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Colorimetric detection with aptamer-gold nanoparticle conjugates coupled to an android-based color analysis application for use in the field

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ABSTRACT

The feasibility of using aptamer-gold nanoparticle conjugates (Apt-AuNPs) to design colorimetric assays for in the field detection of small molecules was investigated. An assay to detect cocaine was designed using two clones of a known cocaine-binding aptamer. The assay was based on the AuNPs difference in affinity for single-stranded DNA (non-binding) and double stranded DNA (target bound). In the first assay, a commonly used design was followed, in which the aptamer and target were incubated to allow binding followed by exposure to the AuNPs. Interactions between the non-bound analytes and the AuNPs surface resulted in a number of false positives. The assay was redesigned by incubating the AuNPs and the aptamer prior to target addition to passivate the AuNPs surface. The adsorbed aptamer was able to bind the target while preventing non-specific interactions. The assay was validated with a number of masking and cutting agents and other controlled substances showing minimal false positives. Studies to improve the assay performance in the field were performed, showing that assay activity could be preserved for up to 2 months. To facilitate the assay analysis, an android application for automatic colorimetric characterization was developed. The application was validated by challenging the assay with cocaine standards of different concentrations, and comparing the results to a conventional plate reader, showing outstanding agreement. Finally, the rapid identification of cocaine in mixtures mimicking street samples was demonstrated. This work established that Apt-AuNPs can be used to design robust assays to be used in the field.

1. Introduction

Nanomaterials have revolutionized the area of sensor development, due to the ability to tune their properties based on the materials size and shape, offering multiple means to design sensing platforms for different applications [1,2]. The combination of biorecognition elements with nanomaterials allows the design of hybrid sensing materials with great target specificity and selectivity and tunable outputs including colorimetric, electronic and others [3]. Despite all these advantages and the great promise of nano-bio sensing systems, the feasibility of using these sensing platforms in-the-field remains to be demonstrated. In the case of colorimetric sensors, the integration of automated analysis tools that simplify data analysis for on-the-spot decision making is critical to help in the transition of this technology to the field.

Metal nanoparticles show size-dependent color change that can be exploited for the design of colorimetric detection systems. In particular, gold nanoparticles (AuNPs) show a red color when dispersed in solution, but change to blue when their aggregation is triggered [4]. The transition from a dispersed state (red) to an aggregated state (blue) has been engineered to result as a response to an external stimulus, creating colorimetric sensors for a myriad of targets [5].

Aptamer based sensing platforms for the detection of small molecules, peptides, proteins, and cells have gained a great deal of interest due to their high selectivity and sensitivity [6,7]. Aptamers are DNA/RNA molecules that bind a target with binding affinities

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in the nanomolar level [8,9]. Advantages specific to aptamers in sensing applications include ease of synthesis, controllable chemical modification, and chemical stability [10,11]. These properties make aptamers ideal candidates for recognition elements in selective target sensors [12].

The use of aptamers to control the aggregation state of AuNPs due to target binding has been realized for a number of aptamer/target pairs [13–15]. In general, an aptamer binding its target involves a conformational change that affects the AuNPs stability, and promotes AuNP aggregation. Despite the many reports on the use of these bio-nanomaterials for sensing different targets, no information is available on the feasibility of their use in field-related settings. Most of the sensing approaches reported are "proof-of-concept" studies that work in a laboratory setting and with a small set of hand-picked controls.

The main objective of this work was to investigate whether aptamers can be used in combination with AuNPs (Apt-AuNPs) to design a colorimetric assay to detect an analyte in field-relevant settings. To show the applicability of our approach to field-testing, an assay to detect cocaine was investigated. For this work, two well-characterized clones of a cocaine-binding aptamer (CBA) were used [16]. Both clones are known to bind cocaine, although with different affinities, but have not been demonstrated in AuNPs colorimetric sensing. In the case of Apt-AuNPs, a conformational change by the aptamer when binding its target has been proposed as critical to produce a colorimetric outcome [17]. The aptamer clone MN6 (short cocaine-binding aptamer) has been identified to transition from an open conformation (single stranded-like) to a more closed conformation (double stranded-like, with extensive base pairing) upon cocaine binding. On the other hand, the MN4 aptamer clone (long cocaine-binding aptamer) was demonstrated to maintain a closed structure in the presence or absence of cocaine, offering no significant conformational change upon binding. These properties of the CBAs were taken into consideration when designing the cocaine detection assays. Importantly, it was demonstrated that the assay design could be optimized to minimize responses to non-target analytes. It was proposed that these false positive responses were due to interactions between the analytes and the AuNPs′ surface which affected their stability, resulting in a color change similar to the one observed by target binding by the aptamer. These unwanted interactions were eliminated by protecting the AuNPs with the DNA prior to exposure to test analytes, resulting in an assay with minimal response to typical cutting agents, filler compounds or other controlled substances. Additionally, conditions that included storing the Apt-AuNPs for prolonged periods of time while maintaining the assay activity were investigated and optimized. Finally, to prevent ambiguities related to color determination by naked-eye analysis of the assay, a fully functional android-based color analysis application (app) was developed. This app was designed to analyze the color of an unknown sample and compared it to the color of positive references, to determine if the substance tested positive for cocaine.

2. Experimental section

2.1. Materials

All the materials were purchased as analytical grade and used without further purification from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Standard 1 mg/mL methanol solutions of ecgonine methyl ester hydrochloride (EME) and cocaine hydrochloride were purchased from Lipomed Inc. (Cambridge, MA). DNAse/RNAse Free water and Quant-iT® OliGreen® ssDNA Reagent and Kit were purchased from Invitrogen Corporation (Carlsbad, CA). HEPES buffer was purchased from Amresco Inc. (Solon, OH). The substances listed in Table 2 were obtained from the United States Army Criminal Investigation Laboratory (USACIL-Forest Park, GA). Centrifuge tubes were purchased from Axygen, Inc. (Union City, CA). The aptamers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNA batches were purified by standard desalting. The aptamer sequences were 5′-GGC GAC AAG GAA AAT CCT TCA ACG AAG TGG GTC GCC-3′ (long cocaine-binding aptamer, MN4) and 5′-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-3′ (short cocaine-binding aptamer, MN6).

