ABSTRACT: We have developed a disposable point-of-care (POC) aptamer-based biosensor for the detection of salivary cortisol. Nonstressful and noninvasive sampling of saliva compared to that of blood makes saliva an attractive biological matrix in developing POC devices for biomarker monitoring. Aptamers are attractive as recognition elements for multiple reasons, including their specific chemical synthesis, high stability, lack of immunogenicity, and cell-free evolution. A duplex aptamer conjugated to the surface of Au nanoparticles (AuNPs) by Au-S bonds is utilized as the sensor probe in a lateral flow assay (LFA) device. The addition of salivary samples containing cortisol makes the cortisol-aptamer undergo conformational changes and dissociate from the capture probe. Increasing cortisol concentration in the dispensed saliva sample results in increased dissociation and leads to increased binding of AuNP conjugate on the test line. Therefore, the color intensity of the test line on the LFA is a direct function of the concentration of cortisol in saliva. This simple and fast method provides detection in the cortisol range of 0.5–15 ng/mL, which is in the clinically accepted range for salivary cortisol. The limit of detection was 0.37 ng/mL, and the accuracy was confirmed by enzyme-linked immunosorbent assay (ELISA) testing results. High selectivity was observed for salivary cortisol against other closely related steroids and stress biomarkers present in saliva.

1. INTRODUCTION

Point-of-care (POC) devices are increasingly being utilized for monitoring various health-care conditions in a convenient, easy-to-use, and low-cost format. This ranges from pregnancy to flu testing to glucose monitoring. These POC platforms are based on capillary flow through nitrocellulose (paper). This approach has the advantage that it does not require an external force for fluid flow, thus further simplifying the operation and reducing the physical dimensions and the cost of manufacturing. Paper-based lateral flow assay (LFA) devices use several recognition mechanisms (such as antibody/antigen, aptamer/aptamer) for detecting the presence of targeted analytes and displaying the results to the user (primarily as a color change). Recent reviews of the field of paper-based microfluidics for medical diagnostics include those by Yamada et al. and Gong and Sinton.

The detection of stress levels using POC devices is an area of growing importance for both healthy individuals experiencing stressful events (such as athletes, police, and armed forces personnel) and individuals with health issues. Multiple stress-related biomarkers (cortisol, dopamine, serotonin, etc.) present in biological fluids (blood, sweat, saliva, urine, etc.) at various concentrations provide an opportunity for developing microfluidic POC diagnostics (see the review by Steckl and Ray). A variety of biomarker detection mechanisms have been employed for this purpose, including colorimetric, electrochemical, and optical detection.

Targeting biomarkers found in blood has the advantage of being able to correlate with conventional and very accurate laboratory analysis, but it suffers from the fact that it requires penetrating the skin for obtaining samples, which may increase the stress level of patients, and blood properties change rapidly with time after extraction from the body because of coagulation. Therefore, the use of noninvasive biofluids for biomarker detection is quite attractive.

Aptamer-based bioassays are being increasingly investigated in a wide variety of applications in targeted drug delivery, molecular diagnostics, biosensor systems, and biomarker discovery. Aptamers are single-stranded DNA or RNA ligands that are synthesized toward a wide variety of targets, such as proteins, peptides, small ions, and organic compounds. Aptamers are selected from a DNA library by in vitro selection process named systematic evolution of ligands by exponential enrichment (SELEX). Compared to antibodies, aptamers as potential recognition elements have several attractive characteristics: easy chemical synthesis, adaptive modification, small size, high stability, lack of
immunogenicity, and cell-free evolution.\textsuperscript{19} Aptamer-based POC lateral flow assays (LFAs) have been reported for the detection of stress biomarkers, such as dopamine in urine\textsuperscript{20} and cortisol in sweat.\textsuperscript{7}

In this paper, we report on the detection of cortisol in saliva using an aptamer recognition mechanism. Saliva is a complex fluid composed of different peptides/proteins, hormones, nucleic acids, lipids, electrolytes, ions, and several other components, which reflect the body’s health and physiological conditions.\textsuperscript{21} Saliva has received increasing attention in POC detection technologies as it contains a wide array of biomarkers with clinical relevance and liquid samples of sufficient volume can be readily obtained. Salivary cortisol has a relatively high concentration,\textsuperscript{22–24} ranging from 0.6 to 10.4 ng/mL, with an average normal level of ~6 in the morning and 1 ng/mL in the evening. Cortisol is a steroid hormone released by the adrenal cortex to regulate blood pressure, cardiovascular function, and metabolic activities.\textsuperscript{25} The body regulates cortisol production based on its level in the bloodstream, an excess or deficiency of cortisol in the body leading to Cushing’s syndrome and Addison’s disease, respectively.\textsuperscript{26,27} Salivary cortisol levels for patients with Cushing’s syndrome are 12 ng/mL in the morning and 9 ng/mL in the late night,\textsuperscript{28} while for patients with Addison’s disease, it shows 1.5 ng/mL level in the morning.\textsuperscript{29}

