Aptamer-based lateral flow assay for point of care cortisol detection in sweat

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\textbf{ABSTRACT}

A new aptamer-based lateral flow strip assay has been designed and developed for on-site rapid detection of cortisol in sweat. Cortisol in sweat has been identified as a key biomarker to monitor physiological stress. A highly sensitive and specific cortisol sensor was achieved by conjugating cortisol-selective aptamers to the surface of gold nanoparticles (AuNPs). Aptamer-functionalized AuNPs are stable against salt-induced aggregation. When cortisol molecules are present in the sample, they interact with the designed aptamers causing their desorption from the AuNP surface. Free AuNPs can then be captured by reaction with cysteamine immobilized on the test zone of the lateral flow test strip. This enables the visual detection of cortisol within minutes. Important parameters that affect the detection sensitivity in both solution and lateral flow assays, such as the loading density of aptamers per AuNP, salt and cysteamine concentrations, were investigated to provide the optimum assay performance. This hand-held device successfully exhibited a visual limit of detection of 1 ng/mL, readily covering the normal range of free cortisol in sweat (8–140 ng/mL). No significant cross reactivity to other stress biomarkers was observed. The advantages of this paper-based biosensor over previously reported test strips include the use of aptamers (which are more stable, simpler to use and lower cost than antibodies) and a simplified lateral flow assay (LFA) strip design (without the use of complementary aptamers in the test line). The resulting LFA aptasensor provides a rapid, sensitive, user-friendly and cost-effective point of care device for cortisol detection in sweat and other biofluids.

1. Introduction

The level of hormones produced by the endocrine system and neurotransmitters produced by the nervous system are key indicators of body status and response to internal and external stress. The point-of-use detection and quantification of these biomarkers in various biological fluids (e.g. blood, sweat, urine) is an important and expanding field [1]. Cortisol is a glucocorticoid hormone released by the adrenal cortex and plays an important role in the body’s physiological processes and functions [2]. Cortisol levels vary during the day, being the highest in the early morning (30 min after awaking, 50–250 ng/mL) and the lowest before bedtime (30–130 ng/mL) [1,3]. The lack of sufficient cortisol in the body results in Addison’s disease [4]. Elevated cortisol levels with repeated activation leads to Cushing’s syndrome [5], which can result in severe fatigue, depression, anxiety, cognitive difficulties, obesity and cardiovascular disease [6,7].

Studies have shown that the cortisol level increases in response to both physical and psychological stress [8], and has therefore been identified as a key biomarker of stress. Cortisol secretion in response to physical or emotional stress increases blood pressure to provide fat and glucose in muscles and brain for successfully coping with stress. However, prolonged elevation of cortisol can damage health and produce serious problems, such as hypertension, particularly in older and/or unhealthy individuals [9,10]. Monitoring cortisol levels can reveal physiological states for both prognosis and diagnosis purposes.

Cortisol at different physiological concentrations can be found in a variety of biofluids, such as blood, urine, saliva, interstitial fluid and sweat [11]. Blood analysis is the gold standard in monitoring the physiological state of individuals, however it is invasive and drawing blood can increase the stress biomarkers level. Sweat is an attractive alternative to blood, as it contains several biomarkers and health indicators [12]. Moreover, sweat samples can be continuously accessed from various locations on the body surface [13]. Due to the low flow rate of sweat generation, lipophilic proteins such as cortisol with passive transport through the lipid-bilayer membrane of cells have more time to enter sweat with a close correlation with their unbound (free cortisol) concentration in blood [12,14]. As free cortisol accounts for all major activities related with cortisol in body [15], sweat as a
A noninvasive biofluid containing free cortisol, with concentration ranging from ~8 to ~140 ng/mL [16], is a very attractive source for diagnosing and treating cortisol related problems. Conventional laboratory detection techniques for quantifying cortisol are based on laborious separation methods, such as chromatography, or complex antibody recognition, such as immunoassay or electrochemical immunosensing [17,18]. Although these commonly used techniques provide sensitive and accurate detection of proteins, there are some drawbacks, including large sample volume (~0.1–1 mL), lengthy assay time, the need for specialized instruments and skilled personnel. The resulting test complexity and high cost prevent their conversion to a rapid diagnostics format and use as point-of-care (POC) devices. Lateral flow assays (LFA) are simple paper-based devices with a widely adopted platform for POC diagnostic devices without the need for specialized and costly equipment. In LFAs, nitrocellulose membranes are utilized because of their ability to immobilize specific recognition elements (such as antibodies, aptamers) that indicate the presence of target molecules.

