Rapid Colorimetric Sensor Based on Gold Nanoparticles Functionalized 4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole for Cortisol Detection in Saliva Sample

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Abstract: *The rapid, simple, and selective colorimetric sensing method of cortisol has been successfully developed using AuNPs modified with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AuNPs-AHMT). The principle of this method is based on the color change from wine red to purple (redshift) when AuNPs-AHMT interacts with cortisol. The hydrogen bonding between the hydroxyl group from cortisol and the amine group from AHMT induces the aggregation of AuNPs. The modification of the AuNPs surface with AHMT aims to increase its stability. The properties of AuNPs and AuNPs-AHMT were characterized by UV-vis spectrophotometer. The interaction between AuNPs-AHMT and cortisol was studied by UV-vis and FTIR spectroscopies. The proposed method was optimized and validated. Au(III) was reduced to AuNPs at an optimum $NaBH_4$ concentration of 1.0 mM. Validation of the proposed method showed good analytical performance with linearity from 1.0–50.0 nM, accuracy 91.07–102.77%, intra-day precision < 2.22% and inter-day precision < 2.17%, detection limit 0.76 nM, quantification limit 2.54 nM, and sensitivity 0.0112 nM/mL. The proposed method also showed good selectivity with the presence of some interferences in the sample. The proposed method was successfully applied for the determination of cortisol in the saliva by the standard addition method with acceptable recovery.*

Keywords: *AHMT; colorimetric sensing; cortisol; gold nanoparticles*

■ INTRODUCTION

Cortisol is a glucocorticoid steroid hormone that is produced in the adrenal glands. The secretion of cortisol in biological fluid has a circadian rhythm where the concentration of cortisol in the morning is higher than in the night [1-2]. Cortisol is also known as a stress hormone because the secretion of cortisol can be induced by psychological and physical stress [3-5]. Besides that, the increase in cortisol concentration levels is linked to other diseases including stroke. The correlation between stroke and cortisol level shows that acute stroke mortality is related to the increase in cortisol level and is associated with the biomarker of early stroke detection and severity [6]. Cortisol is not only present in serum and urine but is also released in saliva samples. In saliva, steroidal hormones are only present in free form [7], because of their diffusion through the cells of the salivary gland [6]. It was demonstrated that salivary cortisol concentration has a correlation with free cortisol in the serum [2,6,8-9].

Some methods have been developed for cortisol detection in saliva samples, such as an immunochemical assay [10-12] and liquid chromatography-mass spectrophotometry (LCMS) [13-14]. But these methods need laboratory instruments that are relatively

expensive. These methods also require instrumentation carried out by an expert. Additionally, they are a multi-step analysis method and relatively expensive. So, the development of a new method that can be used for the early detection of cortisol that is rapid, simple, and cost-effective, such as colorimetric sensing, is very important.

In recent years, colorimetric sensors have received a lot of attention as a promising method for biological and chemical analyte detection due to their significant advantages, including the ability to be recognized with the naked eye, ease of use, quick assay times, and low cost. The principle of a colorimetric sensor is that color changes when sensing materials interact with an analyte. The color is produced by the variation in absorbance brought on by the optical characteristics of plasmonic material sensing [15-17]. Since the intensity, frequency, and location of the localized surface plasmon resonance (LSPR) bands strongly depend on the size, shape, surface modification, dielectric, and aggregation of material sensing, this optical property serves as the foundation for colorimetric sensing and can be used to detect the color change in colloid [18-19]. Specifically, gold nanoparticles (AuNPs) display an LSPR band within the visible region and have the potential as a colorimetric sensor.

AuNPs are materials or particles that have a size distribution between 1 and 100 nm and have been used in many applications such as biomedicine [20], immunochemical analysis [21], environmental monitoring [22], food safety screening [23], and colorimetric sensing for diagnostics [24]. AuNPs are interesting candidates for rapid colorimetric sensing because of their optical properties, such as their high absorption coefficients and very strong LSPR absorption band in the visible area [25]. The high surface-to-volume ratio and optical properties of AuNPs assist highly selective and sensitive detections [26]. The principle of AuNPs as a colorimetric sensor is based on color change. Colloidal AuNPs are generally red or pink and will change to another color when the aggregation of AuNPs occurs as a result of interaction with the analyte. However, AuNPs need to be stabilized to prevent self-aggregation.

Stabilization of AuNPs is carried out by using stabilizing or capping agents to increase their stability. During the chemical synthesis of AuNPs, ascorbic acid, sodium citrate, or sodium borohydride ($NaBH_4$) are normally used as capping agents. However, many capping agents are also used, such as albumin [27], γ-cyclodextrin [28], chitosan [29], and PEG [30]. In this work, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT) was used as the capping agent to modify the AuNPs. AHMT contains mercapto, amino, hydrazine, and triazole groups and can bind with AuNPs surface via the mercapto groups. AHMT can also bind with cortisol through the hydrogen bonding interaction between the –NH group from AHMT and the –OH group from cortisol.

