DEVELOPMENT OF COLORIMETRIC PAPER-BASED ANALYTICAL DEVICE FOR CORTISOL DETECTION

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ในปัจจุบันนี้การตรวจวัดคอลติซอลมีความสำคัญในการศึกษาเกี่ยวกับโรคที่เกี่ยวข้องกับความเครียดมีหลายงานวิจัยที่ใช้เทคนิคอิมมูโนแอสเสย์แบบแข่งขันสำหรับตรวจวัดฮอร์โมนที่มีโมเลกุลขนาดเล็ก อิมมูโนแอสเสย์เป็นวิธีหนึ่งที่ใช่อย่างแพร่หลายเนื่องจากความใช้ง่ายและความรวดเร็วในการตรวจวัด แต่มีข้อเสียคือตัวตรวจวัดจะต้องการ ตัวตรวจวัดสัญญาณที่ใช้งานยาก หรือ ต้องการสารเคมีเพื่อที่จะเพิ่มสัญญาณมากขึ้น ในงานนี้เราจะทำให้ตำแหน่งที่ตรวจวัดเป็นช่องที่แคบลง เพื่อที่จะเพิ่มสัญญาณในการตรวจวัดจากการเพิ่มความเข้มของสารที่จะผ่านแอนติบอดีที่ถูกตรึงอยู่ที่ตำแหน่งที่ตรวจวัด ซึ่งสัญญาณจะเพิ่มขึ้นเกือบสองเท่าเมื่อเปรียบเทียบกับตัวตรวจวัดปกติโดยในงานนี้จะใช้โทรศัพท์เป็นตัวตรวจวัด โดยได้ช่วงความเป็นเส้นตรงอยู่ที่ 5 ถึง 5,000 นาโนกรัมต่อมิลลิลิตรและได้ค่าขีดจำกัดของการตรวจวัดและขีดจำกัดการวัดเชิงปริมาณอยู่ที่ 1.6 และ 4.8 นาโนกรัมต่อมิลลิลิตรตามลำดับจากผลสามารถสรุปได้ว่าตัวตรวจวัดนี้สามารถใช้ในการตรวจวัดคอลติซอลในเลือดได้อย่างมีประสิทธิภาพและเราเชื่อว่าจะสามารถนำไปใช้กับสารประกอบโปรตีนชนิดอื่นๆได้

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Nowadays, the detection of cortisol can play a key role in understanding stress-related diseases. Several competitive immunoassay formats have been reported for the detection of this small hormone molecule. Lateral flow immunoassay (LFIA) is one of the most widely used platforms that offer its simplicity and rapidity for such analysis. Unlike previous sensors for cortisol sensing that require complicated readers, specialized reagents, or even post-treatment methods to amplify the obtained signal, in this work, we employed a concave test zone constructed on the LFIA device (cLFIA) which can directly enhance the signal response as the flowing sample was substantially concentrated before passing through the immobilized antibody. Nearly 2-fold sensitivity enhancement was obtained using this straightforward immunoassay format. A smartphone was used as a detector and the linear calibration plot of this cLFIA was established in the range of 5 to 5,000 ng mL^{-1} with an LOD and LOQ of 1.6 and 4.8 ng mL^{-1}, respectively. From the result, it could be concluded that this cLFIA device could be applied to detect cortisol in human serum effectively. In addition, This cLFIA device is believed to explore new possibilities for the determination of other protein compounds and exploit them in point-of-care testing.

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Student's Signature .....
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CHAPTER I

INTRODUCTION

1.1 Introduction

Cortisol is a steroidal hormone that is released from the adrenal cortex. This hormone can control blood pressure, metabolic activities, and cardiovascular function. Since cortisol production is influenced by physical and psychological stress, monitoring cortisol levels can play a decisive role in understanding stress-related diseases [1]. Typical cortisol levels in the blood are between 50–230 ng/mL in the morning and slowly decreased to 30–160 ng/mL throughout the day [2]. Unusual high and low cortisol levels in the body lead to Cushing's syndrome and Addison's disease, respectively [3].

Currently, various standard methods to detect cortisol are usually ground on immunoassays coupled with a series of detection techniques such as chemiluminescent [4], surface plasmon resonance (SPR) [5], or electrochemical detection [6]. These detection methods generally rely on the utilization of sophisticated apparatuses, time-consuming and tedious procedures, impeding their practical implementation as a point-of-care (POC) testing in actual clinical use. Although enzyme-linked immunosorbent assay (ELISA) with colorimetric, fluorescent, or luminescent methods have been widely reported with sufficient sensitivity, their limitations arise from the use of the labor-intensive and expensive (microplate) readers,

as well as the complexity from the measurement of enzyme activity [7]. Hence, a more straightforward, faster, and cheaper method is of primary interest, especially for the on-site screening test. This has led to the uptake of the paper-based lateral flow immunoassay (LFIA) as the most prominent POC diagnostic device due to its compromising sensitivity, simplicity, speed, and cost [8]. Therefore, the LFIA device is of interest in this study.

There are two commonly used immunoassay formats: competitive and non-competitive. Specifically, competitive immunoassays have been used effectively to measure small analytes such as drugs or hormones [9]. Indeed, cortisol is considered a low molecular weight hormone that fits the competitive assay [10]. In the competitive format, the unlabeled analyte competes with a fixed amount of labeled similar analyte (often gold nanoparticle (AuNP) labeled analyte). Only a small amount of analyte is efficient to produce a noticeable response. However, the sensitivity of this format is not considerably high compared to the sandwiched format and often yielded an inadequate limit of detection. Hence, the sensitivity enhancement of the competitive immunoassay is still challenging and yet to be explored.

Generally, there are two main strategies to improve the sensitivity: (i) chemical modification and (ii) geometrical modification of the device. For (i) chemical modification, several reports have demonstrated the post-treatment method to amplify the obtained signal. For example, the reduction of gold ion (Au^{3+}) catalyzed by the gold nanoparticle (AuNP) at the test line of LFIA in the presence of the reducing

agents has been used in a few studies [11]. The larger size of AuNP caused by Au ion metallization can effectively intensify the color of the colloidal gold (red) into a darker shade (dark violet). However, this technique required additional steps and reagents and eventually prolonged the total analysis time, which is undesirable. Therefore, a geometrical modification (ii) of the LFIA structure is far more promising for end-users. Different architectural modifications of the LFIA device have been proposed, mostly done by the constriction of the lateral flow channel to increase the binding event between immobilized antibody and analyte of interest. The fabrication of the flow path barriers via wax [12] or polymer [13] printing in the flow path is two of the most common methods to increase the immunoreaction between the immobilized antibody and the analyte. Although a significant signal enhancement was remarkably obtained using the strategies, the requirement of materials (such as solid wax, photopolymers, or photoresists [12] and specific equipment (such as a wax printer, plasma machine, laser beam) [14] is still a major obstruction for such fabrication.

In this work, we developed LFIA with a concave test line (cLFIA) fabricated using a straightforward office tool hole puncher. The cortisol presented in the sample and a fixed amount of AuNP-cortisol/BSA will be used in this competitive immunoassay. In the absence of cortisol, AuNP-cortisol/BSA will solely be captured by the immobilized antibody constricted at the concave test zone, thus revealing a red spot from AuNP within 10 min. Conversely, in the presence of cortisol, a small-size cortisol compound will compete with AuNP-cortisol/BSA to capture the antibody. This incoming cortisol

will initially migrate to the test zone while less AuNP-cortisol/BSA can be bound with the antibody on the test zone, thus manifesting a less intense color. Benefitting from this concave flow path architecture, the increased binding events between antibody and antigen are demonstrated. Furthermore, a reduced amount of deposited antibody applied on the constricted test zone can also signify the advantages of the cLFIA proposed in this study. In addition, the ability of the sensor to detect the cortisol level in complex media was also demonstrated using certified human serums (level 1 and level 2). The obtained results were also compared and validated with the standard electrochemiluminescence (ECL) method.

1.2 Objectives

The two main goals of this work are as follows:

1. To develop a novel biosensor for the determination of cortisol
2. To apply the proposed sensor for biological sample analysis

1.3 Scope of the research

To achieve the research objective, the following scope was set:

1. Research and study information of immunochromatography, gold nanoparticles conjugation, cortisol, and optical detection.

2. Study the optimal concentration of cortisol-BSA for conjugation with gold nanoparticles and characterized by UV-visible spectrometry and Dynamic light scattering.
3. Optimize the running buffer component, design of strip, and concentration of antibody.
4. Prepare and optimize competitive immunochromatography for cortisol detection.
5. Result and discussion

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Immunoassays[15]

Immunoassays are bioanalytical methods in which the quantitation of analyte depend on the reaction between antigen and antibody. Urine and serum samples are normally used in immunoassay. The basic principle of detection in immunoassay is based on the binding reaction between target antigen (Ag) and antibody (Ab). The antibody binds specifically with the corresponding antigen which is similar to an enzymatic reaction and involves electrostatic and hydrophobic interaction. The stability of immunoassay reaction is dependent on the complementary shape of the binding site of the antibody and antigen. The combination of antigen and antibody is a reversible process. The strength of the binding of antibody and antigen can be defined by the equilibrium constant (K) as demonstrated in the Equation 1.

$$K = \frac{[AgAb]}{[Ab][Ag]} \qquad \text{(Equation 1)}$$

K = Equilibrium constant of antigen and antibody binding

[AgAb] = Antigen-Antibody complex concentration

[Ab] = Antibody concentration

[Ag] = Antigen concentration

To date, immunoassays have been extensively employed in numerous applications, however, this method is mostly implemented in medical diagnostic area to satisfy the increasing demands of healthcare. The result interpretation is dependent upon combined detection methods, which can either be optical spectroscopy, electrochemical method, fluorescence, or mass spectrometry.