2.2. Gold nanoparticle synthesis

A 100 mL solution of 1 mM $HAuCl_4$ was heated and refluxed at its boiling point with stirring, and 10 mL of a 38.8 mM sodium citrate solution was added. The solution continued to boil with mixing for 20–25 min. The sample was cooled to room temperature, kept in the dark, and filtered using a 250 mL Corning Filter System with 0.22 μm pore size. The sample was stored at RT wrapped in aluminum foil until used.

The AuNPs were determined to be 15 nm in diameter by dynamic light scattering (DLS). DLS measurements were performed in a Zetasizer Nano-instrument (Malvern Instruments, Westborough, MA) utilized in backscatter mode (173° detection angle) with the temperature set at 20.0 ± 0.1 °C. The final AuNPs concentration was determined to be 10 nM using a Cary 300 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA) based on the extinction measured at 520 nm, using $\varepsilon = 2.4 \times 10^8$ $Lmol^{-1}$ cm^{-1}.

2.3. Free aptamer cocaine colorimetric assay

The AuNPs were diluted with buffer by mixing 7.5 mL of AuNPs synthesized in sodium citrate, as described, with 7.5 mL of 20 mM HEPES 2 mM $MgCl_2$ pH 7.4 buffer in a 50 mL conical vial, and stored in the dark at room temperature, overnight. Test solutions were prepared by adding 1.8 μL of 30 μM CBA, dissolved in water, to 18.2 μL of the desired concentration of the analyte dissolved in buffer. After incubation for 30 min, these solutions were added to 180 μL of the buffer treated AuNPs, and incubated for 30 min. Under these conditions, the aptamers were loaded at a ratio of 60 DNA/AuNPs. Finally, NaCl was added to promote AuNPs aggregation followed by quantification of the color change. Typical NaCl concentrations used in the assay were 25 mM and 40 mM for MN4 and MN6, respectively. The exact NaCl concentration varied slightly on a daily basis and was adjusted to obtain consistent background values.

The assay response was monitored by measuring the AuNPs extinction at 650 nm (aggregated AuNPs, blue color) and 530 nm (dispersed AuNPs, red color) 150 s after NaCl addition in a Spectra Max M5 plate reader (Molecular Devices, Sunnyvale, CA). The data was plotted as the ratio of aggregated-to-dispersed AuNPs (E_{650}/E_{530}, blue/red), and a calibration curve was obtained. The standard deviation (SD) was used to represent the error in the measurements of four replicates. The detection limit (DL) was calculated as three times SD above the blank for all calibration curves. The DL was used as a measure of the sensitivity of the assay. The data was normalized to the blank for ease of comparison and to account for batch variations. Images were taken 150 s after the addition of NaCl with a Canon SLR camera and a smartphone.

2.4. Adsorbed aptamer cocaine colorimetric assay

The CBAs were adsorbed on the AuNPs simply by mixing them and allowing them to incubate in buffer. Two different DNA densities were used 60 and 300 DNA/AuNPs, CBA-60-AuNPs and

CBA-300-AuNPs, respectively. The CBA-60-AuNPs were prepared by mixing 7.5 mL of AuNPs synthesized in sodium citrate, as described, with 45 μL of 100 μM CBA, dissolved in water, in a 50 mL conical tube. The aptamer was allowed to incubate with the AuNPs for 4–5 h, and 7.5 mL of 20 mM HEPES 2 mM $MgCl_2$ pH 7.4 buffer was added to the vial. To obtain the CBA-300-AuNPs (300 DNA/AuNP), 22.5 μL of 1000 μM CBA dissolved in water was added to the AuNPs and the same procedure as the MN4–60-AuNPs was followed. The assay was performed by adding 20 μL aliquots of the substances to be tested, prepared in buffer or methanol, to 180 μL of the AuNP assay. After incubation for 1 min, NaCl was added to induce the color change. Typical NaCl concentrations used in the assay were 75 mM and 130 mM for MN4 and MN6, respectively. The exact NaCl concentration varied slightly on a daily basis and was adjusted to obtain consistent background values. Data treatment and collection was the same as for the Free Aptamer Cocaine Colorimetric Assay.

2.5. Aptamer surface coverage

The amounts of aptamer associated with AuNPs were determined using the Quant-iT® OliGreen® ssDNA Reagent and Kit. The fluorescence intensity of different dye-aptamers mixtures were measured and fit to a calibration curve following the manufacturer specifications. For the Adsorbed Aptamer Assay, 500 μL of the CBA-AuNPs were centrifuged using an Amicon® filter with MWCO of 50 kDa. The MWs of the MN4-CBA and MN6-CBA were 11,128.3 Da and 9288.1 Da, respectively. The free DNA passed through the membrane, and the filtrate was used to determine the amount of free DNA (or DNA not associate with the AuNPs). The Free Aptamer Assay samples were treated the same way but the volumes were adjusted to account for dilutions introduced when preparing the assay. The measurements were performed in duplicate.

2.6. Transmission Electron Microscopy

Transmission Electron Microscope (TEM) images were taken with a Hitachi H-7600 TEM (Hitachi Ltd., Tokyo, Japan) on 200 mesh copper grids with a Formvar Carbon film (Electron Microscopy Sciences, Hatfield, PA).

2.7. Shelf-life Studies

The AuNPs were treated with diethylpyrocarbonate (DEPC) overnight and autoclaved before performing the assay characterization studies reported through the text. To characterize the effect of this treatment on the assay shelf-life, the colorimetric response of these AuNPs was compared with samples that were not treated post-synthesis (no DPEC-autoclave). In one case the non-treated AuNPs were stored at RT in the dark after the DNA was added, and a different batch was stored at 4 °C after aptamer addition. The assay response was monitored at different time points for a period of up to 4 months. The assay was tested with 20 μL aliquots of methanol, EME and cocaine dissolved in methanol at a final concentration of 300 μM.

2.8. Android-based color analysis application

The color analysis app was designed to run on the android operating system versions 2.3 and above. The app analyzed the colors of a picture of the assay run with a sample of interest and compared it to the colors of known cocaine standards to determine if the assay is positive or negative. For this analysis, the app establishes a "positive color domain", through user input, by first analyzing the lowest positive concentration (LPC) in the assay, and then the highest possible cocaine concentration (HPC, 1 mg/mL cocaine standard) tested in a 1 mg/mL unknown sample.