In this research, a novel colorimetric sensor for cortisol using AuNPs modified with AHMT was developed. The AuNPs produced, the interaction between AuNPs and AHMT, and the interaction between AuNPs-AHMT and cortisol were characterized and studied by UV-vis spectrophotometers. Fourier transform infrared (FTIR) was used to characterize the bonding between AuNPs and AHMT. The reaction between AuNPs-AHMT and cortisol was optimized, and the proposed method was validated. Finally, the proposed method was applied to the determination of cortisol in saliva samples.

■ EXPERIMENTAL SECTION

Materials

Chloroauric acid ($HAuCl_4$) was made from gold bars that dissolved in aqua regia (nitric acid and hydrochloric acid at a ratio 1:3). Sodium borohydride ($NaBH_4$, 99%) from Sigma-Aldrich with the CAS number 16940-66-2, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT, ≥ 99%) from Sigma Aldrich with CAS number 1750-12-5), and cortisol (Hydrocortisone) from Sigma Aldrich with CAS number 50-23-7 were used in this study.

Instrumentation

The instrumentals used were a UV-visible spectrophotometer Shimadzu-1800 from Germany to measure the wavelength and absorbance of AuNPs, AuNPs-AHMT, AHMT, and AuNPs-AHMT-cortisol. Particle size analyzer (PSA) and zeta potential

measurements were performed on Zetasizer Ver. 7.01 (Malvern 1061025, Germany) to measure the size of particles and size distribution of AuNPs formed, respectively. The FTIR spectral measurements were carried out by Shimadzu IR Prestige-21, Germany.

Procedure

Synthesis of AuNPs

AuNPs were synthesized by reduction of $HAuCl_4$ with $NaBH_4$ and sodium citrate as a reduction agent, according to the literature [31], with a little modification. The procedure is as follows: 10 mL $HauCl_4$ 1 mM was stirred in a beaker glass, then added with 10 mL trisodium citrate 2 mM. While stirring, 10 mL of $NaBH_4$ 1 mM was added drop by drop into the mixture of solutions. The mixture of solutions turned into a red color after the addition of $NaBH_4$. After stirring was stopped, the stir bar was removed, and the solution was centrifuged and filtered. The result of this synthesis is a red color of AuNPs. The red colloidal AuNPs were then stored in the vial bottle at 4 °C.

Optimization of $NaBH_4$ concentration

The quantity of $NaBH_4$ as a reduction plays an important role in the production of gold nanoparticles with high stability. The concentrations of $NaBH_4$ used in this research were 0.50, 0.75, 1.00, 1.25, and 1.50 mM. The procedure is that 10.00 mL $HAuCl_4$ 1.00 mM was stirred in a beaker glass, then added to 10.00 mL of trisodium citrate 2.00 mM. While stirring, 10.00 mL of $NaBH_4$ 1.00 mM was added drop by drop into the mixture of solutions. The mixture of the solution turned a red color after the addition of $NaBH_4$. After stirring was stopped, the stir bar was removed, and the solution was centrifuged and filtered. The same procedure was carried out for the $NaBH_4$ concentrations of 0.50, 0.75, 1.25 and 1.50 mM.

Surface modification of AuNPs with AHMT

Gold nanoparticles were modified as follows: AHMT modified by 30.00 mL of AuNPs was added to 1.00 mL of AHMT at 0.05 mM. This solution was stirred for 2 h at room temperature and then centrifuged at 12000 rpm for 20 min. The AuNPs modified by AHMT were characterized by UV-vis and FTIR spectrophotometers.

The stability of AuNPs before and after modification with AHMT was observed for 2 months. The absorbance of AuNPs and AuNPs-AHMT was measured by UV-vis spectrophotometer at intervals of 15 min, 30 min, 60 min, 2 h, 3 h, 5 h, 24 h, 7 d, 30 d, and 60 d.

Colorimetric detection of cortisol using AuNPs-AHMT

The principle of AuNPs for cortisol detection is based on the color change of AuNPs-AHMT solution. As much as 2.0 mL of cortisol 10.0 nM was added into a test tube that contained 2.0 mL of AuNPs-AHMT and the 1.0 mL buffer phosphate (pH 7.0) was also added. Then the color change was observed, and the absorbance was measured by a UV-vis spectrophotometer in the range of 300–800 nm.

Method validation of AuNPs-AHMT as cortisol detection

The analytical parameter was optimized, and the proposed method was validated. The method validation was performed by analyzing some parameters such as the linearity, limit of detection (LOD), accuracy, precision, selectivity, and sensitivity. The linearity was determined with cortisol concentration ranging from 0.0 nM to 50.0 nM. LOD was evaluated from the equation $3S_b/m$, where S_b is the standard deviation of the blank and m is the slope from the calibration curve. The limit of quantification (LOQ) of an analytical method validation can be defined as 3.3 × LOD. Precision was determined from the coefficient of variance that was analyzed during the intra-day and inter-day. Accuracy was determined by the recovery of cortisol standard solutions. Recovery was determined by the standard addition method and the selectivity of this colorimetric sensor was evaluated by the addition of other substances that are normally present in the saliva, such as amylase and some hormones such as testosterone, progesterone, cortisone, and adrenal hormones.