2.1.1 Types of immunoassays

There are two types of immunoassays: competitive and sandwich immunoassays. The competitive immunoassay exploits the competition between nonlabelled and labeled antigen to binding with a limited number of antibodies. The second mode is the sandwich immunoassay, a platform mostly applied with antigens having at least 2 binding sides. These two types of assays inherently have their own characteristics and are employed for a particular purpose.

2.1.1.1 Competitive immunoassays

The detection principle of competitive immunoassays is relied upon the competition between antigen and labeled antigen to react with a certain amount of antibody on the test line. The obtained signal is inversely proportional to the analyte concentration in the sample, in which the faded signal will be observed in the presence of target analyte in a concentration-dependent manner. In other words, it can be stated that the signal intensity is initiated by the labeled antigen bound to the antibody

on the test line. The quantity of analyte in the sample can be determined when the concentration of labeled antigen and antibody are steady. The labeled and non-label antigen will be mixed and bound with antibodies that are coated on the surface. After a fixed period, the signal created by successfully bound labeled antigen is used to construct a calibration or determine the concentration of analyte in the sample. It is important to note that the probability of binding for labeled antigen and antigen is similar. The trend of a normal standard curve for competitive immunoassay is demonstrated in Figure 1, and it shows that, as the concentration of antigen increases, the obtained signal in the bound fraction decreases.

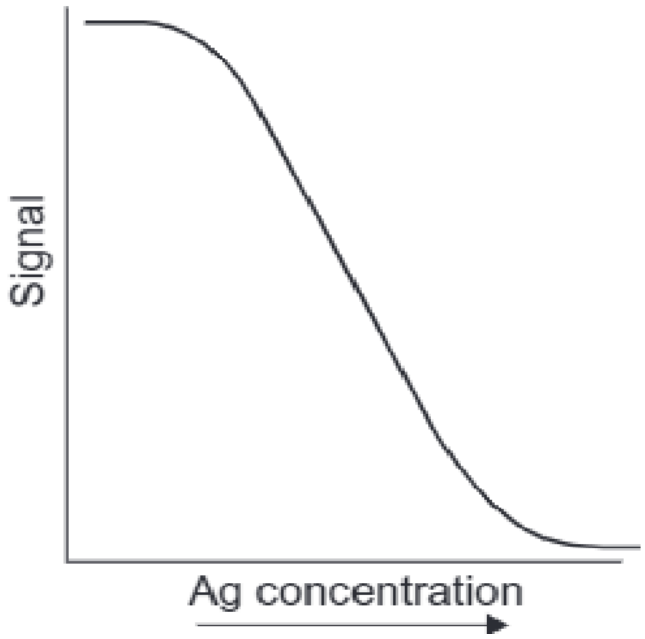

Figure 1 Typical standard curve of competitive immunoassay.

2.1.1.2 Sandwich immunoassay

Sandwich immunoassay uses two capturing antibodies to determine the analyte concentration. This type of assay is generally adopted to detection of target analytes that are large enough to bind with two separate antibodies at the same time. The first antibody is coated on a solid support called the capture antibody. This antibody is used to extract analyte from the matrix. The second antibody is labeled antibody known as a conjugated antibody. This antibody binds with antigen at the different sides of antigen from the capture antibody. The signal is directly proportional to the concentration of analyte in the sample. The normal standard curve for sandwich immunoassay is shown in Figure 2. When the concentration of antigen is increased, the signal is also increased. This is because increasing number of conjugated antibodies are being captured at the test line.

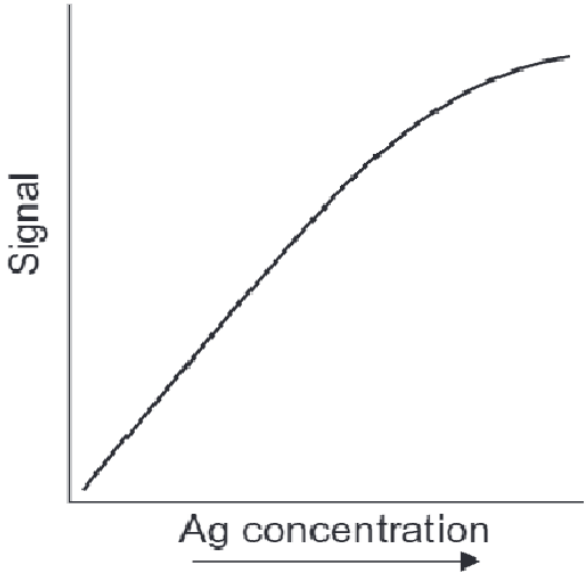

Figure 2 Typical standard curve of competitive immunoassays

2.2 Lateral flow immunoassay (LFIA)

LFIA is a common platform intensively integrated with immunoassay reactions. The detection platform can either be competitive or sandwich assay. LFIA is mostly involved with optical detection and is performed on several substrates such as nitrocellulose membrane (NCM) assembled on a backing card and paper-based device. The specific interaction between antigen and antibody is a key principle of this assay. Based on the previous reports, LFIA is widely used in several areas to examine important molecules or markers such as insecticides, cancer biomarkers, drugs and pregnancy, etc. [16]

The LFIAs typically consist of flow systems and immunoassays reactions. The movement of the sample is driven via capillary force. Four main components in LFIA are sample pad (the area that sample is applied on), conjugated pad where AuNPs or labeling agent that is conjugated with antibody or antigen and dropped on this part, test line (TL) and control line (CT). As depicted in Figure 3, reaction membrane contains TL and CT lines. Typically, a capture probe or antibody that is specific to antigen is immobilized on the TL line. Finally, an absorbance pad is located at the end of the LFIA strip to make the solution flow complete and continuous. All those components are assembled on a backing card.

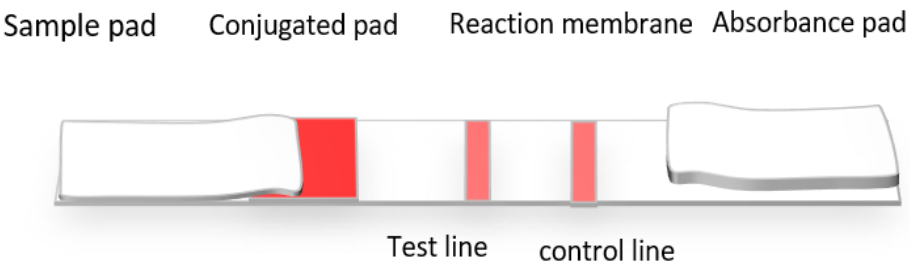

Figure 3 Components of lateral flow immunoassays

In a LFIA assembly, nitrocellulose is a key material to drive reaction and serve as a reaction membrane. Because of the strong dipole of nitrate ester on nitrocellulose and peptide bond of antibody, electrostatic interaction occurs. The electrostatic force is assigned to provide direct immobilization of antibodies or selected probes on the nitrocellulose membrane. Biorecognition element is deposited on the TL and CL. The sample solution dropped on the sample pad will move via capillary force. The sample pad is made from a cellulose acetate membrane that has a low affinity to capture protein. AuNPs conjugated with antibody is dropped on the conjugate pad. The absorbance pad maintains continuous flow and prevents backflow of the sample. All of the components are fixed on the backing card. Quantitative and qualitative analysis of the analyte can be obtained by the naked eye.

For interpreting the results, signal intensity produced by AuNPs is determined using an Image J software. The pore size of nitrocellulose has a strong effect on the sample flow rate. The large pore size of nitrocellulose causes a low flow rate.

Consequently, it is particularly suitable for a low reaction rate of immunoassay. This thing suggests that the rate of reaction should be primarily considered when choosing nitrocellulose.

2.3 Important factors in lateral flow immunoassays [17]

There are many factors that need to be considered when developing LFIA. Elaborate investigation of these important factors is typically required to achieve the desirable performance. The essential parameters are summarized as follow: If the reaction rate between antigen and antibody is slow, the slow flow rate of solution is required to successfully establish antigen-antibody interaction. The increasing flow rate mainly causes a decrease in assay time, sensitivity of the test, and background signal. Optimization of lateral flow immunoassay operating factors should be performed to get good results. The most important parameter of lateral flow immunoassay is the flow rate. The flow rate is restricted by the size of the labeling element and pores size of nitrocellulose. The immunoreaction rate is prescribed by the type of nitrocellulose and buffer migration time. The fast buffer migration will increase signal to noise ratio. The number of antibodies on the test line should be optimized because an excess amount of antibodies causes nonspecific absorption on the test line. Excess amount of antibody increases the stereo-hindrance effect which decreases antibody binding affinity.

Another factor is the amount of colored detector reagent. The color intensity on the test line is proportional to the amount of colored detector reagent and activity of the antibody. Colored detector reagents such as magnetic particles, color latex beads, and gold nanoparticles are used as a label in lateral flow immunoassay. The key factor to choosing a colored detector reagent is that it should be stable and does not change the biorecognition element properties. Gold nanoparticles are mostly used as colored detector elements due to their good optical property and easy to functionalize with antibodies.

Several factors, including the incubation temperature, the incubation time, and buffer condition have major influence on the antigen-antibody binging affinity. These relevant parameters are considered as a primary concern and needed to be optimized to achieve the best condition in immunoassay. Based on previous findings, raising the incubation temperature will increase the rate of antigen and antibody reaction. Lowering the affinity constant due to a high temperature will cause the dissociation of antigen-antibody binding. Incubation time is also an important factor that needs to be investigated. Longer incubation time will allow antigen-antibody to have a higher chance to react to each other. However, this can lead to longer analysis time as well as lower sensitivity as the labeled antigen is easy to degrade. To avoid this problem, assays could be performed at shortened incubation time at nonequilibrium conditions.