Many of the current cortisol POC detection approaches rely upon antibody (Ab) recognition. To date, Ab-based cortisol detection in LFAs has been reported for plasma with a limit of detection (LOD) ~3.5 ng/mL [19] and for saliva with an LOD ~1–10 ng/mL [20].

An alternative is the use of aptamers, which are single strand DNA or RNA oligonucleotides that can bind with high affinity and selectivity to their target molecule. Compared to Abs, aptamers provide a simpler handling method, cost-effective production, low immunogenicity and high stability. An in-depth review comparing aptamers and antibodies has been presented by Song et al. [21]. Aptamer applications have been investigated extensively and showed a similar or better performance over Abs in vitro [22–24].

Recently, aptamer-based detection methods [25] for different biomarkers in various biofluids have been successfully adopted [26–28] as counterparts to Ab detection techniques, such as electrochemical microfluidic devices [29], surface plasmon resonance [30] and ion-sensitive field-effect transistor [31]. Aptamer conjugated gold nanoparticles (AuNPs) provide colorimetric detection that is cost effective compared to fluorescence or radioactivity-based assays [32]. AuNPs in colloidal solution present a reddish color. Upon AuNP aggregation by introduction of salt, the solution experiences a color change that is visible by naked eye. The color of aggregated AuNPs ranges from fading out/disappearance of red color to the appearance of a blue/purple color, depending on the AuNP diameter and salt concentration. Citrate-stabilized AuNPs can interact with different molecules and proteins, shielding the Au surface from interaction with salt molecules and preventing aggregation [33].

Martin et al. [34] demonstrated the first-known aptamer for cortisol (with a dissociation constant of ~7–16 μM) and its detection in a buffer at physiological concentrations. The aptamer-AuNP approach has also been reported [26] as a fast and selective sensor platform for serotonin detection in buffer, with minimal response to other neurotransmitters with similar chemical structures. In that approach, adding serotonin to the mixture of AuNPs-aptamer decreases the solution susceptibility to aggregation in the presence of salt. Here, a similar but much simpler method is reported with successful colorimetric detection of cortisol in artificial sweat by aptamer-AuNP based conjugate assay.

The basic concept of AuNP color change in aptamer-based sensors is illustrated in Fig. 1. Citrated AuNPs conjugated to aptamers carry a negative surface charge generating a repulsive force that prevents their aggregation. This results in a reddish color in solution due to absorption at green wavelengths (typically at ~520 nm). In the presence of cortisol, the aptamers undergo conformational changes and/or are removed from the AuNP surface. Upon the addition of NaCl solution, the surface charge and electrostatic repulsion of “naked” AuNPs are reduced, resulting in AuNP aggregation. In turn, this produces a red shift of the peak absorption wavelength and a change in color to gray/blue [34,35].

In the present study, we have designed and developed an aptamer-based strip biosensor for the detection of cortisol in sweat (and other biofluids) by taking the advantage of the assay sensitivity and selectivity found in solution phase. AuNP-aptamer based sensors have been previously reported for various targets (such as adenosine, α-amylase) [36,37], proteins [38,39] and biomarkers [28]. To the best of our knowledge, this is the first aptamer-based strip biosensor for cortisol detection in sweat.

In this strip biosensor, AuNPs serve as a color probe and provide a visible, rapid and sensitive one step detection without the need of using capture (complementary) aptamers on the LFA test zone. Cortisol present in the sample desorbs cortisol-matching aptamers from the surface of the AuNP and release “naked” AuNPs. Cysteamine that is immobilized in the test zone of the sensor contains a thiol group that enables the capture of the free AuNPs. The intensity of the red color resulting from AuNP aggregation in the test line is directly proportional to the concentration of cortisol in sweat samples.

2. Materials and methods

2.1. Materials and solutions

Cortisol-binding DNA aptamer (40-mer) sequence [34] (ATGGGGCA ATGCCGGGTTGGAGAATGGTTGCCGCACTTCGGC) MW = 12474.1 g/mol and IDTE™ buffer, pH 8 were purchased from Integrated DNA technologies (Coralville, Iowa). In our work, 40 nm Au nanoparticles were purchased from Nanocomposix (San Diego, CA). Cortisol protein (≥98%) was purchased from Fitzgerald (Acton, MA). Artificial sweat was obtained from Pickering Laboratories (Mountain View, CA).

Sodium phosphate tribasic dodecahydrate (Na2HPO4·12H2O), sodium chloride, magnesium chloride, Tween 20, Triton X-100, bovine serum albumin (BSA), cysteamine, sucrose, serotonin (≥98.0%), neuropeptide Y (NPY; Human; ≥95%) and HCl were purchased from Sigma-Aldrich (St. Louis, MO). Tris buffer (pH 8), nitrocellulose membrane (Millipore HFL135), and cellulose fiber sample pad (CFSPO01700) were purchased from MilliporeSigma (St. Louis, MO). Glass fiber pads (8950) were purchased from Ahlstrom (Helsinki, Finland).

