without dilution, acetonitrile and dimethylsulfoxide (DMSO) adsorb to the AuNP surface causing unintended aggregation. Acetonitrile was problematic even at dilutions of less than 1% (final volume). Solvents should be tested with AuNPs before use with the colorimetric assay. The temperature at which the assay is performed can have an impact on whether a response is observed with the assay. This has more to do with the aptamer structure and melting temperature, or stability of the aptamer structure at a given temperature, than with the nanoparticles. In our work, we have determined that there is a delicate balance between having the aptamer in a preformed structure with the DNA in a folded form, and also not completely in a single stranded structure. This is the case for the adsorbed aptamer assay form of these colorimetric assays (**Figure 1**). When considering structure, the aptamer sequence must also be contemplated because much of the aptamer structure is the result of the individual oligonucleotide sequences. However, further investigation into this aspect is needed.

In general, the approach we use in developing an aptamer-AuNP based colorimetric assay is the same for all aptamer/target pairs. First, obtain an aptamer for a target of interest. This can be achieved through searching the literature or the selection of an aptamer for a molecule of interest. At this stage, there is no way of knowing whether the aptamer is a good candidate for creating a colorimetric assay. This can only be determined through experimentation. Next, the aptamer is incubated with AuNPs overnight to fabricate the adsorbed aptamer assay (**Figure 1**). The standard approach is to begin testing with 60 aptamers/AuNP; however, multiple coverages are prepared for the next stage of testing. When the assays are ready for testing, perform the titration curve investigations as described (**Figure 2**). The midpoint of the titration curve serves as a reliable starting salt concentration to be used for the target, assay blank, and control tests. The salt concentration is fine-tuned to provide a maximum color difference between the target and assay blank samples. To test the response is real and caused by aptamer-target binding and not due to non-specific target/solvent interactions, perform the assay with controls. Simultaneously, assay color development times are investigated at 5 min intervals. Followed by target-assay incubation times at 5 min intervals, initially 15+ min incubation times are used until this step is optimized. Depending on the results, further optimization of the salt concentration, aptamer coverage, incubation time and color development time may be necessary (**Figure 3**). This approach describes a protocol for the design and development of aptamer-AuNP colorimetric assays for a target/aptamer pair. Further investigations into the aptamer sequence and structural effects on the improvement of adsorbed aptamer colorimetric assays are of great interest. There is a need for understanding the interactions associated with the aptamer, target, and AuNPs. This knowledge could lead to tailoring aptamers for better functionality and even predicting which aptamers will be active in the adsorbed aptamer assay format.

Disclosures

The authors declare they have no competing financial interests.

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