Buffer condition is also an important factor in assay optimization. In some cases, antigen-antibody interaction is pH-dependent. The pH effect will sometime vary with

temperature; so, these two factors should be optimized together. Cationic salt dissolved in buffer solution will inhibit antibody binding due to decreasing ionic radius and increasing radius of hydration. Both cationic and anionic salts really have strong effect on antigen-antibody binding affinity. Those ions and their respective effects are as follows:

$Cs^+ < Rb^+ < NH_4^+ < K^+ < Na^+ < Li^+$ $\qquad$ $CNS^- < NO_3^- < I^- < Br^- < Cl^- < F^-$

2.4 Antibody-based lateral flow immunoassay

The antibody is collocated on the test line and control line. The antibody can form immunocomplexes with a specific antigen. Two types of antibodies are used on lateral flow immunoassay. A primary antibody (Ab 1°) is an antibody that can only form a complex with a specific antigen. A secondary antibody (Ab 2°) is an antibody that binds to an antibody with an antigen or another antibody. Primary and secondary antibodies are immobilized on the test line and control line, respectively.

2.5 Labeling agents for lateral flow immunoassays

Various types of labeling agents and systems are implemented in immunoassays such as enzyme labeling agent and metal labeling agent. A labeling agent is used to generate a detectable signal which is mostly related to colorimetric detection. Enzyme or metal labeling agents can be directly attached to capturing element via covalent interaction. [18]

2.5.1 Enzyme label

Enzymes are widely used to label with antigens for the detection of analytes. The enzyme can be measured at a very low concentration due to its catalytic properties to generate colored, fluorescent, or luminescent compounds. Enzymes utilized in immunoassays should have the following characteristics. Importantly, they must have a high specific activity for verifiable substrates and easy to be conjugated with antigen or antibody without losing their activity. Also, the conjugated enzymes should naturally have long shelf lives, and if stored properly, can be kept for several months or years when they are not in use. The two enzymes commonly employed are alkaline phosphate and horseradish peroxidase. Using enzyme as a labeling reagent, substrates are typically required to complete an enzymatic reaction such as phenyl phosphate, 3-indoxyl phosphate, and 2-phospho-phenyl phosphate.

2.5.2 Metal label

A metal label [19] that is commonly used as a labeling agent includes gold nanoparticles. Gold nanoparticles have many advantages such as easily controllable size distribution, long-term stability, and easy attraction with antigen, antibody, proteins, and DNA probe. There are many methods to attach the biorecognition element with gold nanoparticles. Colloidal gold carries the negative charge that allows binding with a positive charge of protein directly. The covalent bond is created

between antibody and gold nanoparticles. Normally, gold nanoparticles interact with heterobifunctional chemistry that is thiol. This allows for the subsequent reaction with amino-, carboxyl-, or glycosyl- groups of antibodies. Advantages of covalent over noncovalent bonding are a low-risk aggregation of gold nanoparticles, the binding does not depend on ionic strength of conjugation buffer, and covalent binding allows for orientation of antibody. The dual-layer method is used to attach antibodies and gold nanoparticles via a mediator. The mediator is a protein that randomly attaches to a gold surface and forms biochemical layers. The biochemical layer stabilizes gold nanoparticles and fixes the orientation of antibodies. There is reported the use of staphylococcal protein A (SpA) as a mediator. In this report, they compared the activity of gold nanoparticles to attach with antibodies by direct adsorption and by the dual-layer method. The results showed that only 25% of adsorbing antibodies reacted with antigen, whereas more than 90% of antibodies bound through a protein A can bind with antigen. Those mentioned methodologies are depicted in Figure 3.

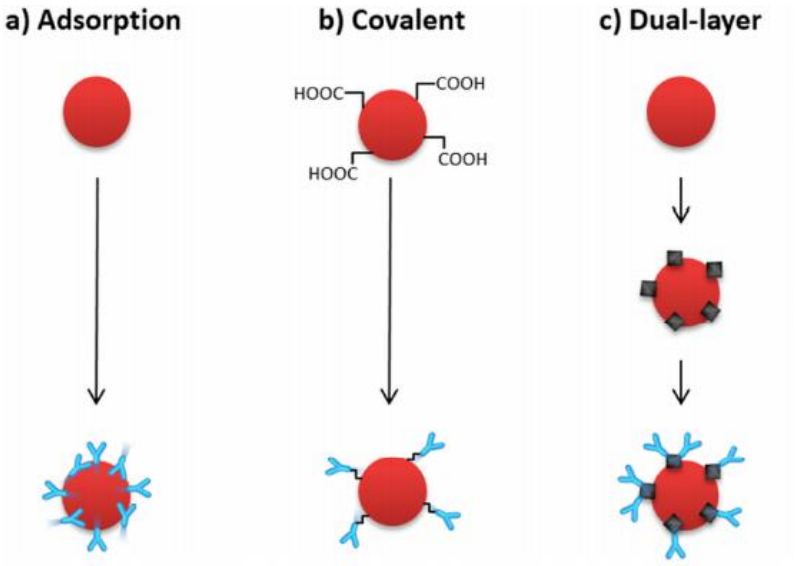

Figure 4 Methods for coupling antibodies to gold nanoparticles: a) through direct adsorption b) by covalent attachment to functional groups of polymers that adhere to GNP surface via S-G interaction; and c) by covering GNP with a protein receptor (Staphylococcal protein A, SpA), which then binds to the antibody.

The application of gold nanoparticles has several pathways including heavy mental ion determination, small molecules detection, DNA detection, and drug delivery.

2.6 Optical detection [20]

Optical detection is a colorimetric assay using a camera or scanner for image capturing. The image can be transmitted to a digital file, which can be analyzed by a software, and the result returns ideally in real-time. In general, quantitative analysis of

colorimetric paper-based requires the following steps: the image capturing, the processing of the image, and the construction of the calibration curve. The most problematic issue of camera detection is to maintain a constant condition for image capturing due to the color intensity of the image depending on the spectrum and intensity of the light source. The colorimetric results are captured by camera or scanner into a digital file. The images undergo treatment by image processing software such as image J or photoshop which in turn convert them into grayscale value or RGB intensity to evaluate their color. The color intensity is proportional to the analyte concentration and then the color intensity can be used to plot the calibration curve. The advantages of this method are simplicity, rapidity, inexpensive instrument, and on-field measurement capability.

2.7 Cortisol [21]

Cortisol has been used in psychological studies as a biomarker of stress, anxiety, and depression. Cortisol has many effects on various functions throughout the body. The major function of cortisol is the mobilization of fat and amino acid from cells to generate energy and synthesize the new compound. Cortisol also has an anti-inflammatory effect on traumatized tissues, and it suppresses the immune system. Besides, the presence of cortisol could directly affect humans' behavior such as sleep pattern, mood, and the reception sensory input.

Cortisol is the final product of the hypothalamic-pituitary-adrenal (HPA) axis. Hypothalamus secretes corticotrophin-releasing hormone (CRH). The pituitary gland responds to CRH by secreting adrenocorticotrophic hormone (ACTH) which stimulates the secretion of cortisol from the adrenal cortex. The HPA axis is regulated by negative cortisol feedback that leads to suppression of CRH and ACTH release. Cortisol level is changed during the day in response to pulsatile trophic hormone stimulation with cortisol levels peaking early in the morning and decreasing progressively during the day. Cortisol circulates in the blood in both free and bound forms. Cortisol is generally bound with albumin.

Psychobiological research interests in the measurement of cortisol because its concentration is mostly related to many pathological states. Hypercortisolism leads to Cushing's syndrome whereas hypocortisolism leads to Addison's disease. A half of Cushing's syndrome patients also show a degree of psychological disturbance. Sixty percent of cases of major depression are associated with hypercortisolism. Abnormal HPA axis from hypercortisolism has been proposed as a mechanism that may develop from chronic stress.

The determination of cortisol concentration is mainly related to blood, urine and saliva. The analytical methods used to measure cortisol are chromatographic and immunometric techniques. These methods have different features and problems related to the matrix of samples. A blood sample can provide information on diurnal rhythm, while urine level measurement represents cortisol production interval of time.

Salivary cortisol measurement is a noninvasive technique and cortisol in salivary is free cortisol that is non-binding with albumin-like blood samples.

Serum cortisol can be measured by forming an organometallic complex, detectable by infrared spectroscopy, electrochemical technique, chromatographic methods, and radioimmunoassay. These methods improve sensitivity and are less time consuming compared to conventional chromatography techniques that are time consuming, labor-intensive, and expensive for routine.

2.8 Literature reviews

2.8.1 Cortisol detection

In 1979, Kabra et al. [22] used liquid chromatography (LC) to detect cortisol concentrations in human serum. In this work, LC was equipped with a UV-visible detector and detected at 254 nm. The limit of detection was found to be 2 ng/mL.

In 2010, Chen et al. [23] used an electro spun nanofiber was used in a solid-phase extraction tip for the extraction of hydrocortisone, cortisone acetate, ethinylestradiol, and estradiol. This sample pretreatment technique was used to achieve high throughput sample extraction in a saliva sample. The detection limit was found to be 0.01 mg/mL with 0.22-0.75 mg/mL range for determination of cortisol.

In 2011, Klopfenstein et al. [24] Employed a liquid chromatography for separation and mass spectrometer for identification and quantification of cortisol in human serum. The author measured the cortisol production rate by steady-state

infusion of deuterated cortisol and analysis of stable isotope dilution. This method showed improved selectivity because interference such as 20β-dihydrocortisol was eliminated. The limit of detection was 0.6 ng/mL.

All in all, the chromatography technique showed a low limit of detection and can be applied for various types of samples. However, this technique has some drawbacks such as the requirement of a sample pretreatment step for improving selectivity.

2.8.2 Immunoassay techniques

In 1972, Abraham et al. [25] used the radioimmunoassay (RIA) technique to examine cortisol concentration in plasma after precipitation of protein with ethanol. This technique exhibited the detection length from 10-500 ng/mL. Nonetheless, the RIA technique was not practical used due to harmful effect of radioisotope.