■ RESULTS AND DISCUSSION

Synthesis of AuNPs

AuNPs were synthesized by using the chemical reduction method of the $HAuCl_4$ solution with $NaBH_4$

and sodium citrate as a reduction agent. The addition of $NaBH_4$ to the Turkevich method was established in an attempt to simplify the synthesis by eliminating the heating process [32]. The success of AuNPs colloidal formation was confirmed by the color change from yellow to wine-red colloidal solution. Then, it was characterized by a UV-vis spectrophotometer to know the surface plasmon resonance of AuNPs and by PSA to know the size distribution of AuNPs colloidal formation. The absorption spectrum of AuNPs is shown in Fig. 1(a), with the maximum surface plasmon resonance at about 540 nm, which resembles the surface plasmon resonance band of AuNPs [33]. PSA analysis shows the size distribution of AuNPs at 38.67 nm within the nanoparticle size range [34] (Fig. 1(b)).

Optimization of $NaBH_4$ Concentration

The formation of stable AuNPs colloidal is influenced by the concentration of the reducing agent. Thus, in this work, the absorption of AuNPs produced at different $NaBH_4$ concentrations was investigated. The concentrations of NaBH4 are an important factor in controlling the size of AuNPs [35]. The variations of $NaBH_4$ were 0.50, 0.75, 1.00, 1.25, and 1.50 mM. The color and size of AuNPs colloidal results were confirmed by UV-vis spectrophotometer and PSA to determine the optimum $NaBH_4$ concentration. The absorbance of UV-vis spectra with variations in $NaBH_4$ concentration is shown in Fig. 2. The highest absorbance is 0.546 at 1.0 mM of $NaBH_4$ concentration. The higher absorbance and narrower peak indicated that AuNPs formed a small

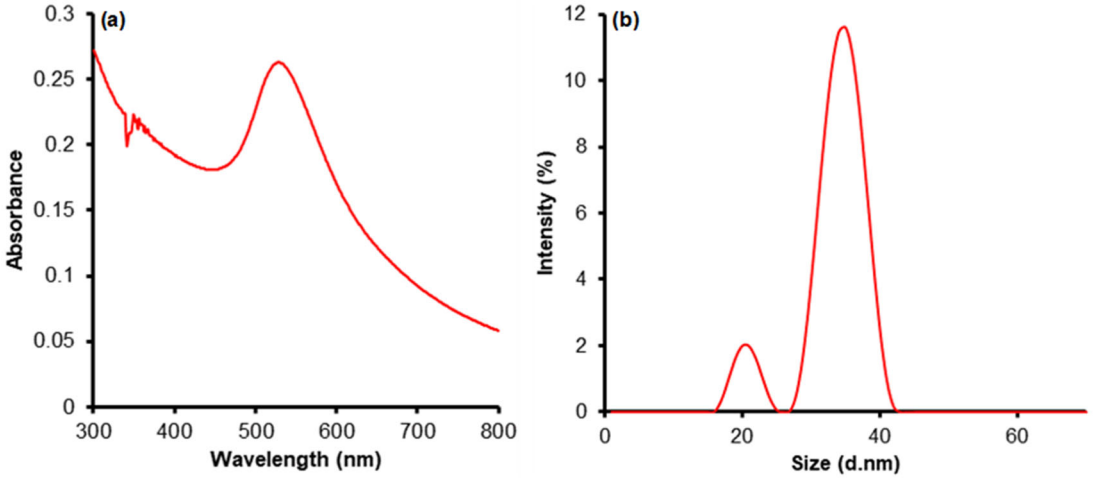

Fig 1. (a) The UV-vis spectra of AuNPs colloidal and (b) the size distribution of AuNPs colloidal

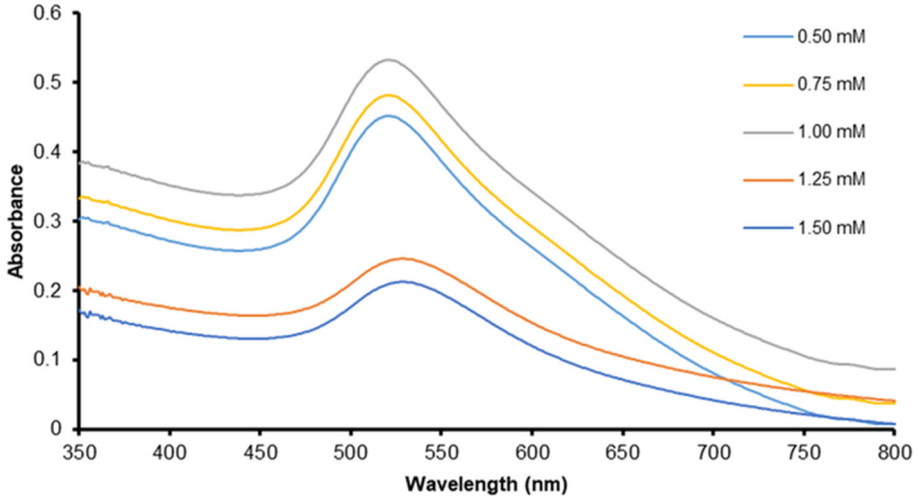

Fig 2. The UV-vis spectra of AuNPs colloidal with variation of $NaBH_4$ concentration