To characterize the sample's color, the operator selects an area representative of the well using the on-screen "selection square". The application then analyzes every pixel within the selection square and calculates an average red, green, and blue value (RGB). This average was calculated using an incremental averaging technique, as shown in

$$AVE_N = AVE_{N-1} + \left(\frac{Pixel_N - AVE_{N-1}}{N}\right) \quad (1)$$

Once the average RGB value for the color was determined, it was converted from the sRGB color space to the CIExyY color space, considering $AVE_N = C_{srgb}$. This calculation was performed with the following equations

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

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

$$x = \frac{X}{X+Y+Z} \; y = \frac{Y}{X+Y+Z} \quad (4)$$

Eq. (2) was used to convert the exponential RGB values to linear RGB values. The *X*, *Y*, and *Z* values of the CIE color space were then calculated by using the matrix specified by Eq. (3). Finally, the *x* and *y* chromaticity values were calculated using Eq. (4). The *x* and *y* color coordinates obtained from Eq. (4) represented the average color of the pixels within the selection square (CIE *X* and CIE *Y*, respectively). These coordinates are the numerical values that were saved to the database of "positive color" points. After the app was calibrated with the standards, the app evaluated the color of any selected sample and considered any samples showing lighter shades of red than the LPC or darker shades of purple-blue than the HPC as non-cocaine (negative). A sample with color values between the LPC and HPC was considered as cocaine-containing (positive).

3. Results and discussion

3.1. Colorimetric assay characterization and optimization

The main objective of this work was to investigate whether a robust colorimetric assay based on AuNPs and aptamers could be designed to be used in the field. The first detection scheme, the Free Aptamer Cocaine Colorimetric Assay, was designed following literature reports that established that an aptamer could be exposed to its target to promote binding, followed by introduction of AuNPs as a colorimetric probe for the binding event [13,14]. Fig. 1a shows a schematic representation of the assay design. The aptamer was mixed with the target or test-analyte and incubated in binding buffer for 30 min. Subsequently, the AuNPs were introduced followed by 30 min incubation. Finally, a colorimetric change was induced by addition of NaCl. Addition of the salt increased the ionic strength of the media, masking the charges of the DNA adsorbed on the AuNPs, promoting their aggregation. This process resulted in a color change of the suspensions from red to blue. The cocaine assay mechanism was based on the fact that single-stranded DNA protected the AuNPs from salt-induced aggregation due to its ability to adsorb on the AuNPs surface, providing a high density of negative charges. Double-stranded DNA, on the other hand, interacted less strongly with the AuNPs

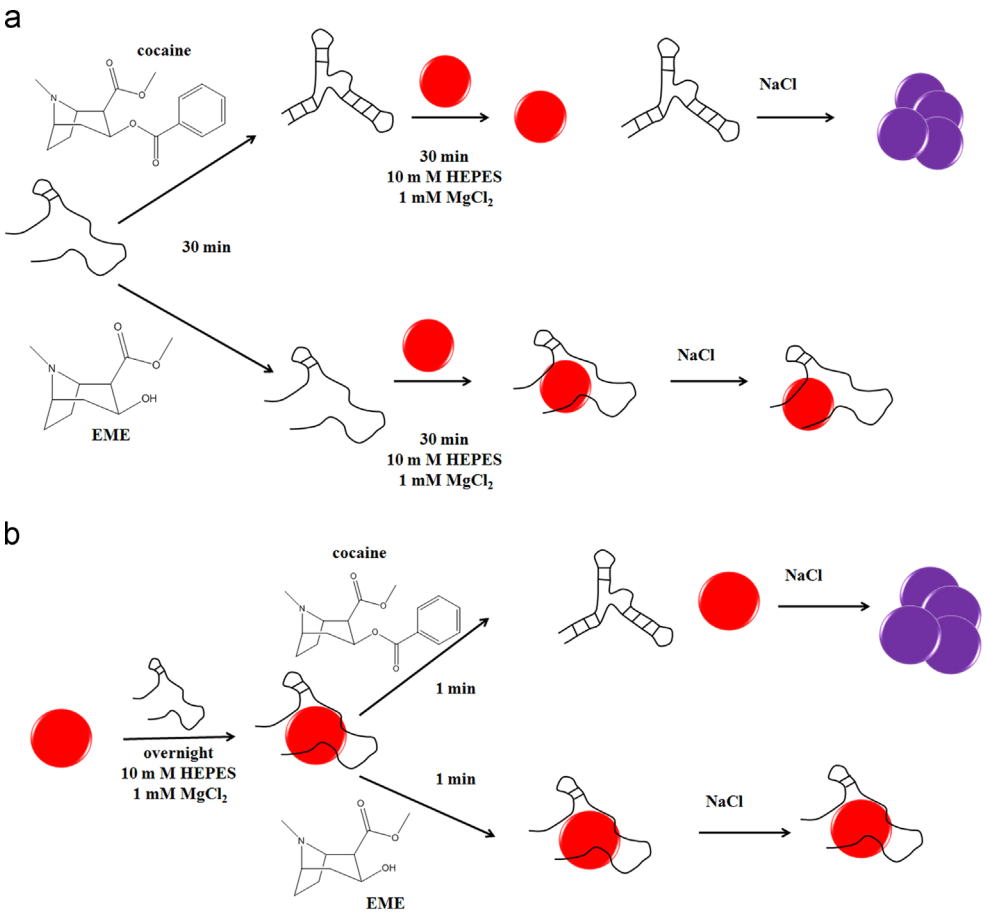


Fig. 1. Schematic representation of the cocaine colorimetric assays. (a) Free Aptamer Assay and (b) Adsorbed Aptamer Assay. In both cases, the response with the MN6 aptamer clone is shown.

surface, due to its more rigid structure, providing little stabilization to the AuNPs [18].

Based on NMR studies [16], the aptamer clone MN6 was expected to go through a conformational change upon cocaine binding, from an open (ss-DNA-like) conformation to a more paired (ds-DNA-like) conformation which translates to a color change from red to blue in the assay when NaCl was added. When a non-target analyte was introduced no major conformational changes were expected, resulting in no color change, as shown in Fig. 1a. On the other hand, MN4 maintained a three-way junction conformation in the presence or absence of cocaine. Due to the lack of change in conformation between the binding and non-binding states, this clone was not expected to result in a significant colorimetric response of the assay [17].