In 2003, Van [26] reported an electrochemiluminescence immunoassay (ECLIA) for cortisol determination. This established method was fully automated detection with noninotropic assays for cortisol detection in human saliva samples. In this work, sheep polyclonal immunoassays were applied, and a competitive electrochemiluminescence assay was adopted for the detection of cortisol. The result can be obtained within 20 min. The detection limit was found to be 8 ng/mL. This

method has been further applied for the assessment of the hypothalamic-pituitary-adrenal axis activity.

In 2011, Manenschijn et al. [27] reported the use of an enzyme-linked immunosorbent assay (ELISA) for determining cortisol concentration in hair to measure long-term cortisol levels. Hair samples were collected from 195 healthy individuals, nine of which were hypercortisolemic, and one hypocortisolemic patient. They found the correlation between hair cortisol concentration and both waist circumference and waist to hip ratio. However, there is no correlation between hair cortisol concentration and age or blood pressure.

2.8.3 Point of care cortisol detection

In 2007, a high sensitive and selective cortisol microfluidic immunosensor using functionalized gold nanowires modified electrode was reported by Kumar and co-workers [28]. Additionally, cortisol antibody was immobilized on gold nanowire for used as working electrode and 3 **α**-hydroxysteroid dehydrogenases were injected into reaction cell in each measurement to convert (reduce) ketosteroid into hydroxyl steroid for example cortisone to cortisol (inactive form to active form). The linear range from 3.7 to 12 mg/mL were established for cortisol detection in serum and other biological fluids.

In 2009, Yamaguchi et al. [29] developed the immune-chromatographic test strip for salivary cortisol detection. Glucose oxidase-cortisol was synthesized as molecular

recognition of cortisol. Under the optimal condition, colorimetry was carried out to detect cortisol within 25 min. The method showed a linear range from 1 to 10 ng/mL.

CHAPTER III

Experimental

This chapter summarizes all procedures and equipments utilized to fabricate and validate the proposed paper-based lateral flow immunoassay (LFIA) device. Also, this includes apparatuses, chemicals, and reagents, and the preparation steps that are essential in this thesis.

3.1 Chemical and reagent

3.1.1. Gold nanoparticle solution 20 nm (AuNPs) (Sigma-Aldrich)

3.1.2. Sucrose (Sigma-Aldrich)

3.1.3. Phosphate saline buffer tablet (PBS) (Sigma-Aldrich)

3.1.4. Bovine serum albumin (BSA) (Sigma-Aldrich)

3.1.5. Cortisol (Sigma-Aldrich)

3.1.6. Progesterone (Sigma-Aldrich)

3.1.7. Cortisone (Glentham)

3.1.8. Corticosterone (Glentham)

3.1.9. Tween 20 (Glentham)

3.1.10. Monoclonal cortisol antibody (CUSABIO)

3.1.11. Cortisol-BSA conjugated (CUSABIO)

3.1.12. Sodium chloride (Mallinck rodt)

3.1.13. Sodium hydroxide (Merck)

3.2 Instruments and equipments

3.2.1. Nitrocellulose (unicardsCN140) (SARTORU)

3.2.2. Conjugated pads (GF33Glass) (SARTORU)

3.2.3. Plastic backing card (SARTORU)

3.2.4. Sample pad (Fusion5) (GE healthcare Whatman)

3.2.5. Absorbance pad (GE healthcare Whatman)

3.2.6. UV–visible spectrometer (Agilent technologies)

3.2.7. Milli-Q deionized water (>18.2 MΩ cm) (Millipore)

3.2.8. Strip cutting machine (BIODOT)

3.2.9. Smart phone iPhone 7 (Apple, USA)

3.3 Preparation of solution

3.3.1. 0.01 M PBS solution, pH 7.4 (200 mL)

0.01 M PBS solution was prepared using 100 mg of PBS buffer tablet diluted in 200 mL of Milli-Q water. The solution was sonicated until the tablet completely dissolved and then checked with a pH meter.

3.3.2. Cortisol BSA at 300 µg/mL (100 µL)

A stock solution of cortisol BSA was stored at -20 °C. Before using cortisol BSA, it should be defrosted by leaving cortisol at room temperature for around 5 min. The concentration of stock cortisol BSA was 1 mg/mL in 0.01 M PBS. A 30 µL of the stock solution was pipetted into 0.25 mL tube, then diluted with 70 µL of 0.01 M PBS.

3.3.3. Cortisol stock solution (50 µg/mL) (20 mL)

1 g of hydrocortisone powder was diluted in 1 mL of methanol and then the solution was vortexed until fully dissolved. Then, 19 mL of Milli-Q water was added to the solution.

3.3.4. 3% BSA solution (1 mL)

3% BSA solution was prepared by dissolving 30 mg of BSA in 1 mL of Milli-Q water.

3.3.5. 1% sucrose solution

To prepare 1% sucrose solution, 10 mg of sucrose was weighed and diluted with 1 mL of Milli-Q water**.**

3.3.6. Running buffer

Running buffer consisted of 0.1% tween and 0.1% BSA dissolved in 100 mL of PBS. First, 100 mg of BSA was accurately weighted and diluted with 100 mL of 0.01 M PBS. This solution was then mixed with 100 µL of Tween 20 and stirred until the solution is homogenous.

3.3.7. 10%w/v NaCl solution (1 mL)

10%w/v NaCl solution was prepared using 100 mg of NaCl dissolved in 1 mL of Milli Q water.

3.3.8. 0.1 M NaOH stock solution (500 mL)

NaOH stock solution contains 2 g of NaOH dissolved in 100 mL Milli-Q water in a 250 mL beaker. The solution was then transferred into a 500 mL volumetric flask and the final volume was adjusted using Milli Q water.

3.4 Conjugation of cortisol-BSA with AuNP

A cortisol-BSA was conjugated with AuNP following the literature [30] with a slight modification. Initially, the pH of the AuNP solution was adjusted to pH 8 by 0.1 M NaOH to modify the charge at the surface of AuNP. After that, 1 mL of AuNP pH 8 was pipetted into a 1.5 mL PCR microcentrifuge tube to avoid the adsorption of nanoparticles on the microtube wall. Then, 100 µL of cortisol-BSA at concentration of

200 µg/mL was added to the AuNP solution. The solution was incubated under the stirring condition for 30 min at room temperature. After incubation, 100 µL of 3% BSA was added to the solution to reduce nonspecific absorption. The mixed AuNP solution was then centrifuged at 12000 rpm (4 °C) for 30 min. Lastly, the supernatant was removed while the residue was made up to the volume of 100 µL with 3% of BSA. Overall procedure is schematically illustrated in Figure 5.

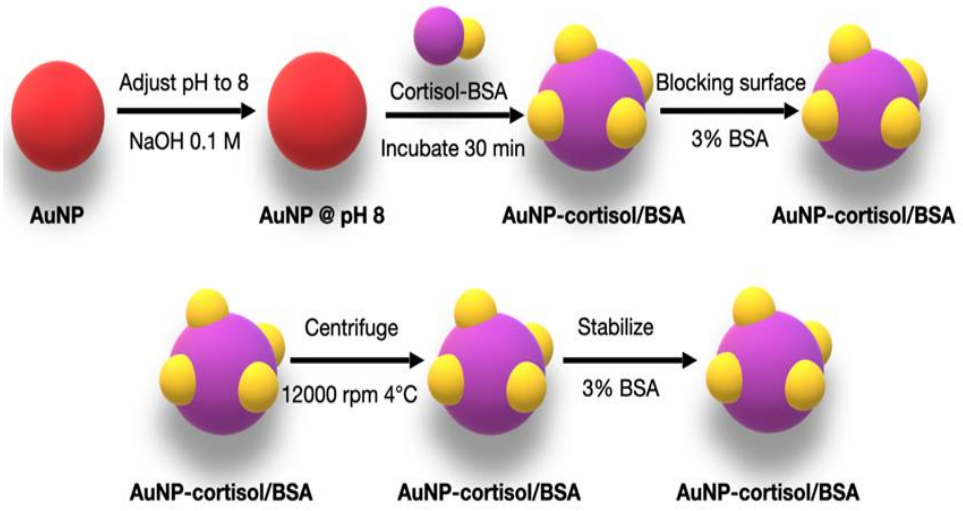

Figure 5 *Schematic illustration of a conjugation procedure for AuNP-cortisol/BSA*

3.5 Fabrication of the cLFIA test strip for cortisol detection

The strip proposed in this work is composed of (i) glass-fiber sample pad, (ii) glass-fiber conjugated pad, (iii) nitrocellulose membrane (NC), (iv) absorbent pad, and (v) backing card. Every component is mounted on the backing card with a 2 mm overlap between components. The strip was cut into 4 mm by a cutting machine. To

construct the concave test zone, the LFIA strip was punched with an office hole puncher into two semicircles. The width between the two semicircles is 1 mm, while the distance between the conjugate pad and test zone was set at 2 cm. Notably, the back of the plastic backing card was marked with 2 straight lines to control the size of the semicircles. 1 %w/v sucrose was applied on the test line to stabilize AuNP and then sucrose was dried in an oven at 37 °C for 30 min. 0.2 µL of monoclonal cortisol antibody was applied on the concave test zone of the nitrocellulose membrane. The control line, which is an anti-BSA antibody (600 µg/mL), was printed next to the test zone by ZX1010™ Dispense Platform to confirm the functionalization of the AuNP-cortisol/BSA. Next, 4 µL of sucrose solution (1% w/v) was applied on a conjugate pad and dried at 37 °C for 1 hr to increase the stability for AuNP-cortisol/BSA. Lastly, 4 µL of AuNP-cortisol/BSA was applied on the conjugate pad and dried at 37 °C for 30 min. Preparation of the cLFIA test strip with a concave test zone is demonstrated in Figure 6.