2.2. Experimental methods

The work is aimed at detecting cortisol in sweat samples by colorimetric assay and developing a test on LFA. As this is a new approach...
for cortisol detection, it is necessary to evaluate the LOD in solution phase first, and then develop the test on paper-based LFA devices. Many factors have been optimized in order to increase the assay sensitivity in both solution and lateral flow cases (see Supplementary material). Optimizing the aptamer:AuNP loading density (Tables S1–S3) is the first step in order to determine the minimum number of aptamers needed to stabilize AuNPs against salt-induced aggregation. In order to determine the efficiency of this approach, this concept was tested with artificial sweat, which is a close representation of human eccrine sweat [40] containing all of the key ingredients (amino acids, minerals, metabolites, etc.) in appropriate concentrations.

2.2.1. Preparation of aptamer-AuNP probe

The colorimetric procedure of cortisol detection in sweat started by adding 5 μL cortisol aptamer into 40 μL AuNPs (40 nm diameter, 1 μM) with a loading density of 5886:1 aptamer:AuNP (Table S3), followed by 2-h incubation at room temperature. The optimum loading density of DNA aptamers to AuNPs was obtained by monitoring the assay color change from red to blue in the presence of cortisol. Optimization of variables and incubation time studies were performed, the data are available in Supplementary material. To test the response of the aptamer-AuNP assay, 5 μL solutions of artificial sweat containing cortisol in different concentrations (0, 1, 5, 10, 50, 100 and 300 ng/mL) were mixed with aptamer-AuNP suspensions and incubated for 30 min in ambient conditions). Cortisol binding buffer (CBB) was made of 50 mM Tris, 137 mM NaCl, 5 mM MgCl2, pH 8. A cortisol protein stock solution was prepared by dissolving 1 mg in 1 mL of buffer (10% ethanol, 30% CBB and 60% deionized (DI) water), and then subsequently diluted with DI water for desired concentrations. Spectral analysis of AuNPs was performed in the range of 400 nm–700 nm using Nanodrop One Spectrophotometer (Nanodrop Inc, Thermo Fisher Sc.).

2.2.2. Preparation of test strips

Lateral flow technology is a robust technique that could convert current cortisol detection technologies into a user-friendly test kit. The lateral flow assay for cortisol detection in artificial sweat consists of four components: sample pad, blocking agent pad, nitrocellulose membrane and wicking pad. Test strips cards were prepared by attaching nitrocellulose membrane (Millipore HF135 × 35 mm × 20 cm) on adhesive backing cards, followed by mounting wicking pad (19 mm × 20 cm) on it with 2-mm overlap to ensure liquid transfer between the membrane and wicking pad. Individual test strips with dimensions of 10 mm × 65 mm were obtained using a guillotine cutter (CM4000, BioDot, Irvine, CA). The test zones on the nitrocellulose membrane were produced by dispensing cysteamine 60 mg/mL in a line with 3-mm overlap on nitrocellulose membrane and a sample pad with 2-mm overlap on blocking pad.

2.2.3. Assay procedure in lateral flow phase

In lateral flow assays, the accumulation of AuNPs on the test line provides a clear red band, thus reducing the need for higher concentration of AuNPs for visual detection. Initial lateral flow experiments were performed using conditions optimized in solution phase for aptamer-AuNP loading density and incubation time. Forty microliters of 1 nM AuNPs incubated for 2 h in ambient conditions with 5 μL aptamer at a loading density of 5886:1 aptamer:AuNP, followed by adding 5 μL artificial sweat and leaving in room temperature for 30 min. The appearance of weak intensity red band at the test line for a control sample (with no cortisol in artificial sweat) required further studies to find the optimum loading density of aptamer-AuNP for lateral flow phase. Due to relatively weak non-covalent binding between aptamers and AuNPs, it is possible for aptamers to be trapped while flowing through the membrane in spite of the use of blocking buffer. Dissociation of aptamers from the surface of AuNPs resulted in binding in the test line region between immobilized cysteamine capturing molecules and AuNPs even in the absence of cortisol as a target. Therefore, determining the minimum number of aptamers needed to shield AuNPs while flowing through the membrane was required. The loading density of aptamer that prevented capturing of shielded AuNPs by cysteamine on the test line was obtained at 7100 aptamers per AuNP.