Normal cortisol levels in blood are high in the morning (50–230 ng/mL) and low at night (30–160 ng/mL).\textsuperscript{3} Related to levels of cortisol in blood, salivary cortisol levels also change during the day. Yamakana et al. showed that salivary cortisol changes during the day with the highest concentration (~3.7 ng/mL) in the morning and lowest in the evening (0.3 ng/mL).\textsuperscript{30} Cortisol levels increase in response to both physical and psychological stress, such as illness, injury, or depression.\textsuperscript{31,32} Monitoring cortisol levels has become an important diagnostic indicator of stress and physiological state. Cortisol is found primarily in a conjugated (bound) form with proteins while circulating in the blood. The major binding proteins are corticosteroid-binding globulin and albumin, which bind approximately 90 and 7% of cortisol, respectively.\textsuperscript{33} Cortisol is found as a prominent biomarker molecule in the other main biofluids.\textsuperscript{3} However, obtaining accurate cortisol levels in urine is negatively affected by possible diuretic medication and pregnancy status. For sweat, the collected sample volume is relatively low and its biomarker levels are affected by skin conditions.\textsuperscript{7} Saliva provides a simple noninvasive and nonstressful source of sample fluid for analysis. Interestingly, salivary cortisol concentration has been reported\textsuperscript{34} to be independent of the saliva generation rate and the amount of mucus present. Furthermore, the transfer of free cortisol from blood to saliva has been reported\textsuperscript{34} to reach equilibrium with a coefficient of 0.97 in ~5 min. The strong correlation between the concentrations of free cortisol in blood plasma and salivary cortisol indicates the absence of cortisol-binding proteins in saliva.\textsuperscript{35} Therefore, the measurement of salivary cortisol is a promising approach to assess free cortisol in the blood.

A variety of methods have been reported for the detection of cortisol in saliva, including chemiluminescent lateral flow immunoassay,\textsuperscript{36} radioimmunoassay,\textsuperscript{37} enzyme-linked immunosorbent assay,\textsuperscript{38} surface plasmon resonance,\textsuperscript{39} liquid chromatography,\textsuperscript{40} and antigen–antibody immunoreactions.\textsuperscript{41} Lateral flow assays (LFAs) are simple paper-based devices that meet all requirements expected from POC biosensors: low-cost, user-friendly, one-step assay performance, high sensitivity, and selectivity with a low limit of detection (LOD). In LFAs, the recognition antibodies are immobilized on the nitrocellulose membrane, which provides a substrate for both reaction and detection. Several groups\textsuperscript{7,42–44} have demonstrated salivary biosensors in the clinically accepted range using various detection elements and methods. Competitive LFA devices using antibodies have been developed for salivary cortisol detection, including a commercially available product, as listed in Supporting Information Table S1. Because cortisol is a very small molecule (362.46 Da), it does not provide multiple binding sites and, therefore, the less-sensitive competitive assay has been utilized rather than the more sensitive sandwich assay. In the competitive assay, a test line is formed in the absence of cortisol in the sample. To increase the sensitivity in the competitive assay LFA, Zhang et al. and Shortliffe et al. adopted the chemiluminescence\textsuperscript{45} and fluorescence\textsuperscript{45} signal probes for the assay, respectively. However, these more complex detection methods require additional considerations, such as enclosed adaptors for the signal reader unit and/or the use of a smartphone camera. For naked-eye detection, Apilux et al. adopted\textsuperscript{44} the silver enhancement technique to precipitate silver ions on gold nanoparticle surfaces, leading to a 3.6× increase in the colorimetric signal intensity but requiring additional steps after forming the test line on LFA devices. Oh et al. developed\textsuperscript{43} the modified competitive assay with depletion and detection zones, which gives higher sensitivity and wider detection range than the conventional competitive assay by evaluating the ratio of two zones. In this approach, signal interpretation can be less intuitive, which causes a usability issue without the use of an external analyzer.

Here, we present an aptamer-based POC lateral flow assay\textsuperscript{46,47} that measures cortisol concentration in saliva by providing a colorimetric signal in the test strip. We have previously reported\textsuperscript{3} the aptamer-based detection of cortisol in sweat using the mechanism of salt aggregation. In this article, we report on the aptamer-based detection of cortisol in saliva using the mechanism of duplex DNA dissociation previously used\textsuperscript{20} for dopamine detection in urine. Unlike the urine case, we have optimized our protocol for the use in salivary detection, which is more practical for frequent cortisol evaluation. For this work, in addition to an improved detection mechanism, the sequence of the cortisol-binding aptamer and the specificity of the DNA capture probes are optimized. All analytical parameters were investigated to obtain high

### Table 1. ssDNA Sequences Used in This Study\textsuperscript{48}

<table>
<thead>
<tr>
<th>name</th>
<th>sequence 5′−3′</th>
<th># of bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA\textsubscript{c}, capture probe</td>
<td>thiol-\textsuperscript{T}TTT TTT TTT TTT TTT TTT TTT TTT TTT TTG TCG TCC CGA CAG</td>
<td>33</td>
</tr>
<tr>
<td>DNA\textsubscript{c}, cortisol−aptamer</td>
<td>CTC TCG GGA CGA CGA CGG CAT GTT CCA TGG ATA GTC ACT AGT CGT CCC-</td>
<td>51</td>
</tr>
<tr>
<td>T DNA (test line)</td>
<td>biotin- CTC TCG GGA CGA C-</td>
<td>13</td>
</tr>
<tr>
<td>C DNA (control line)</td>
<td>biotin- AAA AAA AAA AAA AAA AAA AA</td>
<td>20</td>
</tr>
</tbody>
</table>

Italic and underlined bases indicate complementary sequences.
sensitivity, selectivity, and reproducibility. This includes the optimization of the loading density of aptamers on AuNP surfaces, the ratio of cortisol aptamers (DNA2) to DNA capture probes (DNA1), AuNP concentration, amount of NaCl needed for aging of the conjugated AuNP–aptamers, amount of 6-mercapto-1-hexanol (MCH) for blocking the free space on the AuNP surface, and the concentration of complementary DNA immobilized on the biosensor.