and homogeneous size distribution. The PSA result indicates that the smallest size distribution is 13.30 nm at a 1.00 mM concentration of $NaBH_4$, as shown in Fig. 3. The high absorbance of UV-vis spectra on 1.00 mM of $NaBH_4$ with the narrower full-width half maximum (FWHM). The FWHM criterion is potentially a very sensitive measurement for monodispersity. The narrower peak has a better signal-to-noise ratio, allowing for the detection of a smaller change in the colorimetric sensor [36].

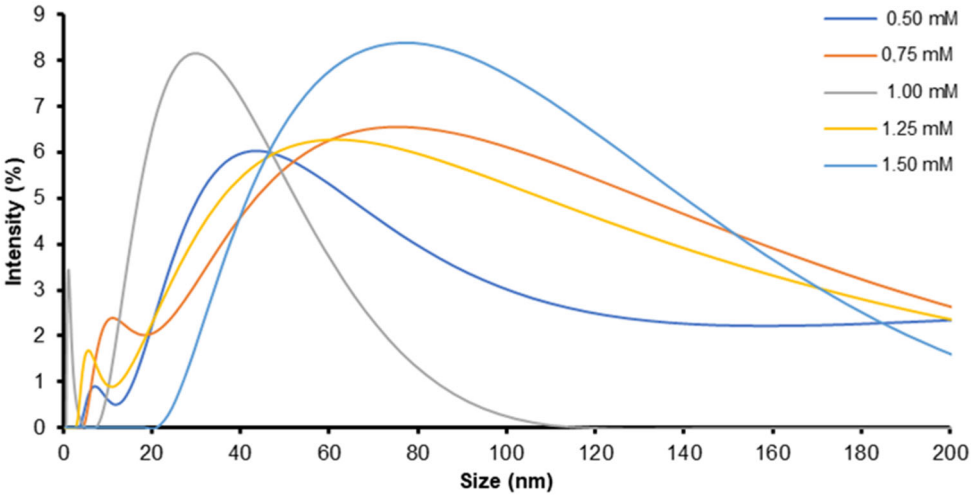

Fig 3. The PSA result of AuNPs colloidal with variation of $NaBH_4$

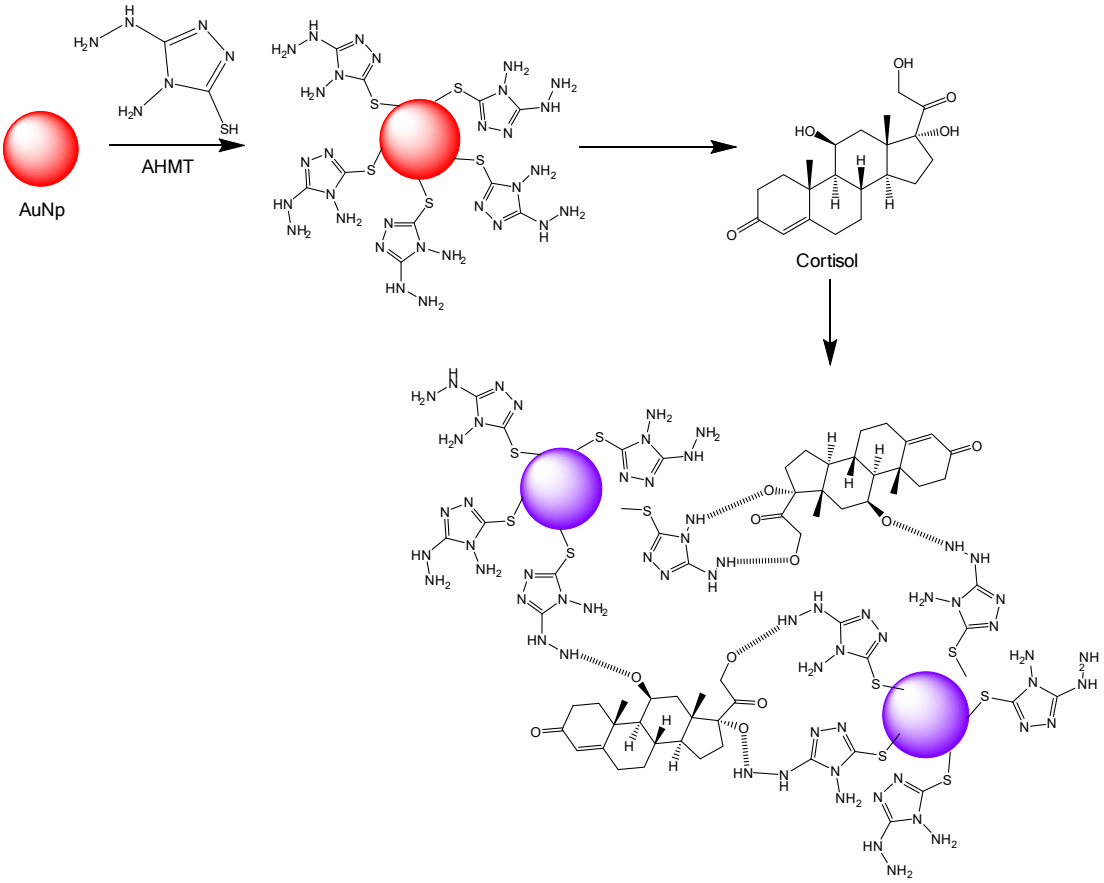

Fig 4. Illustration of reaction mechanism from colorimetric detection of cortisol using AuNPs-AHMT

Surface Modification of AuNPs with AHMT

Generally, AuNPs without modification very easy to cause aggregation and form a larger size distribution. Therefore, it is necessary to modify the surface of AuNPs with a capping agent to increase their stability. The surface attachment of mercapto groups and electron-rich nitrogen to the surface of AuNPs has been well developed [37]. One of the materials that has the ability to be a capping agent is AHMT. AHMT has one mercapto