3. Results and discussions

3.1. Cortisol detection in sweat (solution phase)

It was essential to initially find the optimum aptamer:AuNP loading density in the tests, which would affect the sample volume and concentration used. Reduction of sample volume and enhanced visual color differences were the key parts of the assay for target detection. The loading density of 5886 aptamer:AuNP was selected as optimum based on observed visual color changes for 100 and 1000 ng/mL cortisol in mixture, with no aggregation observed for the control test (Table S3). Initial experiments used 20 μL of 0.1 nM AuNPs (40 nm) solution with a loading density of 5886 aptamer:AuNP and cortisol in buffer. Subsequent experiments were performed with 5 μL aptamer solution (with the same aptamer:AuNP loading density) added to 40 μL of 1 nM AuNP solution. This was followed by adding 5 μL cortisol in artificial sweat at different concentrations (0, 1, 5, 10, 50, 100 and 300 ng/mL) after 2 h incubation in ambient condition. The combined mixture stayed in ambient conditions for 30 min (Fig. S1), after which 1.5 μL of 1 M NaCl solution was added. The interaction between the aptamer nitrogen bases and AuNPs provides a physical adsorption of aptamers on the surface of the AuNPs [41]. Aptamer-modified AuNPs are stabilized against salt induced aggregation. The mechanism of salt-induced aggregation in the presence of the target is caused by cortisol binding with the aptamer molecules and releasing the AuNPs in the process. Na+ ions neutralize the negatively charged citrate molecules on the AuNP surface, which allows particles to come into closer proximity. The reduced inter-particle distance enables plasmon coupling between particles, with a resulting observable color change from red to blue [42]. As the artificial sweat samples already contained various salts, 0.5 μL aliquots of 1 M NaCl were incrementally introduced up to a final added salt concentration of 0.03 M in solution. The assay response can be observed with the naked eye, as shown in Fig. 2. The color changed from red to purple/blue for the samples containing the cortisol target (Fig. 2c–h) while no aggregation occurred for the control sample (slight change in Fig. 2a, b due to introduction of salt).

3.1.1. Optical absorbance spectra for various cortisol concentrations

UV-visible absorption spectra shown in Fig. 3a were obtained using samples with cortisol at different concentrations taken before adding NaCl. The spectra exhibit a nearly identical absorbance peak at λ = 525 nm, indicating dispersed AuNPs. After the addition of the NaCl solution, the resultant spectra (Fig. 3b) show a decreasing absorbance peak with increasing cortisol concentration. This effect is due to the increasing binding of cortisol to the aptamer and the subsequent salt-induced aggregation of the resulting free AuNPs. This demonstrates the high affinity of the aptamers toward the cortisol target.
Cortisol binding to the aptamer is thought to produce DNA conformational changes on the surface of AuNPs, releasing AuNPs for salt-induced aggregation. As can be seen in Fig. 3b, the minimum amount of cortisol measured by spectrophotometry is $\sim 1$ ng/mL, while $\sim 5$ ng/mL is the LOD by the naked eye (Fig. 2d).

The degree of aggregation is defined as the ratio of optical absorbance at 640 nm (indicating aggregated AuNPs) to absorbance at 525 nm (for dispersed AuNPs). Fig. 4 shows the ratio of $A_{640}/A_{525}$ versus cortisol concentration. For cortisol, the absorbance decreases monotonically with concentration (from 1 to 300 ng/mL), whereas the absorbance for NPY is essentially flat with concentration, confirming no assay cross-reactivity.

3.2. Assay selectivity

To assess the selectivity of the current method to cortisol detection in sweat, we investigated the response of the assay to neuropeptide Y (NPY) and serotonin. NPY is a stress biomarker present in sweat with a larger molecular weight (MW = 4272 g/mol) than cortisol (362 g/mol). NPY is a 36-amino acid peptide that is one of the most abundant neuropeptides in the central and peripheral nervous systems [43]. NPY plays a pivotal role in physiological functions and has been correlated with stress resilience [44]. Various psychological and pathophysiological conditions affect the NPY physiological levels [45,46]. NPY healthy physiological concentration varies between 0.8–2.9 pg/mL in sweat [47]. NPY stock solution (1 mg/mL) was prepared in deionized water and then diluted to desired concentrations.

Fig. 6a. A comparison of the optical absorption at 525 nm after adding NaCl as a function of cortisol and NPY concentrations is shown in Fig. 6a. For cortisol, the absorbance decreases monotonically with concentration (from 1 to 300 ng/mL), whereas the absorbance for NPY is essentially flat with concentration, confirming no assay cross-reactivity. We have...
performed tests to determine possible interference by NPY on cortisol signal at physiological range in sweat for both biomarkers: cortisol 50 ng/mL; NPY 1–10 pg/mL. As seen in Fig. 6b, the presence of NPY (even 10 pg/mL, which is $3 \times$ the normal concentration in sweat) does not affect the cortisol signal.