2. EXPERIMENTAL SECTION

2.1. Materials and Solutions. Cortisol-binding DNA aptamer sequences with complementary biotinylated DNA probes, shown in Table 1, and nucleic-acid (NF) water were purchased from Integrated DNA Technologies (Coralville, Iowa). Au nanoparticles (40 nm diameter) were obtained from Nanocomposix (San Diego, CA). Cortisol protein (≥98%), progesterone (≥99%), and β-estradiol (≥98%) were purchased from Fitzgerald (Acton, MA). Sodium phosphate tribasic dodecahydrate (Na2HPO4·12H2O), sodium chloride, magnesium chloride, Tween 20, Triton X-100, Tris (2-carboxyethyl) phosphine (TCEP), bovine serum albumin (BSA), neuropeptide Y (NPY; human, ≥95%), sodium saline citrate (SSC) 20X, 6-mercapto-1-hexanol (MCH) and phosphate-buffered saline (PBS), Tris buffer (pH 8), nitrocellulose (NC) membrane (Millipore HF135), cellulose fiber sample/wicking pad (CFSP001700), Amicon ultrafilters, cysteamine, and sucrose were purchased from MilliporeSigma (St. Louis, MO). Glass fiber pads (8950) were purchased from Ahlstrom (Helsinki, Finland). Streptavidin was obtained from IBA (Göttingen, Germany). Cortisol-competitive human enzyme-linked immunosorbent assay (ELISA) kit was purchased from Thermo Fisher Scientific (Waltham, MA).

Artificial saliva was obtained from Pickering Laboratories (Mountain View, CA). The Pure-SAL saliva collection kit was purchased from Oasis Diagnostics (Vancouver, WA). Human saliva samples were obtained from healthy volunteers using Pure-SAL, and the samples were treated prior to usage using Amicon ultrafilters.

2.2. Preparation of Duplex Aptamer–AuNP Conjugates. The 40 nm AuNPs with initial optical density (OD = 1) were concentrated to OD = 7.5. The consistency of concentrated AuNPs was validated by UV–vis spectrum measurements at 525 nm (NanoDrop One spectrometer, Thermo Fisher Sci.). Conjugation of duplex aptamer–AuNP was performed after hybridization between DNA1 and DNA2 with a ratio of 2:1. DNA1 contains poly T and a short sequence that is a partial complement to DNA2 (cortisol–aptamer). DNA1 (10 μM) and DNA2 (10 μM) were mixed at a 2:1 ratio and then heated for 5 min at 95 °C. The mixture was cooled down at room temperature (RT). After hybridization, 50 μL of thiolated duplex aptamer (3.3 μM) is activated with 2.5 μL of TCEP (20 mM) and then incubated for 1 h at RT. TCEP was used to reduce the disulfide linkage between thiol and sulphydryl groups in DNA2. Three microliters of the reduced-duplex aptamer was added to 40 μL of concentrated AuNP solution under mild shaking for 1 h followed by incubation at RT for ~8 h. Solutions were aged with NaCl (1 M) to increase the aptamer loading density without AuNP aggregation. NaCl was slowly added to solutions dropwise until the final concentration of 70 mM (Figure S1), and then solutions were incubated at 4 °C overnight. To block the unmodified sites and eliminate the nonspecific adsorption on the surface of AuNPs, 2 μL of MCH (28 μM) was added to 50 μL of aged AuNP–aptamer solution and incubated for 1 h. Excess thiolated aptamer and MCH were removed by withdrawing the supernatant after centrifugation at 8000 rpm for 10 min, and the AuNP conjugates were resuspended in NF water. This washing process was repeated three times. The final conjugated AuNP–aptamer samples were then stored at 4 °C for future experiments.

2.3. Preparation of Streptavidin–Biotin–DNA Conjugates. The test line DNA (T DNA) was designed to capture the AuNP–DNA3 complex in the test zone. A streptavidin–biotinylated T DNA conjugate was prepared to be immobilized on the test zone. Streptavidin was dissolved in 10 mM PBS (pH 7.4) to a final concentration of 1 mg/mL. Streptavidin and T DNA (8 μM) with a ratio of 1:3 was incubated at RT for 1 h to provide stable binding. Sample solutions were filtered (30 kDa, Millipore Amicon Ultra) for 30 min at 6000 rpm to remove the remaining free T DNA. Conjugated streptavidin–T DNA, which remained in the filter due to the large molecular size of streptavidin (55 kDa), was removed from the filter membrane and resuspended with PBS up to its initial volume. The mixture was stored at 4 °C for future use. To prepare the capture probe for the control line, 40 μL of C DNA (10 μM) was mixed with 40 μL of streptavidin (1 mg/mL). After 1 h incubation in ambient conditions, 60 μL of PBS (10 mM) and 20 μL of SSC 20X were added to the mixture.