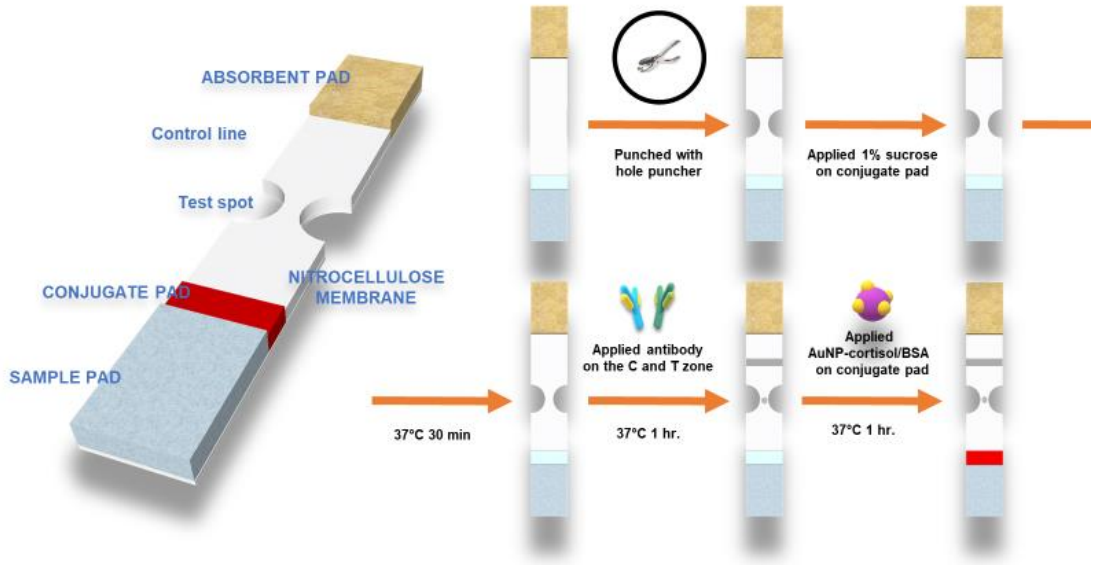

Figure 6 Preparation of the cLFIA test strip with a concave test zone

3.6 Cortisol detection procedure

To perform cortisol detection, 20 µL of the cortisol solution (working standard or serum sample) was loaded onto a sample pad, followed by 70 µL of running buffer containing 10 mM PBS, 0.1% tween, and 0.1%BSA. After 10 min, the LFIA test zone was photographed by iPhone 7 under an in-house light-controlled box with a movable strip tray (Figure. 7) using the Manual shot application. The color intensity (green channel intensity) of the test spot was then analyzed using Image J software.

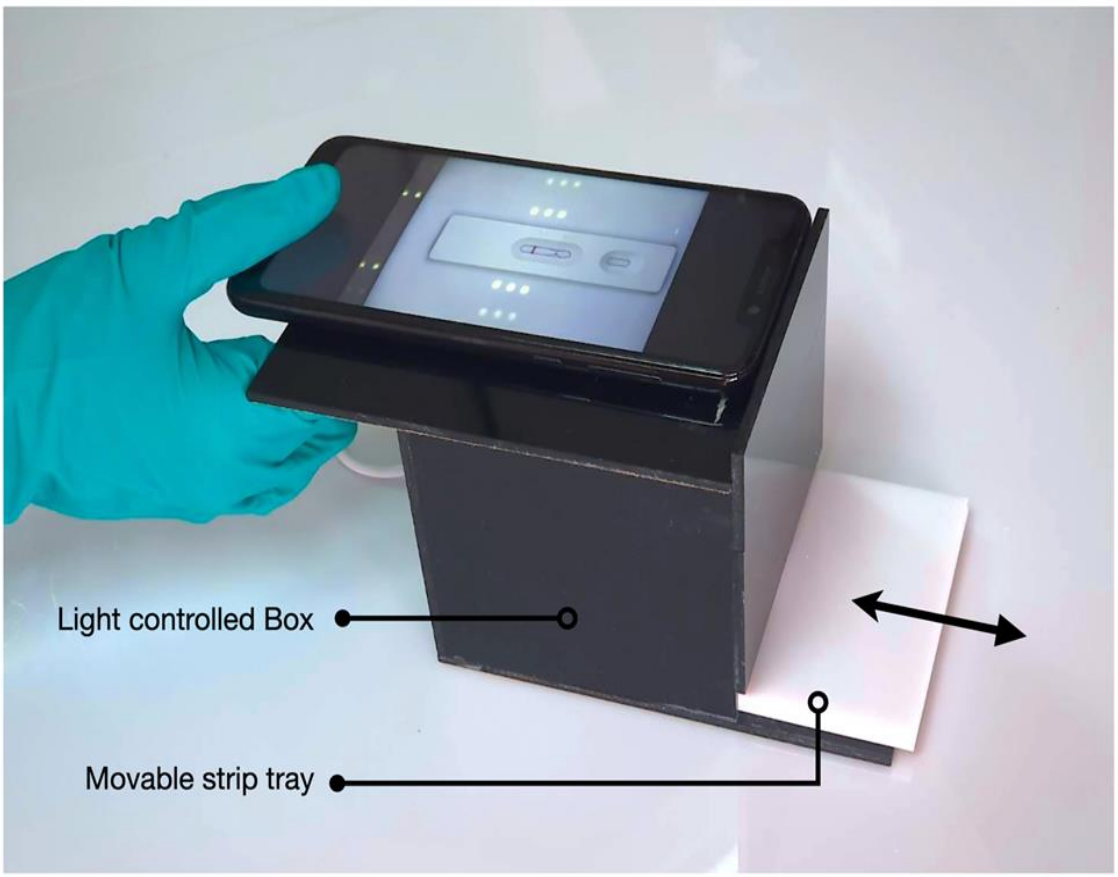

Figure 7 An in-house light control box with a movable strip tray for LFIA device

3.7 Serum sample analysis

To verify the sensor applicability in real-world samples, the developed LFIA test strip was also tested in certified human serums at level 1 and level 2 (overall components are shown in Table 1). The certified serum samples were obtained from pointe scientific (USA). These samples were diluted 2-fold with PBS prior to the analysis. The samples were then analyzed for cortisol level with the proposed LFIA test strip in succession. To confirm the reliability of this developed sensor, the samples were also tested with the conventional method of

electrochemiluminescence (ECL) by National healthcare systems Co., Ltd (Bangkok, Thailand). The results obtained from these two methods were then analyzed using paired t-test method.

Table 1 Summarized table of the components found in certified serum (level I and II).

Component	General Assay Range		Unit
	certified serum Lv I	certified serum Lv II	
Acid phos	8 ± 1.6	18.2 ± 3.6	U/L
Albumin	2.3 ± 0.2	4.3 ± 0.4	g/dl
Alk Phos	94 ± 28	216 ± 65	U/L
ALT(SGPT)	57 ± 11	142 ± 29	U/L
Amylase	247 ± 74	494 ± 148	U/L
AST(SGOT)	65 ± 13	257 ± 52	U/L
Direct Bilirubin	0.9 ± 0.4	5.1 ± 1.0	mg/dl
Total Bilirubin	1 ± 0.4	6.2 ± 1.2	mg/dl
BUN	16 ± 2	52 ± 5	mg/dl
Calcium (CPC)	10 ± 1.0	13.2 ± 1.0	mg/dl
Calcium (AR-III)	9.6 ± 1.0	13 ± 1.0	mg/dl

Chloride	101 ± 5	125 ± 6	mEq/L
Cholesterol	145 ± 15	300 ± 30	mg/dl
Carbon Dioxide	8 ± 5	26 ± 5	mEq/L
CK/CPK	137 ± 41	301 ± 90	U/L
Creatinine	1.19 ± 0.30	5.15 ± 0.77	mg/dl
GGTP	47 ± 14	141 ± 28	U/L
Glucose Hex	79 ± 8	297 ± 30	mg/dl
Glucose Ox	72 ± 7	285 ± 29	mg/dl
HDL(auto)	108 ± 32	193 ± 58	mg/dl
HDL(PEG)	53 ± 16	41 ± 13	mg/dl
Iron	76 ± 15	166 ± 34	µg/dl
Lactate	1.7 ± 0.2	2.7 ± 0.2	mmol/L
LDH	118 ± 24	347 ± 69	U/L
Lipase	40 ± 12	142 ± 43	U/L
Magnesium	1.8 ± 0.5	3.4 ± 0.9	mg/dl
Phosphorus	4.0 ± 0.4	8.4 ± 0.9	mg/dl
Potassium	4.3 ± 0.5	6.9 ± 0.5	mEq/L
Sodium	134 ± 4	170 ± 4	mEq/L
TIBC direct	277 ± 70	407 ± 102	µg/dl
Total Protein	3.5 ± 0.4	6.8 ± 0.7	g/dl

Trig-GPO	91 ± 23	194 ± 49	mg/dl
UIBC	134 ± 34	239 ± 60	µg/dl
Uric acid	5.1 ± 0.9	9.5 ± 1.6	mg/dl

3.7 Stability

The stability was characterized by weekly monitoring of the signal while storing the sensor in a desiccator at room temperature using a cortisol concentration of 200 ng/mL and performing three measurements each week.