To determine the assay cross-reactivity to a biomarker with a simpler molecular structure and smaller size than NPY, we have also investigated serotonin. Serotonin is not present in sweat, but it is abundant in blood and urine. Serotonin is an amine neurotransmitter with a simpler molecular structure and a molecular weight (176.21 g/mol) smaller than that of cortisol.

Serotonin stock solution was prepared by adding 1 mL solvent (10% HCl and 90% DI) to 1 mg serotonin, diluting with DI water for lower concentrations. Following the same loading density of 5 μL aptamer into 40 μL concentrated AuNPs (1 nM) with 2-h incubation in ambient conditions, serotonin was added to the mixture in different concentrations (1–300 ng/mL). Optical measurements before and after adding NaCl are presented in Supplementary material Fig. S2.

To illustrate the assay selectivity to cortisol, the aggregation response of separate samples containing equal concentrations of cortisol, NPY and serotonin is plotted in Fig. 7. While a monotonic increase in aggregation is observed for cortisol, the response for serotonin and NPY exhibits smaller, random changes in aggregation, indicating that there is no obvious reaction with cortisol aptamers. All experiments were repeated 4 times. The error bars for cortisol are relatively small and constant, while for NPY and serotonin they vary significantly. These results expand the known selectivity of the aptamers toward the cortisol target compared other stress-related biomarkers, beyond the original comparison [34] to cholic acid, norepinephrine and epinephrine.

3.3. The principle of the proposed strip biosensor

After performing successful aptamer-based tests to determine a low LOD (1 ng/mL) and establish no cross-reactivity to other stress...
biomarkers, the cortisol detection in lateral flow assay format was investigated in order to develop a more user-friendly POC device. Non-invasive POC devices that monitor the cortisol level could provide improved and personalized healthcare [48,49]. Paper-based microfluidic systems (or lateral flow assay devices) provide many advantages, such as operation without the need of external fluid pumping (due to capillary flow in the cellulose materials), low cost of materials, operation with small sample volumes and without the need of skilled personnel [18]. Most of the current generation of lateral flow assay devices achieve high selectivity and sensitivity to their target analytes by incorporating antibodies or DNA/RNA specific sequences [50,51]. Incorporating aptamers in LFA devices eliminates the shortcoming of using antibodies, such as using animals for their production [52,53], difficult and expensive production process [21,54] and variations between batches of antibodies [55].

The advantage of nitrocellulose-based LFA for the detection of cortisol with AuNPs is that a clear signal can be observed at 10× lower AuNP concentration compared to solution phase detection. The accumulation of AuNPs in a narrow band on the nitrocellulose membrane allows a 10× reduction in their concentration to 0.1 nM from 1 nM in solution, as an indicator for the presence of cortisol in samples.

To translate cortisol detection in artificial sweat from solution phase to membrane flow, immobilized cysteamine on lateral flow assay was used as a binding molecule for the test line. The detection process is illustrated in Fig. 8a. As the loading density of the aptamer affects the intensity of the test line, the ratio of 7100:1 aptamer:AuNP was determined as optimal loading in the lateral flow phase. Sample solutions of 150 μL containing cortisol are dispensed on the sample application pad. The solution migrates through the membrane by capillary action and rehydrates the blocking pad. The components (BSA and others, see Preparation of Test Strips section) in the blocking pad flow through the membrane and prevent non-specific binding of conjugated AuNP-aptamer to the nitrocellulose membrane. The naked AuNPs bind with cysteamine molecules in the test line, forming an observable red band (Fig. 8c). In the absence of cortisol, shielded AuNPs in solution migrate through the membrane and do not interact with cysteamine, as illustrated in Fig. 8b.

3.4. Performance of the proposed strip biosensor for cortisol detection in sweat

To obtain quantitative detection of cortisol in the lateral flow assay, intensities of the test lines were measured using the ImageJ program [56] and plotted as a function of cortisol concentrations. Colored photographs in Fig. 9a clearly show the direct relation between cortisol concentration in samples and the corresponding test line color intensity. The final test results on LFA can be estimated directly by naked eye or can be analyzed quantitatively by the absorption of the red band using a smartphone app or a hand-held reader. The LOD was estimated to be 1 ng/mL cortisol in dispensed artificial sweat solution. To determine the reproducibility of the biosensor response experiments were repeated 5 times and all displayed the same trend of increasing color intensity with cortisol concentration.