2.4. Fabrication of the Lateral Flow Test Strips. Test strips are composed of four key components: sample pad, blocking pad, nitrocellulose membrane, and wicking pad. These are mounted on an adhesive backing card with 2 mm overlaps to enable smooth fluid flow between components, as shown in Figure 1. We used nitrocellulose (NC) membranes (Millipore HF135) with a 2.5 cm length. Cellulose fiber membranes (CFSP001700, MilliporeSigma) were used for both the sample pad and the wicking pad at the end of the test strip. The sample pad was soaked in a buffer solution (0.15 mM NaCl, 0.05 M Tris, and 0.25% Triton X-100, pH = 8.0) to facilitate the flowing of the sample solution onto the test strip. The purpose of the “blocking” pad located between the sample pad and the NC membrane is to release stored blocking agents that will reduce nonspecific binding of conjugated AuNPs to
the NC membrane as fluid flows through the membrane. Blocking pads were prepared using a soaked glass fiber membrane (8950, Ahlstrom) with blocking agents (5% BSA, 20 mM Na₃PO₄, 10% sucrose, and 0.25% Tween 20). Both sample and blocking pads were soaked for 30 min in their respective buffers and then dried at 45 °C for 90 min before the assembly of the LFA device. For immobilizing the test and control DNAs on the NC membrane, streptavidin was applied as a fixing agent to react with the biotinylated DNA. T DNA and C DNA were dispensed on the nitrocellulose membrane by a Biojet AD1500 (BioDot, Irvine, CA) to form the test and control lines with a separation of 5 mm. Next, the membranes were dried at 65 °C for ~10 min and stored in a nitrogen-filled box. C DNA immobilized on the control line can capture AuNP conjugated with DNA₁ to confirm the validity of the test strip. An automatic strip guillotine cutter (CM4000, BioDot, Irvine, CA) was used to cut individual 5 mm wide assembled LFA strips.

Test line location was evaluated to form consistent test lines while minimizing any negative interaction between AuNP/aptamer conjugates and the nitrocellulose membrane. While a location closer to the sample/buffer pad was preferred to avoid any negative effect on AuNP/aptamer conjugates, the test line formation was not consistent when it is located too close to the sample/buffer pad (Figure S2). Also, 80 μL of sample solution was selected because it is a minimum volume that can reach the wicking pad consistently.

2.5. Detection Process on Test Strips. Cortisol stock solution was prepared by dissolving 1 mg in 100 μL of ethanol and 900 μL of saliva (real or artificial). Sample solutions containing desired concentrations of cortisol were mixed with conjugated AuNP–duplex DNA and incubated for 30 min at 37 °C. An 80 μL portion of the mixture was dispensed onto the sample pad. Distinct red bands on the LFA strip appeared in ~15 min. Then, the images of the test strips were analyzed using ImageJ analysis software. The output peak area was utilized to quantify the cortisol concentration in the sample.

3. RESULTS AND DISCUSSION

3.1. Principle of Cortisol Detection by LFA Biosensor. The principle for the aptamer-based lateral flow biosensor to detect cortisol in saliva is based on DNA duplex dissociation in the presence of the target analyte. In the presence of cortisol, DNA₁ on the conjugated AuNP–duplex aptamer (Figure 2a) binds with cortisol. In the process, DNA₂ undergoes conformational changes and is dehybridized from DNA₁ as shown in Figure 2b. The concentration of duplex aptamer and its complementary aptamer on the test zone were optimized to 10 and 8 μM, respectively.

Two different complementary sequences of DNA₁ were immobilized onto the NC membrane forming the test (T DNA) and control (C DNA) lines, respectively (Figure 1). As illustrated in Figure 1, when the sample solution is dispensed to the sample pad, the solution wets the sample pad and moves toward the wicking pad driven by the capillary action. The dissociated AuNP–DNA₁ molecules released by the cortisol–aptamer interaction are free to be captured by the T DNA on the test line through binding between complementary sequences (Table 1). Therefore, the color intensity on the test line is directly related to the concentration of cortisol in the solution. As the liquid continues to flow through the NC membrane, excess free and/or duplex AuNP–DNA₁ aptamers are captured by C DNA through 20T–20A binding resulting in the formation of a red band on the control line.

3.2. Cortisol in Artificial Saliva. The analytical performance for the detection of salivary cortisol was studied by adding known amounts of cortisol standard solutions (0, 1, 2, 4, 6, 8, 10, and 15 ng/mL) to artificial saliva. Artificial saliva was diluted 10X in NF water due to its high viscosity, which did not allow the solution to flow through the NC membrane. As shown in Figure 3, it is observed that the test line peak intensity increases monotonically with increasing cortisol concentration, up to 10 ng/mL. The cortisol in artificial saliva was detectable as low as 2 ng/mL by the naked eye.