group, which can strongly coordinate with the AuNPs surface with sulfur atoms present in its assembly to protect AuNPs from aggregation [38]. In addition, AHMT also has two exocyclic amino groups and three nitrogen hybrid rings, which have a good ability to form hydrogen bonds (NH···N and OH···N) with analytes as shown in Fig. 4.

The characterization of AuNPs surface modified with AHMT was confirmed by UV-vis spectrophotometer and FTIR. Fig. 5 presents the spectra of absorption of AuNPs with and without AHMT. The surface modification of AuNPs with AHMT results in narrower peak spectra with higher absorbance compared with AuNPs without surface modification. It indicated that AHMT could lead to homogeneous size distributions and also prevent the aggregation of AuNPs.

To confirm that the surface modification of AuNPs-AHMT is successful, FTIR measurements were carried out. The FTIR spectra of AHMT with and without AuNPs are presented in Fig. 6. The characteristic peak of AHMT is 3447, 2043, and 1636 cm^{-1} which corresponds to –N–H, S–H, and N=N stretching, respectively. When

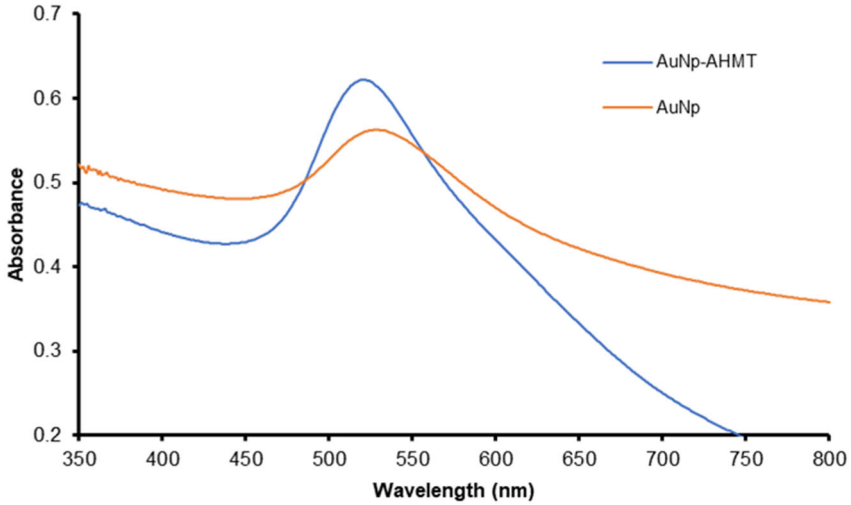

Fig 5. The UV-vis spectra absorption of AuNPs with and without modification with AHMT