The Free Aptamer Cocaine Colorimetric Assay was tested with both MN4-AuNPs and MN6-AuNPs, using cocaine and EME as a negative control. EME is a structurally related cocaine metabolite, which has been used extensively to study the selectivity of the aptamer over chemically related compounds [17]. As expected, the MN6-AuNPs showed a colorimetric response to cocaine with no response to EME, Fig. 2a. Despite its predicted lack of conformational change, the MN4-AuNPs responded colorimetrically to cocaine with no response to EME, Fig. 2a (the reason for this response is currently under investigation). Fig. 2b shows an image with the typical colors observed in the cocaine colorimetric assay. This data suggested that the interactions between the CBAs and AuNPs were more complex than originally believed and could not be predicted based solely on the aptamer conformation in solution.

A systematic characterization of the assay response was performed. Table S1 shows that MN6 provided a better detection limit, although both aptamer clones showed saturation at similar cocaine concentrations. Quantification of the DNA associated with the AuNPs during the assay showed that, despite mixing the DNA and AuNPs in a 60 to 1 ratio, MN4 had a coating density of only 41 DNA/AuNP while MN6 showed that most of the DNA was associated with the AuNPs after a 30 min adsorption incubation period. This was interpreted as early evidence that the CBAs used here interacted differently with the AuNPs, with MN6 having a larger affinity for the AuNPs′ surface.

The Free Aptamer Cocaine Colorimetric Assay results with cocaine and EME were promising and prompted us to test it with substances relevant to field applications [19,20]. As shown in Fig. 2c, the assay showed no response to typical cutting agents and fillers used in street samples (lactose, sodium bicarbonate, flour, etc.). A large response was observed with some other “over the counter” compounds. For instance, procaine and diphenhydramine (DPHA) resulted in a color change *prior* to NaCl addition. Figure S1 shows the chemical structures of the substances that resulted in some response in the colorimetric assay. None have chemical structures similar to cocaine but contain amino groups that can interact with the AuNPs surface in a similar fashion as the DNA nucleotides do. Interestingly, as shown in Fig. 2c, when AuNPs were exposed to these substances but not to the CBAs, the observed change in color was more dramatic (red bars) than the case when the AuNPs were exposed to MN4 (blue bars), suggesting that the CBAs added stability to the AuNPs and prevented some of the interactions with the analytes. Moreover,

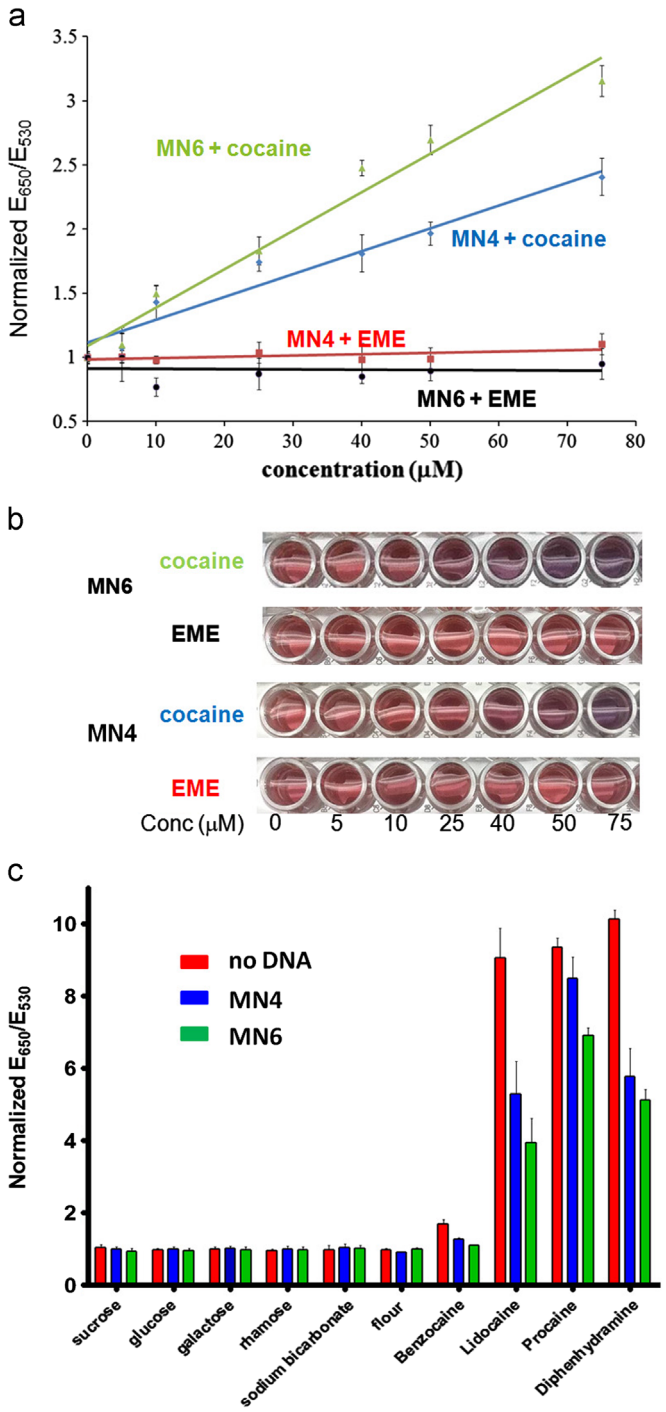


Fig. 2. Free aptamer cocaine colorimetric assay characterization. (a) Quantification of the aggregation response of the assay to cocaine and EME, (b) digital images of the samples analyzed in (a) and (c) aggregation response to typical cutting agents and fillers used in cocaine street samples. All analytes were prepared as 10 mM stocks in buffer. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

MN6, the aptamer with a higher surface affinity (vide supra), showed an even larger stabilization effect (green bars), observed as a lower response intensity of the false positives.