3.8 Selectivity

The selectivity of the test strip was evaluated by measuring three structurally similar steroids: cortisone, corticosterone, and progesterone, which are cholesterol derivatives. The steroid samples were prepared at concentrations of 50 mg/mL.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Microfluidic behavior of the cLFIA device

Microfluidic behavior was studied and compared to that of the conventional LFIA device. In this study, the red dye, used to represent the AuNP-cortisol/BSA, was deposited on the conjugate pad for better visualization. When the running buffer was loaded into the LFIA device, it was observed in Figure 8 that the running buffer could wick the red dye from the conjugate pad down the LFIA channel. During the initial flow (~ 5s), both devices illustrated a linear fluid front like a plug flow profile. However, when the liquid is entering the test zone (~ 10s), it was observed that the red dye was concentrated to pass through the constricted zone of cLFIA. In contrast, a slightly larger fluidic band along the channel was observed in conventional LFIA. It was expected that this concentrated fluid flow would considerably enhance the binding events between immobilized antibodies and antigens as they were forced to pass through a small area and increase the probability of immunoassay reaction on the test spot, thus allowing for superior analytical performance. Next, as the liquid passed through the test zone (> 20s), the red dye clearly spread in the shape of 180° and was gradually

eluted by the running buffer in case of cLFIA. At this stage (~ 20s), a higher fluid front was perceived in constricted LFIA compared to that of conventional LFIA. We believed that an increase in coloring distance could be ascribed by the fan-shaped flowing pattern at the exit of a constricted zone which provides a counterbalance to the capillary pressure [31]. It was evident in this screenshot that the following flow profile from the running buffer was clearly transformed from an almost linear fluid front flow profile to a comet-like shape as it traversed across the concave test zone. Anyhow, the overall time required to complete the assay is insignificantly different (~ 50s) between these two devices.

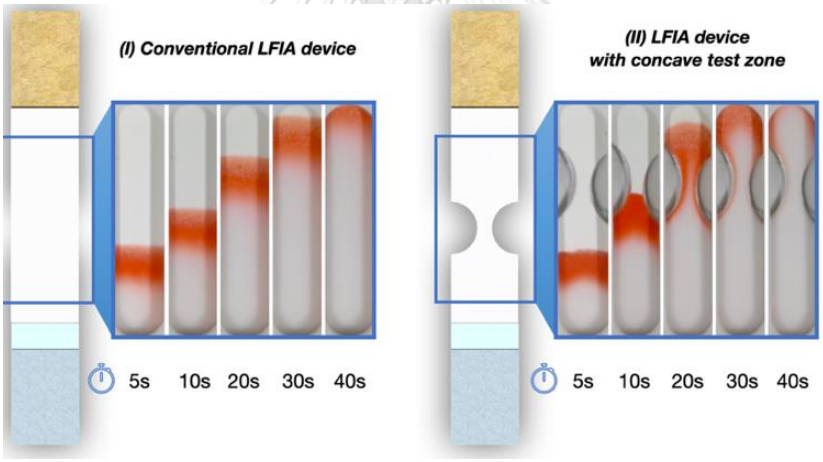

Figure 8 Image showing a microfluidic flow profile of a red dye representing AuNP-cortisol/BSA tested on a (I) conventional LFIA and (II) cLFIA.

We further investigated a signal amplification result by performing two sets of measurements using conventional LFIA and cLFIA. As presented in Figure 9, it can be seen that the concave LFIA markedly increases the sensitivity (slope) toward cortisol

detection up to 2.4 times compared with that from conventional LFIA, simply by punching the paper device. Also, the intensity difference is almost 2.5-fold higher (tested at cortisol concentration of 500, 1000, and 5000 ng/mL and anti-cortisol concentration of 300 ng/mL) compared with the conventional test strip. All in all, it could be deduced that the cLFIA device can be used to enhance the sensitivity without requiring any tedious chemical modification method.

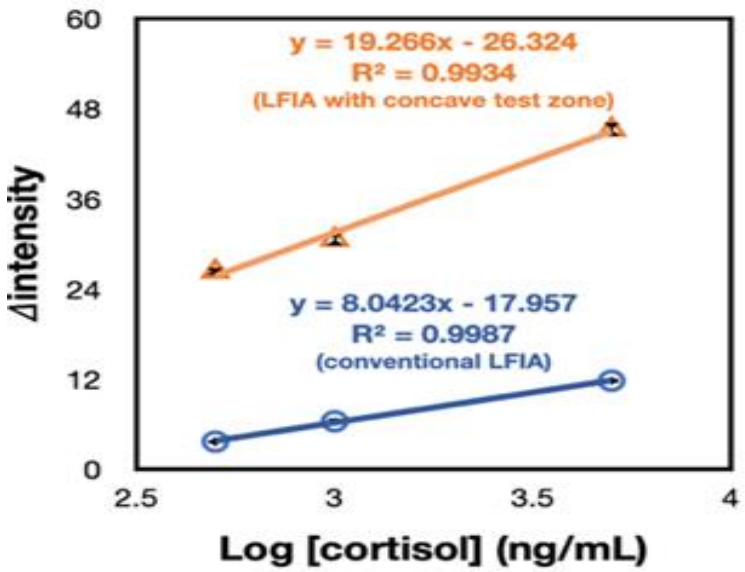

Figure 9 a linear plot between Δintensity ($I_{tested\ cortisol}$ - I_{blank}) and logarithmic concentration of cortisol (500, 1000, and 5000 ng/mL) tested with a conventional LFIA and cLFIA.

4.2 Characterization of the AuNP-cortisol/BSA

In this section, the AuNP characterization (before and after modification with cortisol/BSA) was carried out using UV-Vis spectroscopy and dynamic light scattering (DLS) technique. Firstly, the UV-Vis absorption spectroscopy was studied to monitor the size change and modification of biomolecules to the AuNP. Illustrated in Figure 10 is the UV-Vis absorption spectra of the AuNP before and after modification with cortisol/BSA. It is well-known that the metallic nanoparticle (such as AgNP or AuNP) exhibited a strong absorption band in the visible region. Obviously, this characteristic peak of AuNP was distinctly seen at 518 nm (blue line), corresponding with the supplier's datasheet. Interestingly, when the cortisol/BSA was added and conjugated to the AuNP solution, the absorption peak of AuNP was shifted toward a longer wavelength (red-shift) from 518 to 521 nm due to an increase in the size of gold nanoparticles.

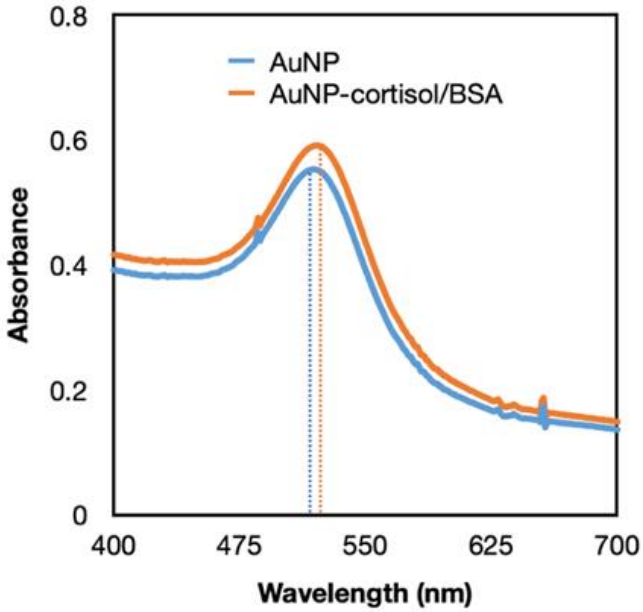

Figure 10 UV-Vis absorption spectra of the AuNP before and after surface modification with cortisol/BSA

Next, the DLS technique was used for determining the size of the nanoparticle. As shown in Figure 11 the hydrodynamic diameter of bare AuNP is 24 nm, which is larger than the given data from a supplier. This is because DLS measures the hydrodynamic radii of the particle, which includes both particle size itself and the ionic and solvent layers in the solution [32]. After AuNP was conjugated with cortisol/BSA, it was observed that the hydrodynamic diameter of the particle increased to 29.54 nm which confirm the conjugation of the cortisol/BSA on the AuNP. Also, the measurements showed a low polydispersity index (PDI), illustrating the homogeneity of the particles in the solution. Furthermore, zeta potential (ζ) measurements were

also conducted to confirm the successful surface modification. Prior to the modification, the AuNP showed a zeta potential of -45.5 mV, attributed to the surface potential of the negatively charged citrate stabilized AuNP. Afterward, the zeta potential changed from -45.5 to -37.5 mV due to the destruction of electrostatic force and charge neutralization on the surface of AuNP caused by the abundant positively charged sites on the protein molecules [33]. All things considered; these results suggested the successful modification of the AuNP surface with cortisol/BSA.

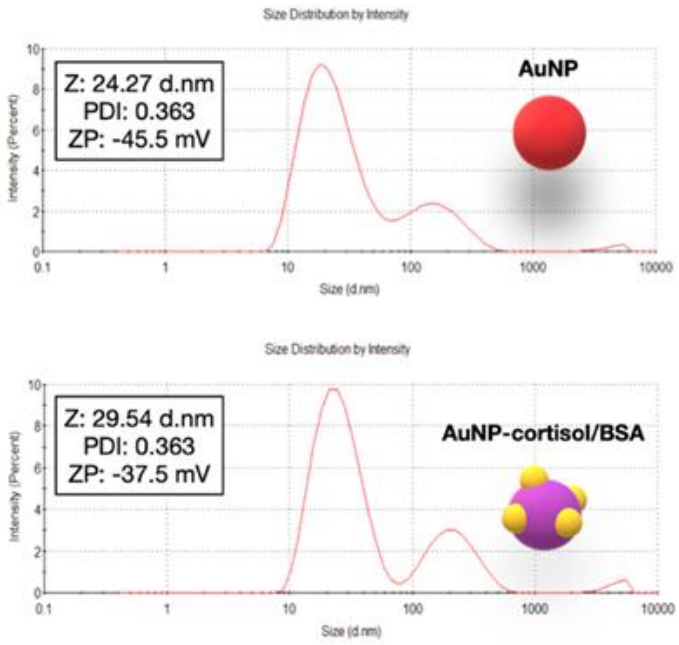

Figure 11 DLS measurements of the AuNP before and after surface modification with cortisol/BSA

4.3 Optimization

Several parameters affecting the detection of cortisol were studied to maximize the detection sensitivity and lower the detection limit. Herein, various assaying

conditions (concentration of cortisol/BSA and anti-cortisol antibody, and detection time) and the geometrical factor (width of a test zone) were optimized.