Further, using ImageJ, the corresponding results were analyzed by measuring the average green channel intensity of the test/control line (nominally red color) against that of the background nitrocellulose (white) surrounding it. Color intensities were averaged vertically and horizontally across both test and background regions to acquire area averages. The green channel of the RGB color space was found to provide superior signal resolution compared to that of the red channel and to grayscale measurements due to the nitrocellulose (white) having high red values in the RGB color space. Therefore, test/control line formation (red) correlates to a decrease in the green and blue channels as opposed to an increase in the red channel. This makes the green (and blue) channel measurements superior to grayscale measurements (average of red, green, and blue channels) due to the removal of the nonchanging red channel. Consequently, a limit of detection <1 ng/mL is achieved for cortisol in artificial saliva using this technique.

Figure 2. Cortisol detection mechanism based on duplex aptamer dissociation: (a) conjugated AuNP–duplex DNA₁−DNA₂ and (b) DNA₂ dissociation from capture strand in the presence of cortisol molecules.

Figure 3. Test to control (T/C) line ratio obtained by ImageJ analysis to measure cortisol concentrations (0–15 ng/mL) in artificial saliva at 15 min after dispensing sample solutions. Error bars indicate the standard errors of three independent experiments (n = 3). Inset: image of corresponding lateral flow strips tested with different concentrations of cortisol (from left to right: 0, 1, 2, 4, 6, 8, 10, 15 ng/mL).
The selectivity of the aptamer-based LFA (apertasensor) was also evaluated by testing with several counter-targets and stress biomarkers: progesterone, β-estradiol, and neuropeptide Y (NPY). The normal physiological concentration of salivary NPY is \(~0.05 \text{ ng/mL}^{5}\). Studies of salivary progesterone show a level of 47 pg/mL in the follicular phase increasing to 94–235 pg/mL in the postovulatory period. The mean salivary β-estradiol concentrations reported by Ham et al.\(^{52}\) were 9.0 \pm 2.6 and 11.7 \pm 4.1 pg/mL in early follicular phase and during ovulation, respectively.

The steroid hormone stock solutions were prepared at a concentration of 1 mg/mL in 10% DMSO and 90% diluted artificial saliva. The concentrations of all biomarkers were set at 100 ng/mL in diluted artificial saliva. As shown in Figure 4, a significant signal response was observed only toward cortisol, while other biomarkers presented minor signals comparable to the control even at these concentrations extremely higher than their normal physiological levels, indicating the excellent selectivity of this aptamer-based lateral flow biosensor for cortisol in saliva.

### 3.3. Salivary Cortisol Measurements.

For the present study, whole saliva samples were collected from four healthy adult volunteers in our laboratory using the Pure-Sal saliva kit\(^{55}\) (Oasis Diagnostics, Vancouver). The Pure-Sal kits were used according to the manufacturer’s instructions by resting the absorbent swab inside the mouth until it was saturated (5 min). Then, the swab was placed into a syringe that contains a filter for immediate compression and filtration. Volunteers were asked to donate morning saliva samples before eating and brushing/rinsing their mouths. For the collection of afternoon saliva samples, volunteers were asked to rinse their mouth and wait for at least 10 min before using Pure-Sal. The saliva samples were initially kept at \(-20^\circ C\) and then transferred into a \(-80^\circ C\) freezer for future study. Saliva is a complex biological fluid that contains large proteins like mucins that could interfere with the assay interactions and contribute to what is known as the matrix effect of saliva. UV–vis optical absorption measurements were performed on the whole saliva (WS) samples taken with/without Pure-Sal and compared to the use of a 50 K Amicon ultrafilter. As shown in Figure 5, the large peak at \(\lambda = 285\) nm indicating protein absorbance is significantly reduced using Pure-Sal, reaching a value closer to that obtained using the 50 K Amicon ultrafilters.

Although the Pure-Sal collector removes a significant proportion of large molecules in human saliva samples, the presence of some remaining proteins still interferes with the assay performance on test strips. High concentrations of ions or proteins in biological samples like saliva increase van der Waals attractive forces between particles, which induce aggregations of AuNPs in our devices.\(^{54}\) We have investigated Amicon ultrafilters with various mass cutoff values (50, 30, 10, and 3 K) for saliva samples to determine the optimum molecular cutoff needed to allow assay interactions. Morning (am) and afternoon (pm) saliva samples were centrifuged at 6000 rpm for 30 min. One hundred microliters of filtrated saliva with different molecular cutoff levels were introduced into the 3 μL conjugated AuNP–apta[...]

**Figure 4.** Selectivity of the aptamer-based LFAs for detecting cortisol vs other biomarkers in artificial saliva (100 ng/mL, 80 μL) at 15 min after dispensing sample solutions, average, and standard error for \(n = 3\) replicates.

**Figure 5.** UV absorbance of whole human saliva samples without filtering and after using either a Pure-Sal collector or a 50 K Amicon ultrafilter.
The T/C ratio for each cortisol concentration (I) is then divided by the T/C ratio of the blank case (I₀, no added cortisol). Finally, the normalized intensity (I/I₀) is plotted vs cortisol concentration to generate a calibration plot (Figure 7).

Figure 7. Calibration curves for cortisol obtained in artificial (LOD = 0.99 ng/mL) and human saliva (LOD = 0.37 ng/mL). Images were analyzed with ImageJ software to quantify the signal intensity of the T and C lines and plotted as described in Section 3.4.