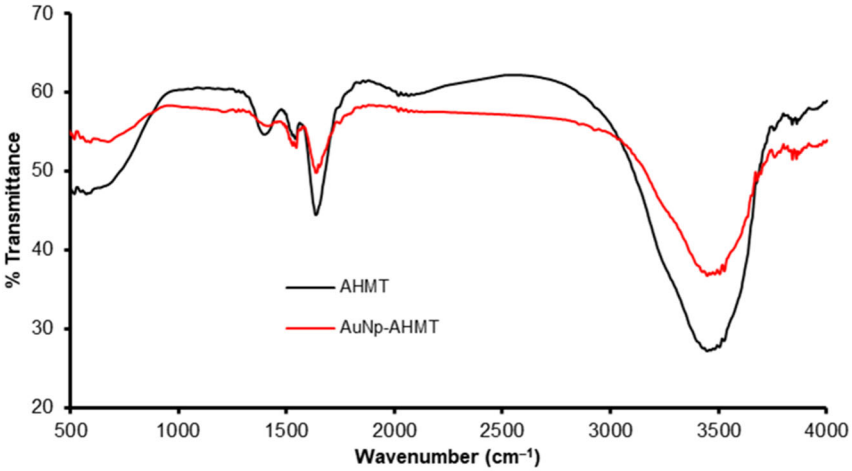

Fig 6. The FTIR spectra of AuNPs with and without modification with AHMT

AuNPs have been modified with AHMT, it causes the peak of the S–H group at 2043 cm^{-1} to disappear. This is an indication that the –SH group of AHMT coordinates with the surface of AuNPs.

The stability of AuNPs before and after modification with AHMT was evaluated during storage for 2 months and characterized with a UV-vis spectrophotometer. The UV-vis spectra in Fig. 7(a) show that AuNPs without modification with AHMT are stable for 7 d, with the highest absorbance at 0.764. The absorbance of AuNPs increases after synthesis for 7 d and decreases continuously for 2 months. This indicates that AuNPs without modification with AHMT are stable for 7 d, and after that, aggregation begins to occur on the AuNPs surface to form larger nanoparticle sizes. Whereas the AuNPs modification with AHMT is stable for up to 2 months. This is evidenced by the results of UV-vis spectra (Fig. 7(b)) that the absorbance always increased until 2 months. This indicates that the presence of AHMT will protect and prevent the aggregation on the surface of AuNPs, so the AuNPs will be more stable.

Colorimetric Detection of Cortisol Using AuNPs-AHMT

The development of AuNPs-AHMT is for the colorimetric detection of cortisol. Cortisol is a steroid hormone and is also known as a stress hormone. The level of cortisol correlates with stroke potency [6], so this compound is widely used as a biomarker of stroke. The principle of AuNPs-AHMT for cortisol detection is the hydrogen bond formation between the –OH group from cortisol and the –NH group from AHMT (Fig. 4). The cortisol structure has three hydroxyl groups that play important roles in binding with AuNPs-AHMT as a probe. Another mechanism is the aggregation of AuNPs-AHMT which causes the formation of a charge transfer complex between AHMT that is rich in electrons and electron-deficient in cortisol, which can induce the agglomeration of AuNPs and cause the color change of AuNPs-AHMT [28].

The colorimetric detection of cortisol using AuNPs-AHMT is based on the color change of AuNPs-AHMT from wine red to purple when there is an interaction

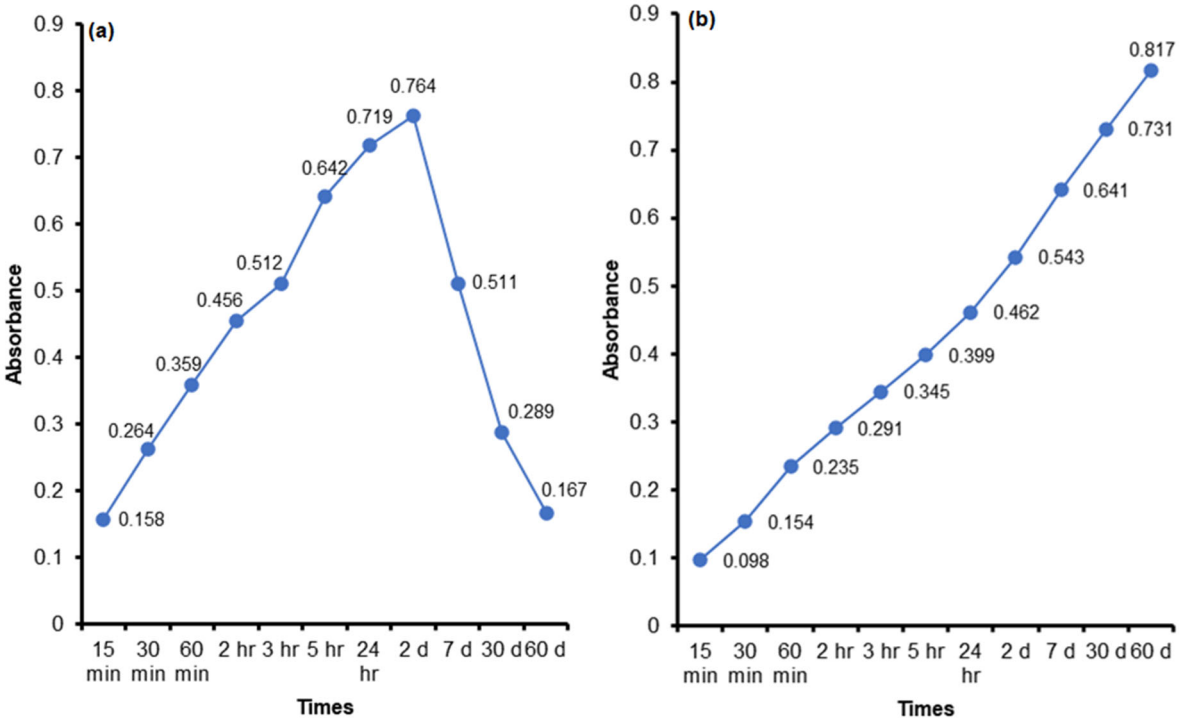

Fig 7. The stability of UV-vis spectra absorption of (a) AuNPs and (b) AuNPs-AHMT

between AuNPs-AHMT and cortisol, as shown in Fig. 8. This color can be easily visualized by the naked eye. The addition of cortisol also causes a new strong red shift at around 640 nm as shown in Fig. 9.