The data from the Free Aptamer Cocaine Colorimetric Assay suggested that the presence of the CBAs helped prevent some of the unwanted interaction between the AuNPs surface and some non-target analytes. To take advantage of these findings, a second assay was designed, the Adsorbed Aptamer Cocaine Colorimetric Assay, as shown in Fig. 1b. In this case, CBA and AuNPs were mixed *prior* to analyte addition, and incubated overnight to allow the aptamer to deposit on the AuNPs surface. Based on the Free Aptamer Assay data (Fig. 2c), this was expected to prevent the AuNPs from interacting with non-cocaine substances. The analytes were added to the Apt-AuNPs followed by NaCl addition after 1 min to promote the color change due to cocaine binding. Fig. 3a shows the assay's quantitative response to cocaine and EME; as shown in Table S2, the DL of this assay was 15 μM. Fig. S2 in the Supplementary information, shows TEM images of MN4-AuNPs challenged with cocaine and EME after NaCl addition, showing typical aggregation in the presence of the target but not when

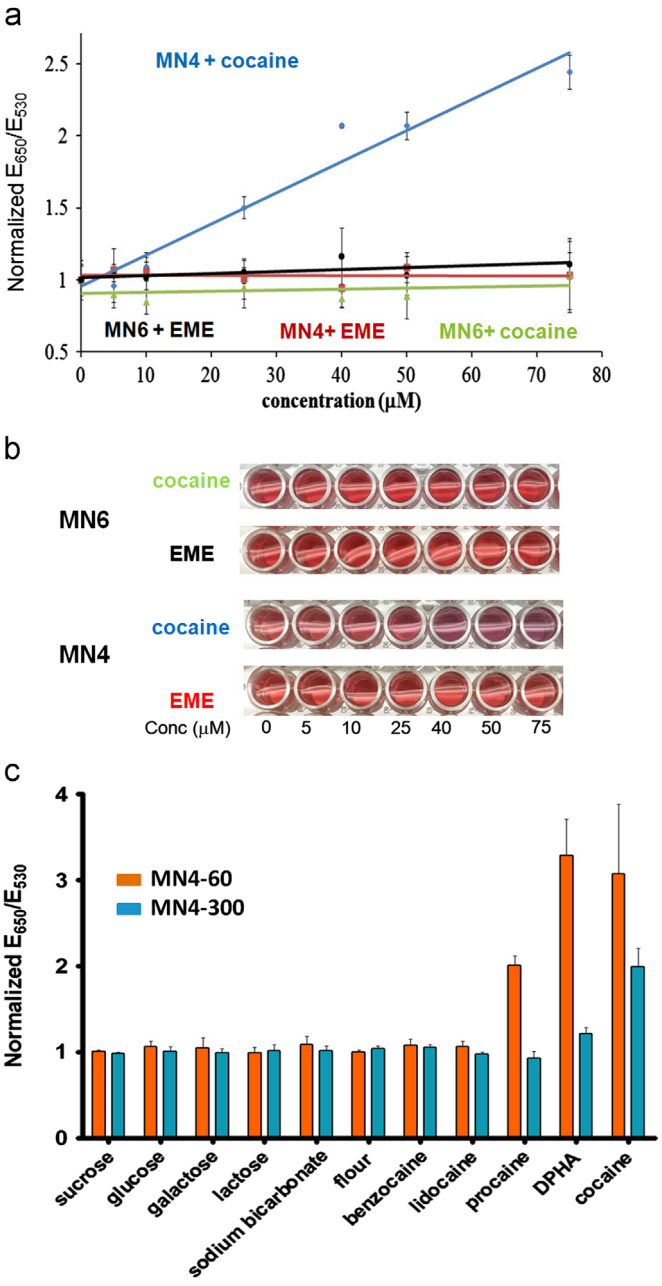


Fig. 3. Adsorbed aptamer cocaine colorimetric assay characterization. (a) Quantification of the assay aggregation response to cocaine and EME, (b) digital images of the samples analyzed in (a) and (c) aggregation response to typical cutting agents and fillers used in cocaine samples, two different DNA loadings were used: 60 and 300 DNA/AuNPs, all samples were dissolved to a 1 mg/mL concentration. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 1
Cocaine assay response to filler molecules and other active compounds.

Substance	MN4–60 AuNPs	MN4–300 AuNPs	Substance	MN4–60 AuNPs	MN4–300 AuNPs	Substance	MN4–60 AuNPs	MN4–300 AuNPs
Buffer	–	–	MDMC	+	–	Citric acid	–	–
Cocaine.HCl	+	+	Pseudoephe drine. HCl	–	–	Inositol	–	–
Benzoylecgonine	–	–	LSD	+	–	Lactose	–	–
Ecgonine methyl ester	–	–	JWH-018	+	+	Mannitol	–	–
Heroin.HCl	+	–	Methamphe tamine. HCl	+, min	–	Dextrose	–	–
d-Amphetamine sulfate	+	–	Oxycodone. HCl	+, min	–	Salicylic acid	–	–
MDMA.HCl	+	–	Ketamine. HCl	+, min	–	Orange gelatin	–	–
Methylone	+	–	Phentermine. HCl	–	–	Strawberry gelatin	–	–
Methadrone	+, min	–	Benzocaine	–	–	Yoohoo powder®	–	–
MDPV	+	–	Lidocaine	–	–	Aspirin®	–	–
Khat	–	–	Procaine	+	–	Tide®	–	–
			HU-210	–	–	AJAX®	–	–

+: positive colorimetric response; –: no colorimetric response; +, min: slight color change observed

exposed to its metabolite. Interestingly, MN6 the CBA with a predicted conformational change upon binding, did not respond to cocaine, while MN4 responded to cocaine with no response to EME. This phenomenon is currently under investigation. The response was followed by the naked-eye, as shown in the images in Fig. 3b. Similar to the previous case, this assay was tested with other substances relevant to field work. Two different DNA loading densities were used on the AuNPs to increase the AuNPs surface protection: 60 and 300 DNA per AuNP (MN4–60 and MN4–300, respectively). As shown in Fig. 3c, when MN4–60 was used, the false positive response observed with benzocaine and lidocaine was prevented (orange bars). However, the responses with procaine and DPHA were still observed. Increasing the DNA density to 300 DNA/AuNPs prevented the responses from procaine and DPHA (blue bars), while still showing response to cocaine. This work shows that the aptamer coating density can be tailored to prevent non-specific interactions between analytes and the AuNPs surface.