The intensities of the test lines were measured using ImageJ [56]. For the results shown in Fig. 9b, the assay time was 5 min and the results were normalized to the signal obtained from the highest concentration (300 ng/mL). All experiments were repeated 5 times, and the mean value and standard deviation (SD) are plotted for each

Fig. 8. Cortisol detection on lateral flow assay using AuNP-aptamers: (a) basic concept; (b) illustration of negative control - no color change in absence of cortisol; (c) color change in test line in presence of cortisol.
concentration. The signal intensity increased monotonically with cortisol concentration, with the lowest detectable concentration being ~1 ng/mL. One can consider as a figure of merit for this type of assay the ratio of analyte LOD to the corresponding AuNP concentration, which would be 2.7 for the solution assay and 27 for the LFA case.

To evaluate their stability, strips were stored in sealed plastic bags and kept at 4 °C for 1 to 10 days and then used for target detection with the same concentration (100 ng/mL) of cortisol in sweat, Fig. S3. To investigate the stability of AuNPs modified with aptamers, samples were stored in vials at 4 °C from Day 0 to Days 5, 10, 15, 20, 25 and 30. The results of subsequent tests were the nearly the same for all samples (see Supplemental material, Fig. S4). The long shelf life of aptamers is a key ingredient for achieving a stabilized solution for long storage periods. The results of our current lifetime tests of the shelf life of the strips (~10 days) and the AuNP solution (~30 days) are quite promising. However, more detailed and longer-term testing is required for commercial product development.

4. Summary and conclusions

In this work, we have presented the first aptamer-based point-of-care paper device to detect cortisol as a stress biomarker. The detection of cortisol in artificial sweat within relevant physiological levels has been demonstrated using cortisol-specific aptamers conjugated to AuNPs. Two approaches were investigated for this purpose: solution phase detection and membrane-based (capillary) lateral flow detection. Physical absorption of aptamer on citrate-stabilized AuNPs (2 h incubation) improves the mixture stability against salt-induced aggregation. Introducing the target analyte (cortisol) into the aptamer-AuNP mixture results in conformational changes of the aptamers induced by cortisol binding and accompanying aggregation and color change. In the solution-based assay, the cortisol LOD was ~1 ng/mL equivalent to ~2.7 nM. The selectivity of aptamer-AuNPs for cortisol detection was tested against other biomarkers (NPY and serotonin) with the same process. No color change was observed for either NPY or serotonin, indicating no (or very little) cross-reactivity between the cortisol aptamer and these other stress biomarkers. Detection in lateral flow format utilized cysteamine (thiol molecule) immobilized on nitrocellulose membranes to produce a test line. In the presence of the target analyte, naked AuNPs attach to the cysteamine and provide a red band. The test line color intensity was shown to have a direct relation to cortisol concentration in sweat solution. For translating experiments from solution phase to lateral flow phase the aptamer/AuNP loading density is slightly increased to reduce the effect of non-specific binding of aptamer in nitrocellulose membrane through migration of mixture. The combination of aptamer-AuNPs provides an easy to use assay probe for detection of cortisol in sweat in both modes. The LFA LOD for cortisol was the same as for the solution assay (1 ng/mL), but at 10× lower AuNP concentration.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix B. Supplementary data

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ASSOCIATED CONTENT

Supplementary Material

Optimization of experimental variables. Aptamer stock solution with a concentration of 654.5\(\mu\)M, AuNPs 0.1nM, cortisol 10 and 100 \(\mu\)g/dL and NaCl 1M were the four variables in the experiment. Based on the effect of salt in AuNP aggregation, investigating the minimum amount of salt needed for AuNPs aggregation was the first step. Equal sample volume (20\(\mu\)L) of all variables was selected. It was found that a minimum salt concentration of 0.1M is requiring to observe a color change of the solution from red to purple or blue. Table S1 shows the minimum amount of salt needed for AuNPs aggregation before introducing DNA.

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**Table S1:** Determining minimum amount of NaCl for inducing AuNP aggregation.