Fig 8. The color change from wine red (AuNPs-AHMT) to purple (AuNPs-AHMT-Cortisol)

Method Validation of AuNPs-AHMT as Cortisol Detection

The proposed method of AuNPs-AHMT for colorimetric cortisol detection was validated. The calibration curve is shown in Fig. 10. The linear response of cortisol is in the range of 1–50 nM with the correlation

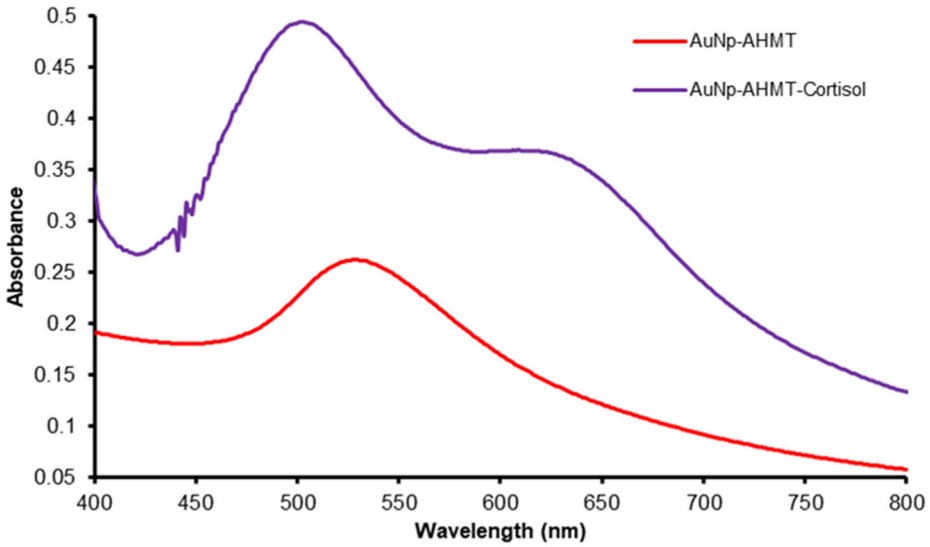

Fig 9. The UV-vis spectra absorption of cortisol colorimetric sensing using AuNPs-AHMT

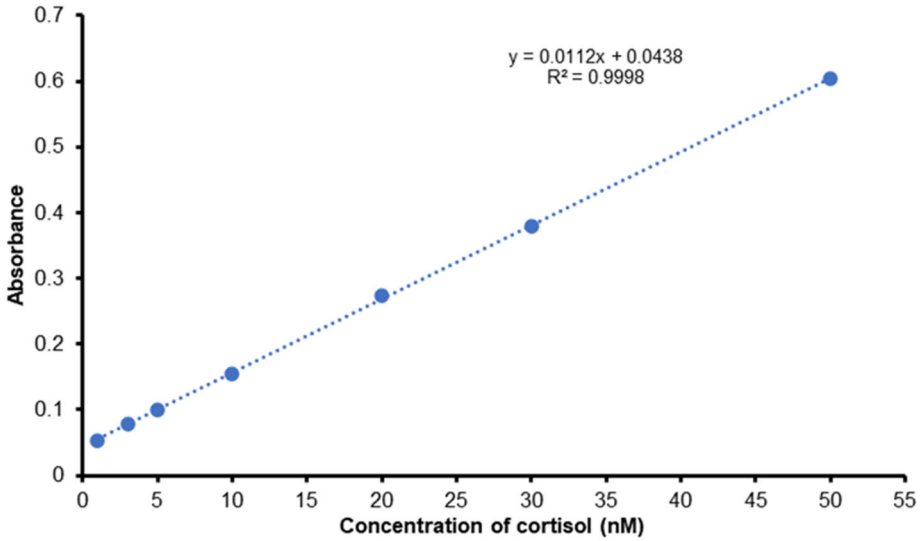

Fig 10. The linear response of cortisol with AuNp-AHMT

coefficient is 0.9998, and the sensitivity is 0.0112 nM/mL (from the slope of the curve). The LOD and LOQ values for cortisol detection using AuNPs-AHMT are 0.76 and 2.54 nM, respectively.