3.2. Assay response to masking agents and controlled substances

The Adsorbed Aptamer Cocaine Colorimetric Assay (MN4-AuNPs) was tested at the US Army Criminal Investigation Laboratory with a set of representative samples of controlled substances, over-the-counter cocaine-related compounds and other household chemicals, as shown in Table 1. The results shown were obtained by naked-eye analysis of the samples with no instrumentation involved, to mimic operational settings. In the field, a fast and reliable test that can determine whether a white powder containing cocaine will be extremely valuable in reducing the number of samples needing to be taken to a centralized location for a more accurate characterization. For this application, the sensitivity of the assay is not critical, since the substances to be tested are available in significant quantities. Due to this, all the samples were prepared at a concentration of 1 mg/mL. As shown in Fig. 3c, the MN4–60-AuNPs did not respond to common household items and sugars tested, which are white powders that can potentially be used as cutting agents. At the same time, most of the substances showing positive results were controlled substances. The stringency of the assay can be tuned to respond preferentially to cocaine by optimizing the DNA coating density. The MN4–300-AuNPs showed no response to any of the other substances, except JWH-018, a synthetic cannabinoid. Control experiments demonstrated that the color change observed was due to the low solubility of this compound, that promoted the AuNPs aggregation and resulted in a distinct deep blue color, easily identified as a false positive by the trained eye. Supplementary Fig. S2 shows TEM images of MN4-AuNPs exposed to JWH-018 after NaCl addition, confirming that the presence of this analyte promoted AuNPs aggregation. These results showed that aptamers can be used in combination with AuNPs to design robust colorimetric sensors that can be used in the field with consistent responses and minimal false positives. However, the assay color analysis by the naked eye requires training and could be difficult, depending on the room lighting and other environmental factors. A portable and automatic assay analysis tool will facilitate transitioning this technology to the field, as discussed in the following sections (vide infra). Another important issue with transitioning these technologies to the field is related to the storage conditions necessary to extend the assay's shelf-life, which is discussed in the next section.

3.3. Assay shelf-life

The assay characterization studies were performed using freshly prepared MN4-AuNPs (used within 3 days after DNA addition to AuNPs), to avoid any issues with either DNA or AuNPs degradation. However, for field use, it is critical to keep the assay components functional for extended periods of time. To improve the shelf-life of the MN4-AuNPs we aimed to prevent the growth of DNases that could degrade the aptamer. To do this, the MN4-AuNPs were treated with DEPC and autoclaved after synthesis, followed by storage at 4 °C until used [21]. Fig. 4 shows a comparison of the MN4-AuNPs response to EME and cocaine (1 mg/mL) after the different treatments used to extend the assay's shelf-life. Methanol was used as a target solvent in our "field testing" validation experiments for improved solubility of free-base and hydrochloride analytes while reducing the solubility of inorganic salts. Fig. S3 shows that using methanol did not change the assay background levels. Fig. 4 shows the response of MN4-AuNPs after storage under different conditions. Fig. 4a and b shows the assay response to cocaine and EME for MN4-AuNPs prepared with AuNPs with no post-synthesis treatment (no DPEC-autoclave). Fig. 4a shows the response after storage at RT. The MN4-AuNPs failed to respond to cocaine after 2 weeks (as observed by signal decay below the DL). Storing the non-treated MN4-AuNPs at 4 °C improved their shelf-life to about 1 month, Fig. 4b. The DEPC-autoclaved treatment extended the assay's shelf-life to 2 months (without significant response decay). This extended shelf-life was probably a result of the DNase growth prevention due to the DEPC treatment [21] and the increase AuNPs stability due to the 4 °C storage, as shown in Fig. S4.

3.4. Android application design

Figs. 2b and 3b show the typical colorimetric output of the cocaine assay. The assay was based on the comparison of the aggregation degree, seen as a change in color from red to blue, of a blank and the sample of interest. Quantification of the aggregation degree is a simple task in a laboratory setting, where a spectrometer is available. However, to transition this technology to the field, a means to take advantage of the AuNPs visual colorimetric response is critical. Recently, the use of smartphone-based color analysis tools has been reported as a way to overcome these issues [22,23]. To transition these Apt-AuNPs-based colorimetric assays to the field, an android-based color analysis application was developed. This analysis tool was designed to run on the android operating system versions 2.3 and above. The basis of the data analysis and color coordinates determination is explained in the methods section and was maintained through the analysis optimization. As was previously demonstrated, using RGB values of images to quantify colors showed severe limitations [22]. To overcome this issue, the CIE 1931 color space was utilized as developed by Shen et. al. [22] Fig. S5 shows a comparison of the fit of a calibration curve obtained with a plate reader and the color analysis of a smartphone picture performed with the app. This data shows the validity of the color analysis approach. Moreover, when analyzing the same data, the app, based on the RGB and color map conversion analysis, provided a better fit than the plate reader data, which used only two wavelengths, as typically done in AuNP work [5]. Analysis of the data obtained from pictures of multiple repetitions of a calibration curve used to set the positive color domain suggested that using both the *x* and *y* chromaticity values to quantify and compare the samples colors was not the best approach. As shown in Fig. 5a, using the change in both coordinates made it challenging to define the borders of the positive and negative color domains. The major issue consisted of the difficulty of setting the domain of the non-positive values (green and orange circles) and the positive range (blue oval) without overlap. The red box in Fig. 5a shows that the width of the positive response range obtained with a certain error value (in this case, one third of the standard deviation of the data, "actual positive response range-1") did not cover the whole area given by positive tests (blue area). Importantly, when the positive response area was increased to include all the positive values obtained in the experiments (by utilizing one half of the standard deviation, "actual positive response range-2", purple box), some of the non-positive values were identified as positive, as seen by the overlap of the purple area with the green area (blank samples) and orange non-positive response areas. A more simple approach was taken by using only one coordinate of the CIE color values for each sample. The CIE*y*-axis value was observed to result in large variations of the data which made the analysis difficult. The CIE*x*-axis data resulted in more reproducible values and reduced the complexity of the data analysis, while maintaining the overall trend of the color coordinates. This simplified color analysis offered the option of defining the boundaries of the positive and negative color value intervals in one dimension with minimal overlap, as shown in Fig. 5b. The image shows the CIE*x* values for a blank and five standard samples, with minimal overlap of the blank and the positive response range.