4.3.1 Optimization of Cortisol/BSA concentration used for AuNP conjugation

Primarily, we investigated the effect of cortisol/BSA concentration used for AuNP conjugation, following the literature [34]. In short, 10 µL of various cortisol/BSA concentration (50, 100,150,200,250 µg/mL) were mixed with 100 µL of AuNP solution and incubated at room temperature for 30 min. Then, 80 µL of 10% NaCl (w/v) was added to the prepared solution. The appropriate concentration of cortisol/BSA will cause no aggregation in the presence of NaCl, while the insufficient concentration of cortisol/BSA will cause AuNP aggregation and change the color from red to blue/violet in the presence of NaCl. Then, a UV-Visible spectrophotometer was used to monitor the absorbance at 530 nm. The result shown in Figure 12 suggested that the lowest cortisol/BSA concentration that produces decent signal and causes no aggregation is 200 µg/mL. Therefore, this cortisol BSA concentration was further used for AuNP conjugation.

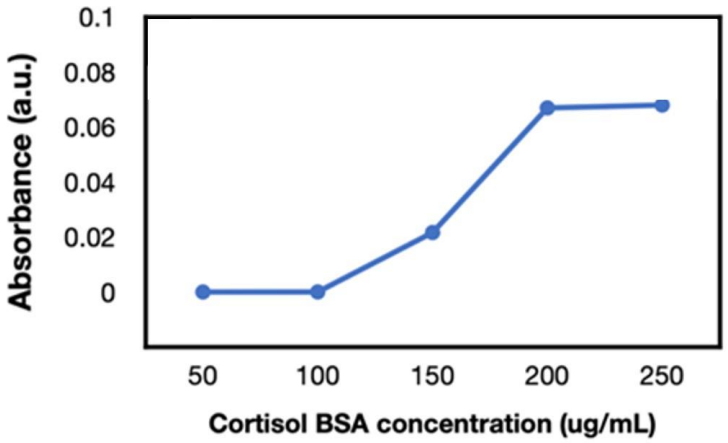

Figure 12 Optimization of essential parameters for cortisol detection: concentration of cortisol/BSA

4.3.2 Optimization of antibody concentration

Antibody concentrations (100, 200, 300, 400, and 500 µg/mL) were tested with LFIA device. As illustrated in Figure 13, it was observed that the different color intensity (Δintensity = $I_{tested\ cortisol}$ - I_{blank}) increased with increasing antibody concentration and reached the highest at 300 µg/mL. This increased antibody concentration will raise the capturing probes, thus allowing for an increased immune reaction between antigen and antibody. Then, the signal gradually decreased as the concentration is continually increased over 300 µg/mL. We believed that the steric effect from the antibodies would play a key role in this phenomenon, therefore causing an adverse effect on the binding reaction. Hence, 300 µg/mL of antibody will be applied to the test zone for further studies.

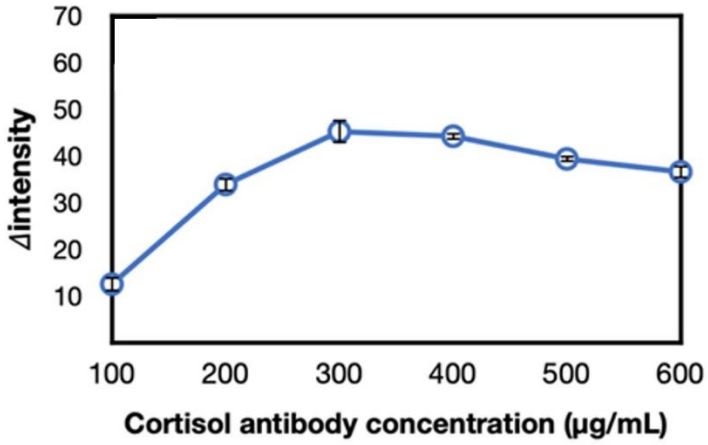

Figure 13 Optimization of essential parameters for cortisol detection: concentration of cortisol/BSA

4.3.3 Optimization of test line width

The geometrical factor from the test line width was also optimized. Differing test line widths (0.5, 1, 2, 3, and 4 mm) on the LFIA device were simply prepared by an office puncher. Note that two straight lines drawn at the back of the plastic backing card were used to control the paper-based device's width; meanwhile, the distance between the conjugate pad and test spot was maintained at 2 cm. The result shown in Figure 14 indicates that the narrower test line width yielded the higher Δintensity. Consequently, a small test line width will concentrate the cortisol in the sample to pass through and increase the binding event between antigen-antibody, therefore enhancing the signal response. However, the test zone width of 1 mm is preferred in

this work since the 0.5 mm width is quickly torn apart. Therefore, 1mm width was selectively used in this work.

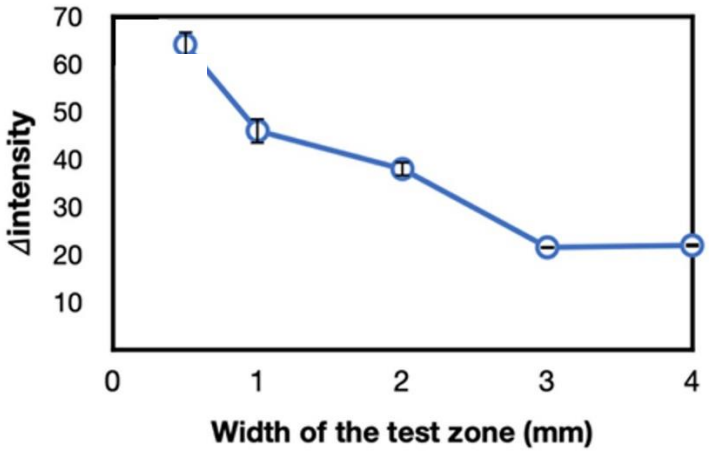

Figure 14 Optimization of essential parameters for cortisol detection

4.3.4 Running time

Minimum time required to complete AuNP-cortisol/BSA binding with the immobilized antibody is 7 min (evidence from the video of the AuNP-cortisol/BSA flowing on the strip test). Thus, the detection time will be studied from 7 min to 15 min. As shown in Figure 15, the Δintensity will increase with increasing time and reach the plateau state at 10 min. Hence, the running time for this device was set at 10 min.

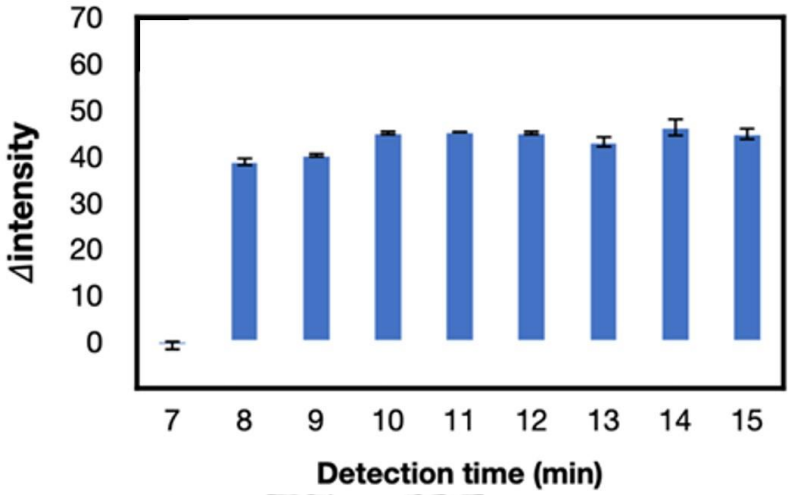

Figure 15 Optimization of essential parameters for cortisol detection: detection time

4.4 Analytical Performance of the cLFIA sensor

4.4.1 Standard curve

The sensing performance of the developed cLFIA was evaluated in this study. AuNP-cortisol/BSA is solely captured by the immobilized antibody on the test spot in the absence of cortisol (blank), thereby resulting in a red spot at the constricted zone. On the other hand, When the cortisol is present in the solution, the red color spot from AuNP-cortisol/BSA is gradually fading (depending on the cortisol concentration) as the cortisol will compete with the fixed amount of AuNP-cortisol/BSA. Therefore, the color of the test spot will progressively change from red to colorless. For semi-quantitative interpretation, image processing software (ImageJ) was used to analyze the color intensity in the green channel of RGB mode. As shown in Figure 16, the Δintensity is increased proportionally with the increasing of cortisol concentration. A

linear relationship between Δintensity and logarithmic concentration of cortisol was established in the range of 1 to 5000 ng/mL (y = 13.365x-7.3154, R^2 = 0.9816) as presented in the inset of Figure 16. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.6 and 4.8 ng/mL, respectively. Interestingly, this LOD level is enough to detect cortisol in actual serum samples, thus showing a promising solution for on-site analysis. Furthermore, it can be summarized that the widest linear dynamic range (3 orders of magnitude) was obtained using this LFIA format. Although the LOD of this developed sensor may be inferior to those other reports, significant advantages such as a straightforward fabrication, no requirement for a post-chemical enhancement or specific apparatus, and less time-consumption strongly denoted the function of the proposed LFIA device.