The precision of the developed method was determined with a standard solution of cortisol during intra-day and inter-day. The precision for intra-day and inter-day are < 2.22% and < 2.17%, respectively, as shown in Table 1. And the accuracy is 91.07–102.77%. The result of AuNPs-AHMT method validation as a cortisol colorimetric sensor are summarized in Table 2.

Selectivity of AuNPs-AHMT for Colorimetric Detection of Cortisol

The selectivity of the colorimetric sensor is very important to study as signal responses on the specific target binding-induced aggregation of AuNPs-AHMT. The probe must be insensitive to the other compound and nonspecific binding. To evaluate the selectivity of the proposed method, some interference compounds were added to the AuNPs-AHMT solution. Some potential interference compounds are testosterone, progesterone, corticosterone, and estradiol. The interference concentration added was 100.0 nM which is much higher than the cortisol concentration (10.0 nM). The result of the selectivity study is presented in Fig. 11, which

Table 1. The precision of AuNPs-AHMT as cortisol colorimetric detection

The concentration of cortisol (nM)	%Precision	
	Intra-day (%)	Inter-day (%)
1.00	1.85	2.17
3.00	2.22	1.54
5.00	1.01	1.22
10.00	1.93	0.39
20.00	0.36	0.43
30.00	0.26	0.41
50.00	0.16	0.27

Table 2. The summary of method validation

Variable	Result
Calibration equation	Absorbance = 0.0112[cortisol] + 0.0438
Correlation coefficient (R^2)	0.9998
Sensitivity (nM/mL)	0.0112
LOD (nM)	0.76
LOQ (nM)	2.54
Precision (%)	Intra-day < 2.22
	Inter-day < 2.17
Accuracy (%)	91.07–102.77

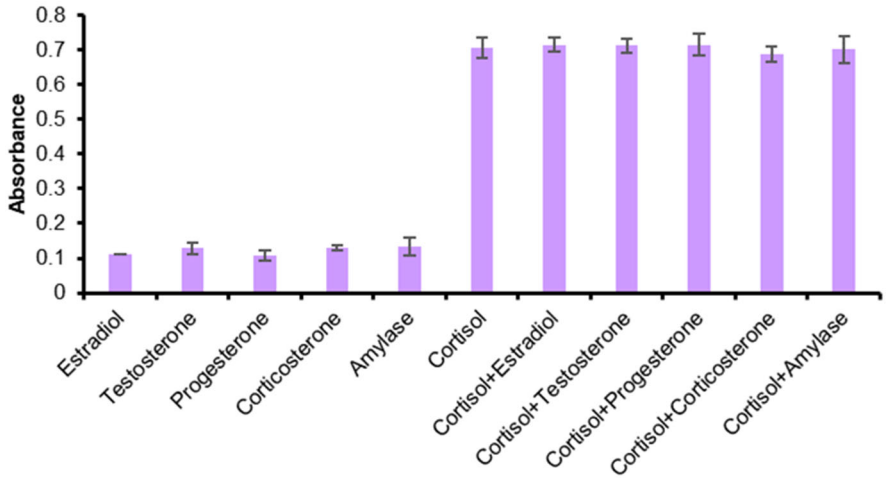

Fig 11. The selectivity of AuNPs-AHMT colorimetric sensing in some potential interferences of cortisol

Table 3. The determination of cortisol in saliva

The concentration of cortisol standard solution (nM)	Found amount (nM)	% Recovery	% RSD	ELISA assay (nM)
5.0	4.55	91.07	0.71	4.91
10.0	9.46	94.64	0.48	10.05

shows that the absorbance of cortisol (individually or mixed with another interference compound) has no obvious influence on the detection of cortisol. Accordingly, this result indicated that AuNPs-AHMT has acceptable selectivity to the cortisol.

Detection of Cortisol in Saliva

To verify the reliability of cortisol colorimetric sensing using AuNPs-AHMT, the content of cortisol in saliva samples was determined. Colorimetric sensing was applied for the detection and determination of cortisol in spiked samples by adding the standard solution of cortisol with different concentrations (5.0 and 10.0 nM) into saliva. The result is shown in Table 3 that the recovery was obtained in the range of 91.07–94.64%. This confirms the success of AuNPs-AHMT application for cortisol detection in saliva samples.

■ CONCLUSION

In this study, cortisol can be detected with a simple, rapid, and selective colorimetric assay using AuNPs modified with AHMT. The hydrogen bonding between AHMT and cortisol induces the aggregation of AuNPs-AHMT as a probe and leads to the color change from wine red to purple. The detection of cortisol was achieved by the naked eye as a colorimetric method and confirmed with UV-vis spectrophotometry which measures the absorbance change of AuNPs corresponding to the cortisol solution. No special organic or additive solvent and no complicated instruments were required in this method. From this work, good linearity, selectivity, precision, accuracy, recovery and low detection limit are obtained for cortisol detection. The rapid colorimetric method for cortisol detection using AuNPs-AHMT in saliva samples has been successfully developed and applied. In the future, it is expected to be a potential method for early detection or monitoring of stroke disease based on cortisol concentration in saliva.

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