After the data analysis protocol was finalized the application interface was designed to match operational settings in field work. Importantly, preliminary data suggested that variations in the environmental lighting could affect the app analysis, due to the presence of shadows or bright spots in the pictures. These issues were mainly observed when ceiling lighting was used. Therefore, the pictures for app analysis were taken with the 96-well plate placed on a portable LED source. This backlighting approach

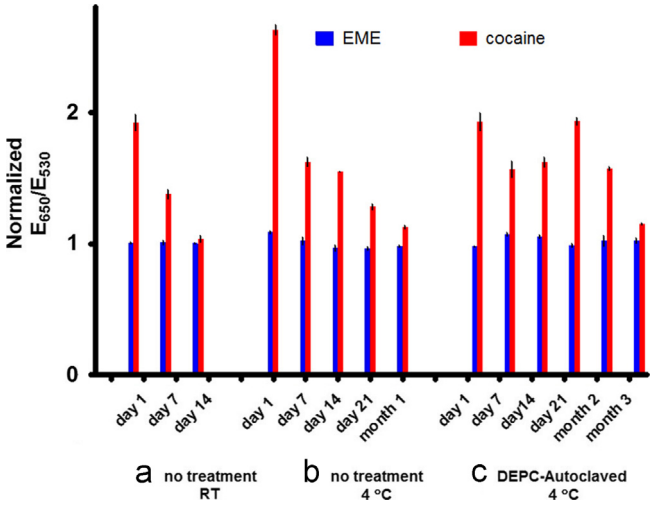


Fig. 4. Adsorbed aptamer cocaine colorimetric assay shelf-life studies. Quantification of assay response to cocaine and EME performed with MN4-AuNPs after different storage conditions.

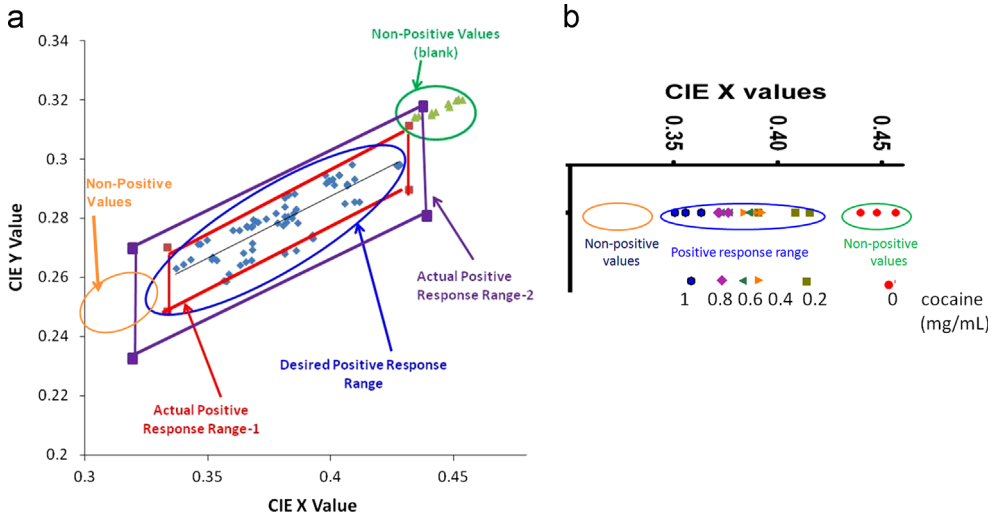


Fig. 5. Colorimetric application data analysis optimization. (a) Data distribution using both the *X* and *Y* CIE coordinates and (b) data distribution using the *X* coordinates only. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

prevented any issues with external lighting conditions, allowing for reproducible results (see Fig. S6). After this optimization, the assay can be used as a presumptive test: an operator found a powder and needed to determine whether the substance contained cocaine. The powder was dissolved (1 mg/mL) and tested with the assay. Finally, a picture was taken and analyzed with the app. In this scenario, a simple and fast "positive" or "negative" answer is needed. To accomplish this, the application requires taking a picture of the assay after testing the sample of interest together with two known positive standards used to define the LPC and HPC of the positive color domain (see the app interface in Fig. S7). This domain sets the color ranges that are considered positive under the conditions the assay is performed (room lighting, etc.). After this color range was set, the color of the unknown sample was determined and compared to the positive standards. If the sample color fell within the positive color range, then the assay result was positive; if the color was off the positive range, then the assay result was negative. A video showing the app analysis of a sample set can be viewed here (Video S1).

3.5. Colorimetric application validation

Validation of the application decision making protocol was performed by testing five replicates of a calibration curve with cocaine standard solutions. The assays results for each calibration curve were analyzed by capturing an image of the color developed to be analyzed with the application followed by immediate quantitative analysis with a conventional plate reader. Table 2 shows the linear fit of the data obtained with the plate reader together with the qualitative analysis of the picture by the application. In each case, the picture was analyzed by setting the LPC with the blank sample and the HPC with the 1.0 mg/mL cocaine standard. In all cases, different cocaine concentrations were identified as positive by the application. To show the versatility of the application developed here, in each data set the LPC and HPC were changed to obtain a narrower positive domain. For instance, the LPC and HPC were set with the 0.2 and 0.8 mg/mL cocaine respectively. Importantly, the application recognized as positive only cocaine concentration between the new LPC and HPC (the blank and 1 mg/mL cocaine samples were negative). This shows that the application can be optimized for specific uses without the need to modify the application data analysis protocol.