Next, the minimum number of aptamers needed to shield AuNPs and prevent aggregation was determined. Aptamer stock solution (654.5µM) was prepared and serially diluted 10-fold (654.5, 65.4, 6.5, 0.65 and 0.06 µM). In each test, 20µL AuNPs (0.1nM) were incubated with 20µL aptamer at 5 different concentrations (Table S2) for 2h, which is the time needed for aptamers to develop non-covalent binding to the AuNP surface. The minimum salt concentration needed for aggregation was found to be 0.1M. Aptamer concentration of 0.65 µM was found to be insufficient to prevent aggregation at 0.1M salt concentration. The higher aptamer concentrations (65.4 and 654.5 µM) did not experience color change at either 0.1 or 0.2 M salt concentration. Assay sensitivity is related to the number of aptamers bound to the AuNP surface, with larger numbers of aptamers requiring higher target and salt concentrations for aggregation. 20µL of 6.54 µM aptamer solution was selected as a proper range. Further investigation conducted to find the exact number of aptamers to shield AuNP against aggregation.
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<td>20µL (H₂O)</td>
<td>20µL (1/5M)</td>
</tr>
<tr>
<td>20µL</td>
<td>20µL (65.45nmol/mL)</td>
<td>20µL (H₂O)</td>
<td>20µL (1/5M)</td>
</tr>
<tr>
<td>20µL</td>
<td>20µL (6.54nmol/mL)</td>
<td>20µL (H₂O)</td>
<td>20µL (1/9M)</td>
</tr>
<tr>
<td>20µL</td>
<td>20µL (0.65nmol/mL)</td>
<td>20µL (H₂O)</td>
<td>20µL (1/9M)</td>
</tr>
<tr>
<td>20µL</td>
<td>20µL (0.06nmol/mL)</td>
<td>20µL (H₂O)</td>
<td>20µL (1/9M)</td>
</tr>
</tbody>
</table>

**Table S2:** Determining the minimum number of aptamers to shield AuNPs from aggregation.

**Cortisol assay optimization - AuNP:aptamer ratio; cortisol concentration.** 20µL of 6.54µM aptamer was the approximate concentration of aptamer in assay with no aggregation, as shown in Table S2. Cortisol as the target is the last variable added to solutions in different concentrations to find the minimum number of aptamers needed to cover the AuNP surface. Cortisol stock solution was prepared by dissolving 1mg in 1mL of buffer (10% ethanol, 30% CBB and 60% DI water), and then subsequently diluted with DI water for desired concentrations. Cortisol binding buffer (CBB) consists of 50 mM Tris, 137 mM NaCl, and 5 mM MgCl₂, pH 8.

To determine the minimum number of aptamers to shield the 40nm AuNP surface, 20 µL aptamer 6.54µM aliquots were diluted with IDTE based on volume (started from 15µL and reduced to 1.6 µL of aptamer) as shown in Table S3.

After 2h incubation of AuNP and aptamer mixture in ambient, 20µL NaCl 0.1M was added to samples followed by adding cortisol at different concentrations (0, 10 and 100µg/dL) with 30 min incubation in ambient. At a loading density of 5886 aptamers per AuNP, salt induced
aggregation occurred for 10 and 100 µg/dL cortisol, while no color change was observed for the control test, as indicated in Table S3. Salt induced aggregation occurred for control samples (no cortisol) with aptamer concentration lower than 0.29 µM, indicating that the loading density of DNA to AuNP is not sufficient to shield the AuNP surface from aggregation. Thus, 0.29µM aptamer concentration was selected as optimum concentration due to the absence of noticeable color change by naked eye in presence of cortisol.

<table>
<thead>
<tr>
<th>AuNPs</th>
<th>Aptamer (µM) 20µL</th>
<th>Loading density AuNPs/Aptamer</th>
<th>Cortisol (µg/dL) 20µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µL</td>
<td>2.4(15µL aptamer+5µL IDTE)</td>
<td>1:49050</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>1.62(10µL aptamer+10µL IDTE)</td>
<td>1:32700</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>1.3(8µL aptamer+12µL IDTE)</td>
<td>1:26160</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.97(6µL aptamer+14 IDTE)</td>
<td>1:19620</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.81(5µL aptamer+15µL IDTE)</td>
<td>1:16350</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.65(4µL aptamer+16µL IDTE)</td>
<td>1:13080</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.49(3µL aptamer+17µL IDTE)</td>
<td>1:9810</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.45(2.8µL aptamer+17.2µL IDTE)</td>
<td>1:9156</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.42(2.6µL aptamer+17.4µL IDTE)</td>
<td>1:8502</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.39(2.4µL aptamer+17.6µL IDTE)</td>
<td>1:7848</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.35(2.2µL aptamer+17.8µL IDTE)</td>
<td>1:7195</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td>0.32(2µL aptamer+18µL IDTE)</td>
<td>1:6540</td>
<td>0, 10, 100</td>
</tr>
<tr>
<td>20µL</td>
<td><strong>0.29</strong>(1.8µL aptamer+18.2µL IDTE)</td>
<td><strong>1:5886</strong></td>
<td><strong>0, 10, 100</strong></td>
</tr>
<tr>
<td>20µL</td>
<td>0.26(1.6µL aptamer+18.4µL IDTE)</td>
<td>1:5232</td>
<td><strong>0, 10, 100</strong></td>
</tr>
</tbody>
</table>