Using the obtained calibration curve, the aptasensor showed an LOD of 0.37 and 0.99 ng/mL for cortisol in human and artificial saliva, respectively. Interestingly, this is similar to the reported LOD for aptamer-based detection of cortisol in sweat of ~1 ng/mL.

It is not totally clear at this point what causes the different signal intensities in human and artificial saliva. It is possible that some critical components present (and not filtered) in human saliva samples enhance the binding between cortisol and the aptamers.

The limit of detection (LOD) was calculated based on the value of the limit of blank (LOB), which is the highest apparent analyte concentration for blank samples (no analyte). The LOD was calculated using the following equations:

\[
\text{limit of blank (LOB)} = \text{mean control} + 1.645 \times \text{SD}_{\text{control}}
\]

\[
\text{LOD} = \text{LOB} + 1.645 \times \text{SD}_{\text{lowest concentration sample}}
\]

The current working range of the LFA assay is from ~0.5 to ~15 ng/mL, which covers the normal range of cortisol in saliva of ~1–10 ng/mL. Signal saturation of the test line intensity is present for concentrations > 10 ng/mL, possibly due to the absence of additional available binding sites on the test line. In the current work, the experimental conditions were optimized to increase the test strip sensitivity under normal concentrations of salivary cortisol. For highly stressful conditions, cortisol levels can be higher than the normal range. To quantify the cortisol concentration for individuals with persistently high levels, one can either lower the sensitivity (e.g., lower AuNP concentration, increasing the aptamer to AuNP ratio, increasing the complementary aptamer length, etc.) or simply dilute the collected saliva samples in the appropriate ratio.

We assume that the presence of proteins and ions in real saliva provided positive matrix interference that led to lower LOD in real saliva. Proteins and ions have provided higher intensities at both T and C lines by inducing small aggregation on conjugated AuNP–DNA1. In addition, human saliva samples in the nitrocellulose membrane presented the higher rate of interactions with the immobilized reagents at the T and C lines.

To investigate the accuracy of the proposed aptasensor, morning saliva samples from four volunteers were analyzed. The saliva samples were pretreated with 10 K Amicon ultrafilters and then added to the conjugated AuNP–aptamer solution. Following incubation at 37 °C for 5 min, samples were dispensed on the test strips. The cortisol concentration of the saliva samples was calculated from the intensity of the I/I₀ ratio of test strips for each subject and the real saliva calibration curve. The experimental results of four independent measurements for each subject were compared to ELISA measurements (n = 2) from the same saliva samples, as shown in Figure 8. The data points and error bars represent the mean and relative standard errors, respectively. A good correlation between the aptasensor values and the ELISA analysis was achieved for all samples (98.6%), with recovery values in the range from 89 to 114%.

3.5. Biosensor Stability Analysis. To investigate the sensor stability both in solution and during lateral flow operation, we tested strips at different storage times. A set of 27 strips was fabricated under identical conditions and stored in a nitrogen-filled box at RT for 2 months. Cortisol at a concentration of 1 ng/mL in artificial saliva was tested on three test strips at storage days 0, 5, 10, 15, 20, 25, 30, 40, and 60 to determine the immobilized biochemical component stability. As shown in Figure 9a, the signal intensity of the test line (peak

Figure 6. Assay results for the detection of cortisol in human saliva: (a) ImageJ peak area intensity for images taken at 15 min after assay start for saliva samples with cortisol concentrations from 0 to 15 ng/mL; error bars represent the standard error from four different saliva samples, each performed in triplicate (n = 12); and (b) photographs of lateral flow strips tested with increasing cortisol concentrations from top: 0, 0.5, 1, 2, 3, 4, 5, 10, and 15 ng/mL.

Figure 8. Cortisol levels obtained from aptasensor assay and standard ELISA method. Cortisol concentrations were obtained from the calibration curve in human saliva.
area) on the test trip was essentially constant and without loss of activity, with the average of signal intensity and SD of 11.1 and 0.18, respectively.

UV−visible absorption spectra (Figure 9b) were obtained from samples (n = 3) of conjugated AuNP−aptamer solutions maintained at 4 °C for 2 months. The spectra exhibit nearly identical absorbance peaks at λ = 260 and 525 nm, representing the DNA and the dispersed AuNPs, respectively. This demonstrates the high stability of the prepared solutions without any aggregation or degradation in a 2 month period.