Finally, the performance of the application was tested with field-relevant samples, including filler agents and mixtures of cocaine and inositol. In these experiments, the LPC was set to 0.3 mg/mL based on the experimentally determined DL of 0.25 mg/mL. Table 3 shows that the application successfully recognized inositol, flour and lidocaine as non-cocaine substances (negative) at a concentration of 1 mg/mL, the highest possible concentration under the assay settings. On the other hand, cocaine mixtures with inositol were identified by the app as cocaine-containing samples (positive). Fig. S8 shows that the plate reader data for the cocaine/inositol samples containing 30% and 40% cocaine were very close to the 0.3 mg/mL cocaine standard (LPC). As discussed in the previous sections, the plate reader data only utilized two wavelengths to perform the color analysis. On the other hand, the colorimetric app, taking into account a larger volume of the color space in the data analysis, was able to identify these mixtures as cocaine-containing samples. These data showed the potential of this android-based tool to perform in-the-field colorimetric analysis. Importantly, the color analysis algorithm should work with any assay resulting in a color change, independently of the color outputs.

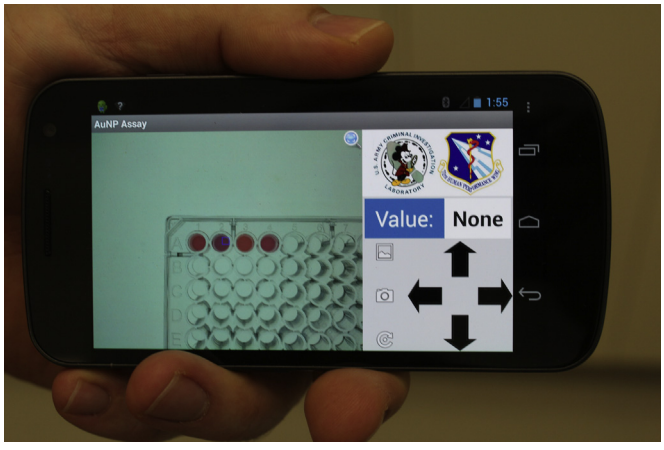
Table 2
Application data analysis validation in comparison to a conventional plate reader.

Plate reader Calibration 1: $a=0.0003$, $b=0.1558$, $r^2=0.9525$					
0	0.2	0.4	0.6	0.8	1.0
LPC, +	+	+	+	+	HPC, +
−	LPC, +	+	+	HPC, +	−
−	−	LPC, +	HPC, +	−	−
Plate Reader Calibration 2: $a=0.0001$, $b=0.1314$, $r^2=0.9505$					
0	0.2	0.4	0.6	0.8	1.0
LPC, +	+	+	+	+	HPC, +
−	LPC, +	+	+	HPC, +	−
−	−	LPC, +	HPC, +	−	−
Plate Reader Calibration 3: $a=0.0001$, $b=0.1787$, $r^2=0.9264$					
0	0.2	0.4	0.6	0.8	1.0
LPC, +	+	+	+	+	HPC, +
−	LPC, +	+	+	HPC, +	−
−	−	LPC, +	HPC, +	−	−
Plate Reader Calibration 4: $a=0.0002$, $b=0.1588$, $r^2=0.9385$					
0	0.2	0.4	0.6	0.8	1.0
LPC, +	+	+	+	+	HPC, +
−	LPC, +	+	+	HPC, +	−
−	−	LPC, +	HPC, +	−	−
Plate Reader Calibration 5: $a=0.0001$, $b=0.1246$, $r^2=0.9752$					
0	0.2	0.4	0.6	0.8	1.0
LPC, +	+	+	+	+	HPC, +
−	LPC, +	+	+	HPC, +	-
−	−	LPC, +	HPC, +	−	−
Plate Reader Calibration 6: $a=0.0002$, $b=0.1417$, $r^2=0.9613$					
0	0.2	0.4	0.6	0.8	1.0
LPC, +	+	+	+	+	HPC, +
-	LPC, +	+	+	HPC, +	−
-	-	LPC, +	HPC, +	−	−

Linear fit obtained with plate reader data, fit to an equation of the form: $y=ax+b$, +: positive, −:negative

4. Conclusions

A colorimetric cocaine assay based on AuNPs and aptamers was designed and optimized to be used in-the-field. A free aptamer cocaine assay was proposed following previous reports on the design of Apt-AuNPs colorimetric sensors. In this assay, the aptamer and analyte were incubated to allow binding and subsequently mixed with the AuNPs. The bound aptamer did not interact with the AuNPs, resulting in a change in color from red to blue when NaCl was added. In the absence of the target, the unbound DNA stabilized the AuNPs preventing the color change. An assay that responded colorimetrically to cocaine but not to EME, a cocaine metabolite, or to typical cutting and masking



Video S1. A video clip is available online. Supplementary material related to this article can be found online at doi:10.1016/j.talanta.2013.12.062.

Table 3
Application assay analysis results with a Google Nexus 4® cell phone.

sample (1 mg/mL in methanol)	Colorimetric app result
Inositol	Negative
Inositol	Negative
Flour	Negative
Flour	Negative
Lidocaine	Negative
Lidocaine	Negative
30% cocaine/70% inositol	Positive
30% cocaine/70% inositol	Positive
40% cocaine/60% inositol	Positive
40% cocaine/60% inositol	Positive
80% cocaine/20% inositol	Positive
80% cocaine/20% inositol	Positive

LPC= 0.3 mg/mL cocaine, HPC= 1.0 mg/mL cocaine

agents was obtained. However, testing the assay with a number of other over-the-counter chemicals and controlled substances showed a high number of false positives. These false positives were prevented by modifying the assay and incubating the AuNPs and the aptamer prior to target addition. Minimal false positives were observed with this detection scheme, even in cases when charged molecules were used as analytes. To transition this assay for field use the shelf-life of the assay was extended to 2 months by post-synthesis treatment with DEPC/autoclave followed by storage at 4 °C. The final step investigated to transition this colorimetric assay to the field consisted of the development of an android-based color analysis tool. The application was based on the color analysis of a picture of the assay after color development. The red, green and blue values of the image were transformed into the CIE 1931 color space. The application compared the color of an unknown substance to cocaine references used to determine the positive color domain. If the unknown color fell within the range, the sample was determined to contain cocaine. The assay was tested with a number of substances and cocaine mixtures and analyzed with the application, showing an excellent ability to detect cocaine even in the presence of cutting agents. In summary, we have demonstrated that Apt-AuNPs colorimetric assays can be optimized to be used in-the-field and the data analysis can be performed with an android-based device. This work establishes that sensors based on AuNPs and aptamers can be robust enough to be used in field applications.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.062.

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