**Table S3**: Optimizing ratio of variables in assay.
INCUBATION STUDIES

**Aptamer incubation.** Incubation studies performed to optimize the minimum time required for the aptamer to bind to AuNPs surface. Incubation studies were done by adding 5µL aptamer with a loading density of AuNP:aptamer 1:5886 to 40µL AuNPs (1nM) in ambient at different incubation times, followed by adding 1.5µL NaCl 1M to solutions. Resultant graph in Fig. S1a shows the amount of aggregation based on time, lowest absorbance at λ=525 for adding salt to assay right after adding aptamers, while longer incubation time to assay makes aptamer bind to AuNPs surface and protects them against salt induced aggregation. Optical measurements done with 30 min increment for each sample to incubate then salt added after incubation. 2h incubation was selected as the optimum time because of minimum aggregation and the same absorbance at λ=525nm for 2h and overnight incubation.

**Target incubation.** Incubation studies carried out to find the minimum time required for the target (cortisol) binds to aptamer and leads to conformational changes to the aptamer on AuNPs surface. 5µL cortisol 10µg/dL was added to the mixture of the AuNPs-Aptamer followed by adding 1.5µL NaCl 1M at different time sequence. At 0min target does not have enough time to dissociate aptamer from AuNP surface, therefore the color of the solution remains red and the optical measurements shows the highest absorbance at λ=525 for 0min. Gradually by giving more time to the suspension of functionalized AuNPs, cortisol binds with more aptamers and leave more naked AuNP for salt induced aggregation. For target incubation studies, 30 min selected as the optimum time due to aggregation in presence of 10µg/dL as shown in Fig. S1b.
Figure S1: Incubation studies to optimize the time required for: (1) aptamer stabilized (shield) AuNPs; (2) cortisol as a target at a constant concentration (10µg/dL) to bind the aptamer.

Assay sensitivity to other stress biomarker (serotonin)

Serotonin stock solution (1 mg/mL) was prepared in 10% HCL and 90% deionized water and then diluted to desired concentrations. 5 µL artificial sweat containing serotonin in different concentrations (from 1 to 300 ng/mL) was added to 5 µL aptamer solution incubated for 2h with 40 µL AuNPs (1 nM). Then 1.5 µL of NaCl (1 M) was added to the suspension 30 min after serotonin was incubated with the mixture. As shown in Fig. S2a and b, optical absorption measurements in samples containing serotonin with and without the addition of 1.5 µL NaCl (1 M) show only minor changes in value and thus no relation between the amount of aggregation and serotonin concentration. The graph of absorption intensity vs serotonin concentration (Fig. S2c) at λ=528 nm indicates no relation between concentration and aggregation.
Figure S2: Optical spectra of mixture of DNA-AuNP and artificial sweat solutions with various serotonin concentrations: (a) without NaCl; (b) with NaCl; (c) peak absorption intensity versus different concentrations of serotonin.

Stability of the test strips

To confirm the stability of the test strips, the as-prepared strips from the same batch were stored in sealed plastic bags and kept in 4°C. The assay was conducted with cortisol (100ng/mL) in sweat at 2 days interval. As shown in Fig. S4, the intensity of the test lines in the first 8 days is roughly the same. From 8 to 14 days a gradual loss of intensity is observed in the test lines. The color change of cysteamine in the test line to yellow makes the intensity becomes slightly weaker. Therefore, using test strips before 10 days storage is the optimum time to have a reproducible result. Adding silica desiccant in the sealed bags could reduce the oxidation of the thiol group in cysteamine and may reduce or delay the color change to yellow.
Stability of AuNP-Aptamer solution

To confirm the stability of the AuNP-Aptamer complex, solutions were prepared and stored in dark at 4°C. Daily optical measurements started after 2h incubation for 35 days after making the complex. As shown in Fig. S3, UV-Vis spectra of the AuNP solution exhibit nearly identical absorbance at $\lambda=525$ nm, indicating dispersed conjugated AuNPs with no aggregations. We did not continue the measurements after 35 days, since we use fresh AuNPs after 30 days of assay testing. The data points in the Fig. S3 inset show the stability for 35 days of the solution consisting 5µL aptamer [$\lambda=260$ nm] into 40µL AuNP [$\lambda=525$ nm] 1nM with a loading density of 5,886:1 aptamer:AuNP.
Figure S3: Stability of conjugated AuNP-Aptamer over time