4. SUMMARY AND CONCLUSIONS

We have presented the first report of an aptamer-based lateral flow biosensor to quantify cortisol levels in human saliva. The low-cost and high-sensitivity LFA assay is intended as single-use, disposable point-of-care test. The assay was designed based on the dissociation of a duplex cortisol−aptamer upon the introduction of the target cortisol in the clinically accepted concentration range. Increasing concentration of the target in the sample leads to increased dissociation of the duplex aptamer and higher intensity at the test line of the LFA. Cortisol at various concentrations was added to cortisol-free human saliva samples to obtain the limit of detection and unknown concentration of human salivary cortisol based on the calibration curve. Under optimal conditions, a detection range of the cortisol concentration within 15 min was from 0.5 to 15 ng/mL and the LOD was 0.37 ng/mL in human saliva. This indicates that the aptamer-based biosensor has similar LOD to antibody-based assays while being able to benefit from the advantages of aptamers (such as lower cost, higher stability, potential improvement with future SELEX procedures). The fact that the calibration of the assays was performed against human saliva (not buffer solution or artificial saliva) makes the results more accurate. To further improve the LFA performance, more sensitive and selective aptamers can be developed by repeating SELEX processes more times with additional modifications. In this study, to eliminate the matrix effect on AuNP aggregation, human saliva collection using Pure-Sal collectors followed by centrifugation was implemented. To develop an onsite detection biosensor, further studies need to be done on the surface chemistry of AuNPs to increase their stability in the presence of ionic solutions. One possible approach is optimizing salt-aging conditions to further increase the aptamer density on AuNP surfaces. Moreover, assay sensitivity could be enhanced by incorporating a different nanoparticle, such as upconverting phosphor (UCP), that has a strong sample tolerance in various matrices. Studies have shown that the sensitivity of the test trips incorporating UCP is increased by 10−100× compared with using AuNPs.56 The drawback of using UCPs is the need for an instrument to read the result, which could be an obstacle to performing POC testing in certain situations. While here we report on the use of aptamer-based detection of salivary cortisol, the approach could be applied for rapid quantitative screening of cortisol and other biomarkers in various biofluids.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03223.

Optimizing loading density of aptamer on the AuNP surface; salivary cortisol analysis by ELISA; and investigation of ultrafilters to treat saliva (PDF)

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Notes
The authors declare no competing financial interest.

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**REFERENCES**


An Aptamer-Based Lateral Flow Biosensor for Rapid Detection of Salivary Cortisol

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\textsuperscript{*}

Supplementary Material

**Optimizing the loading density of aptamer on AuNPs surface.**

NaCl at different concentrations (0, 20, 40, 50, 60, and 80 mM) was added to aptamer-AuNP solutions. To determine the optimum concentration of NaCl needed for aging, 0.5 µL NaCl 1M added to the solutions in a dropwise sequence every 20 min. After aging and incubation for at least 12 h at 4°C the samples were centrifuged at 12000 rpm for 10 min. Then the supernatant was measured by UV-Vis for absorption by free aptamers at 260nm. As shown in Figure S1, increasing the amount of salt in solutions, results in a monotonic decrease in absorbance. The decreasing amount of free aptamers in solution indicates an increasing loading density of aptamers on the surface of AuNPs. The free aptamer absorption reached a minimum at \(~ 70\) mM NaCl concentration, which was then selected as the optimum amount of NaCl in the solution for the aging process.

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Figure S1 UV-Vis absorbance (at $\lambda = 260$ nm) of free aptamers in solution as a function of added NaCl concentration in aptamer-AuNP solutions for stabilizing the conjugated AuNP-aptamer at different concentrations.

As shown in Figure S2, the test line formation was investigated by changing the test line location near to the sample/buffer pad. The solution dispensed is the conjugated AuNP-aptamer with no cortisol. Therefore, no color should appear at the test line. The inconsistency of test line formation could be due to an insufficient effect of the blocking buffer for a test line location that is too close to the sample/buffer pad.

Figure S2. Inconsistent test line formation (green box) located near the sample/buffer pad. Control (no cortisol) solutions were utilized for all strips.
Salivary cortisol analysis by ELISA

Quantitative salivary cortisol measurement was determined using cortisol ELISA testing according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA), see Table S1. The collected saliva samples by Pure•SAL were diluted 4 times with 1× Assay Buffer. Briefly, cortisol ELISA works as a competitive binding assay. The wells of the microplate are coated with monoclonal antibodies to cortisol. First, the saliva samples in a set of standards are added to the wells. Then cortisol conjugated to horseradish peroxidase (HRP) is introduced to each well. Cortisol in saliva samples competes with cortisol conjugated HRP to bind with the antibody binding sites in wells. The reaction produces a yellow color, which reflects the amount of peroxidase detected. Therefore, the absorbance at 450 nm is inversely related to the amount of cortisol in saliva samples.

Saliva samples collected in the morning (am) and afternoon (pm) from four volunteers were tested two times. The mean value of the cortisol concentration represents the amount of cortisol in samples. Cortisol concentration, SD and %CV (coefficient of variation) values in Table S1 for the unknown samples were calculated by the microplate reader.
Table S1 List of the state-of-the-art lateral flow assay devices for salivary cortisol detections.