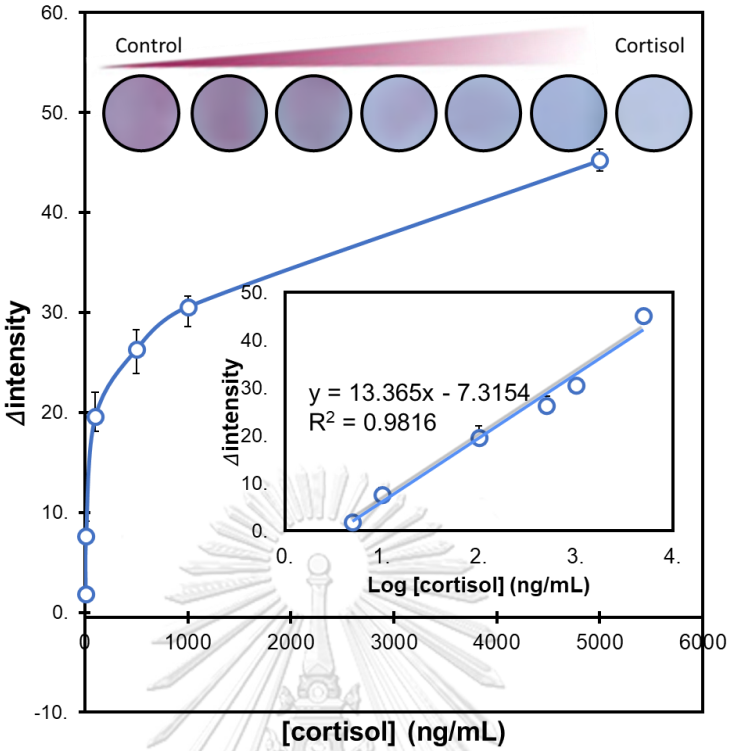

Figure 16 Signal response and linear relationship between ΔIntensity vs. logarithmic concentrations of cortisol (inset) images showing the color of the test spot under the light-controlled box

4.4.2 reproducibility

To investigate the reproducibility of the developed cLFIA, different sensing devices were tested with cortisol at different concentrations (10, 100, and 500 ng/mL). It was found that the percentage of relative standard deviation (%RSD) was in the range of 1.06 to 2.45% (n=3). This result indicates that the sensor offers a good reproducibility for cortisol. Next, the sensor stability, defined as the retention within 95% - 105%, was investigated. Here, the sensors were kept in a desiccator at room temperature and

monitored for a signal change (testing with cortisol concentration of 200 ng/mL, n=3 each week) weekly after that. As shown in Figure 17, the normalized Δintensity was higher than 95% after storage for 6 weeks, indicating excellent stability of the cLFIA test strip.

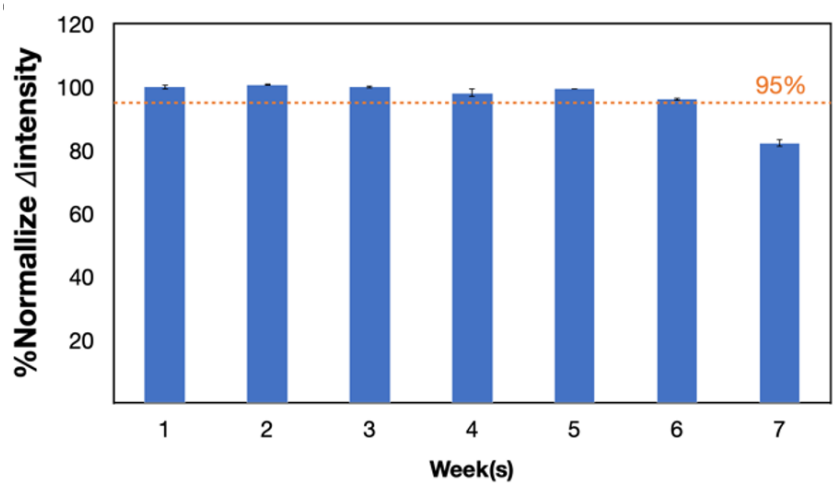

Figure 17 stability test of strip test

4.5 Selectivity study

We further assess the selectivity of the proposed test strip by testing with three structurally similar steroids (cortisone, corticosterone, and progesterone), as they are the derivatives of cholesterol. These steroid hormones were prepared at a concentration of 50 µg/mL. The result is shown in Figure 18, in which all of these steroid hormones caused no interference toward cortisol determination. Hence, it indicates that the proposed cLFIA strip has excellent selectivity for cortisol detection.

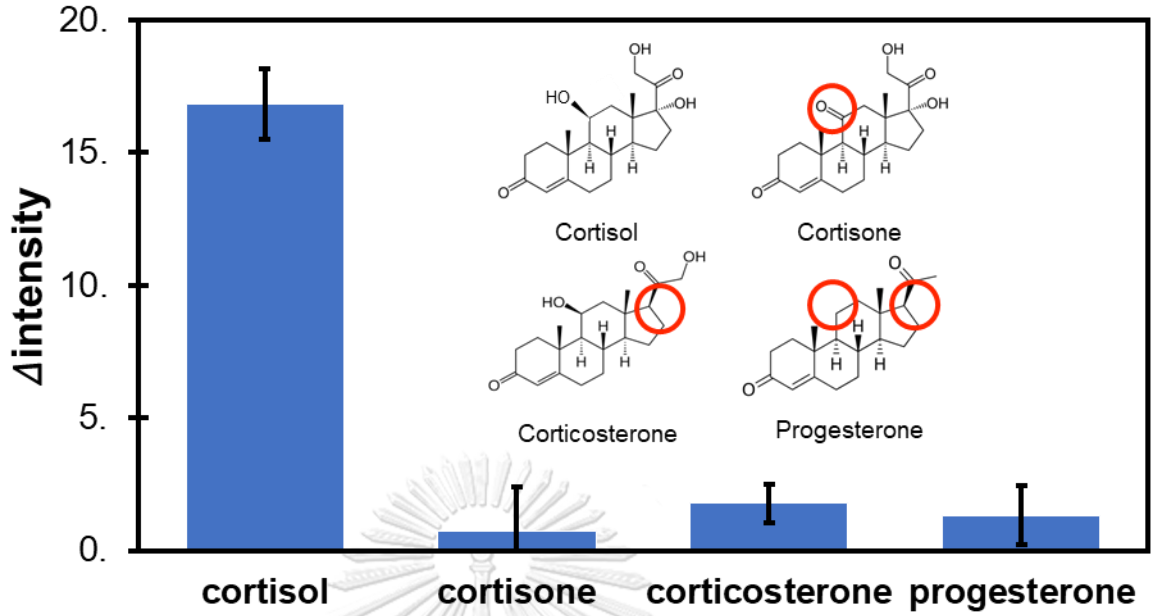

Figure 18 Selectivity of strip test with cortisol, cortisone and progesterone in the concentration of 50 µg/mL

4.6 Detection of cortisol in human serum

Finally, to demonstrate the potential applicability of the sensor, the proposed cLFIA device was applied for the analysis of certified serum samples (pointe scientific lv 1 and 2, lot 717401). In this study, three cortisol levels (concentration of 25, 50, 75 ng/mL), which are the cortisol concentration commonly found in blood samples, were spiked to the serum samples. Recovery testing was performed, and the obtained results are shown in Table 2. Acceptable recoveries were found in the range of 95.8-105.2%, with less than 5.1%RSD. Furthermore, the obtained results were also validated with the standard electrochemiluminescent (ECL) method, as summarized in Table 3. It can be seen that the detectable

concentrations using the cLFIA were in line with those obtained from ECL using paired t-test (at 95% confidential level). On the whole, it can be concluded that the proposed cLFIA showed good accuracy and reliability, thus paving the way for POC testing clinical analysis.

Table 2. Recovery results for the determination of spiked cortisol in certified serum samples

Sample	Concentration (ng/mL)		% Recovery	%RSD
	Spiked	Found		
Certified serum sample (level I)	25	25.4±1.3	101.6	5.1
	50	48.8±0.8	97.6	1.6
	75	75.9±1.1	101.2	1.4
Certified serum sample (level II)	25	24.4±0.9	97.9	3.7
	50	47.9±1.3	95.8	2.7
	75	78.9±0.7	105.2	0.9

Table 3. Summarized results for the determination of cortisol in certified serum samples using the proposed LFIA and standard electrochemiluminescent (ECL) method.

Sample	Concentration (ng/mL)		
	Spiked	Proposed LFIA	ECL[a]
Certified serum sample (level I)	25	25.4±1.3	28
	50	48.8±0.8	50
	75	75.9±1.1	77
Certified serum sample (level II)	25	24.4±0.9	26
	50	47.9±1.3	50
	75	78.9±0.7	73

[a] Electrochemiluminescent method

CHAPTER V

Conclusion

5.1 Conclusions

In this work, A paper based LFIA device with a concave test zone was successfully developed to determine cortisol in human serum. AuNP-cortisol/BSA was used as label that bind with monoclonal cortisol antibody on test spot. The red color spot from AuNP-cortisol/BSA gradually disappeared as the cortisol concentration in the sample is increased. This cLFIA test strip was easily manufactured by the use of an office hole puncher. The sensitivity for the determination of cortisol using this design is enhanced by nearly 2.5-fold because the sample flow is concentrated before entering the test spot, thereby enabling more complete immunoreaction between antigen and antibody compared with the traditional structure.

This method can improve the sensitivity without chemical modification or a post treatment amplification. Standard curve of cortisol concentration in the range of 5 to 5,000 ng/mL was obtain with good linearity. The limits of detection and quantification were 1.6 and 4.8 ng/mL, respectively. Good selectivity and recovery were also obtained. There are no differences between the concentration of cortisol measured by reported method and conventional method. It can be concluded that the proposed cLFIA showed good accuracy and reliability.

The sensitivity of this sensor is lower than some of the bench top instruments. Analytical advantages such as portability, user-friendliness, and naked-eye detection suggest that this sensor is a promising alternative to conventional devices. In addition, this cLFIA is suitable for the determination of cortisol in certified human serum, providing good agreement with a standard electrochemiluminescence protocol.

5.2 Future perspective

For future perspective, we believe that this cLFIA format is also suitable for the determination of other small molecules with good sensitivity. This cLFIA format can be extended for application with a smart device for Point of Care analysis.

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