<table>
<thead>
<tr>
<th>Salivary cortisol detection technology</th>
<th>Authors (Affiliation) [Ref]</th>
<th>Recognition element</th>
<th>Medium</th>
<th>Sample collection</th>
<th>Range (ng/mL)</th>
<th>Assay time</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Flow Assay (IPRO sCORT POC LFD) ¹</td>
<td>Dunbar et al. (IPRO Interactive, UK)</td>
<td>NA</td>
<td>Human saliva &amp; buffer mixture</td>
<td>IPRO Oral Fluid Collector (OFC) – 0.5 mL, 20-50 sec</td>
<td>0.75-15</td>
<td>12 min</td>
<td>Commercially available</td>
</tr>
<tr>
<td>Competitive LFA using smartphone ²</td>
<td>Choi et al. (Yonsei Univ, Korea)</td>
<td>Cortisol–BSA (ref)</td>
<td>Goat anti-mouse IgG (test line)</td>
<td>Buffer and human saliva</td>
<td>Swab in mouth, then mix with buffer</td>
<td>0.01-10</td>
<td>10 min</td>
</tr>
<tr>
<td>Chemiluminescent LFA using smartphone (competitive assay) ³</td>
<td>Zangheri et al. (U of Bologna, Italy)</td>
<td>Anti-cortisol antibody (test line)</td>
<td>Anti-peroxide antibody (control)</td>
<td>Human AM saliva; PM saliva for spiking</td>
<td>Salivettes cotton swabs (60-90sec), then compressed in syringe.</td>
<td>0.3-60</td>
<td>25 min</td>
</tr>
<tr>
<td>VerOFy® fluorescence LFT point-of-care cortisol system with LIAM reader (UV excitation) ⁴</td>
<td>Shircliff et al. (Iowa State Univ; Oasis Diagnostics)</td>
<td>BSA-Cortisol on nitrocellulose. Eu-fluorescent particle conjugate in conjugate pad</td>
<td>Human saliva</td>
<td>Super SAL syringe with filter and pad.</td>
<td>0.5-25</td>
<td>20 min</td>
<td>Two Europium conjugates react with free and conjugate bound cortisol</td>
</tr>
<tr>
<td>Competitive LFA with silver enhancement ⁵</td>
<td>Apilux et al. (Mahidol Univ, Thailand)</td>
<td>Cortisol-BSA (test)</td>
<td>Goat anti-mouse IgG antibody (control)</td>
<td>Artificial saliva &amp; human saliva (centrifuged &amp;10x diluted)</td>
<td>NA</td>
<td>0.5-15</td>
<td>15-20 min</td>
</tr>
<tr>
<td>Trap LFA (wide range highly sensitive positive assay) ⁶</td>
<td>Oh et al. (GIST, Korea)</td>
<td>Cortisol-BSA (deletion)</td>
<td>Anti-mouse IgG antibody (detection)</td>
<td>Buffer &amp; Human saliva w. PBS (1:1)</td>
<td>Salivette® Cortisol</td>
<td>0.01-100</td>
<td>15 min</td>
</tr>
<tr>
<td>SOMA LFD →C (orig. from IPRO on Row 1) ⁷</td>
<td>Soma Bioscience</td>
<td>NA</td>
<td>Saliva &amp; buffer mixture</td>
<td>OFC - Swab &amp; Buffer</td>
<td>0.5-15</td>
<td>12-13 min</td>
<td>Commercially available SOMA LFD Reader</td>
</tr>
</tbody>
</table>

S4
Table S2 ELISA analysis from human saliva samples collected from 4 volunteers in am and pm and tested twice. Samples were diluted to 25% concentration, therefore the final measured concentration values were multiplied by 4.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Mean Conc. of (pg/mL)</th>
<th>Measured Conc. (pg/mL) (×4)</th>
<th>Measured Conc.* (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- am</td>
<td>208.96</td>
<td>835.82</td>
<td>0.84</td>
</tr>
<tr>
<td>1- pm</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2- am</td>
<td>391.78</td>
<td>1567.13</td>
<td>1.57</td>
</tr>
<tr>
<td>2- pm</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3- am</td>
<td>411.68</td>
<td>1646.73</td>
<td>1.65</td>
</tr>
<tr>
<td>3- pm</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4- am</td>
<td>634.86</td>
<td>2539.45</td>
<td>2.54</td>
</tr>
<tr>
<td>4- pm</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Samples diluted 4×

**Investigation of ultrafilters to treat saliva**

Optimum ultrafilter selection was investigated in order to prevent aggregation of AuNPs upon adding saliva samples to the conjugated AuNP-aptamer. Saliva samples collected in the morning (am) and afternoon (pm) from two volunteers were passed through Amicon ultrafilters with different molecular cut-off levels and then introduced into the conjugated AuNPs-aptamer solution following by passing. As seen in Figure S2, utilizing 30K Amicon ultrafilters for sample #1 (am & pm) could prevent the aggregation of conjugated AuNP-aptamer (red color), while using 30K ultrafilters for sample #3 (am & pm) leads to aggregation (blue color). Proteins and other organic matter in saliva vary from person to person and also vary for most individuals during the day. Overall, Amicon ultrafilter with 10K or less molecular cut-off level can prevent from conjugated AuNP aggregation after adding any saliva samples. Therefore, for further experiments, Amicon ultrafilter with 10K molecular cut-off level has been utilized.
<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Amicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-am</td>
<td><img src="a.png" alt="Image" /></td>
</tr>
<tr>
<td>1-pm</td>
<td><img src="b.png" alt="Image" /></td>
</tr>
<tr>
<td>3-am</td>
<td><img src="c.png" alt="Image" /></td>
</tr>
<tr>
<td>3-pm</td>
<td><img src="d.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure S3 Aggregation of conjugated AuNP-aptamer upon adding saliva filtered with Amicon filters of various molecular cut-off values: (a) volunteer 1 – am sample; (b) volunteer 1 – pm sample; (c) volunteer 3 – am sample; (d) volunteer 3 – pm sample.
References


