

## CORTISOL SENSOR AND METHODS OF THEIR USE

### CROSS-REFERENCE TO RELATED APPLICATION

[001] This application claims the benefit of priority of Singapore Patent Application No. 10202301878T filed 30 June 2023, the content of which being hereby incorporated by reference in its entirety for all purposes.

### TECHNICAL FIELD

[002] Various embodiments relate generally to a sensor for detecting cortisol in a sample, and methods of use thereof. In particular, various embodiments relate to a cortisol nanosensor that is able to sensitively and rapidly detect and quantify cortisol in a sample, which may optionally be used in measuring stress levels of subjects.

### BACKGROUND

[003] Stress detection and monitoring is useful for early diagnosis and prevention of related diseases [1]. The cortisol hormone is commonly employed as a biomarker of physiological stress in humans, cattle, fish etc. Cortisol is secreted primarily in blood, and typically also found in other complex biofluids or biological samples, such as saliva, urine, sputum etc. Blood sampling is invasive, but less/non-invasive samples (urine, saliva, sputum etc.) typically has lower concentration (picomolar range) of cortisol than in blood (nanomolar range). Conventionally, cortisol can be quantified by HPLC (an expensive laboratory equipment) or enzyme-based immunoassays (e.g., ELISA) [2]. Large volumes of organic solvents are typically required in HPLC, rendering it a non-environmentally friendly strategy, in addition to the requirement of expensive laboratory equipment. ELISA is a time-consuming method involving environmentally sensitive enzymatic reactions and multiple incubation/reaction steps. For on-site stress monitoring, it is essential to develop rapid, robust, and easy-to-use sensors for determination of cortisol levels that can be operated on-site by untrained personal. Specifically, the sensor should be compatible with complex sample matrices, such as fish tank water, blood, mucus etc (examples of aquaculture samples).

[004] Colorimetric sensors that employ gold nanoparticle (AuNP) and affinity ligands (e.g., aptamers etc) provide a fast, simple, and low-cost detection method. To date, most reported AuNP-based cortisol sensors utilize aptamers with a low binding affinity in free form, i.e., no conjugation [3]. These reported nanoparticles are unable to withstand complex sample matrix and their sensitivities are inadequate for meeting the requirements of environmental-based and non-invasive animal biofluids samples, which are typically in the picomolar range [4].

[005] Therefore, there is still a need in the art for alternative cortisol sensors and methods of their use to address the drawbacks of existing approaches. In particular, there is a need in the art for the provision of cortisol sensors with enhanced specificity and sensitivity of existing approaches in detecting and measuring cortisol in samples.

#### SUMMARY

[006] In a first aspect, there is provided a cortisol sensor comprising or consisting of: i) a metal nanoparticle; ii) a plurality of cortisol binding ligands conjugated to the surface of the metal nanoparticle; and iii) a plurality of peptides conjugated to the surface of the metal nanoparticle by means of a Cysteine (C) residue for reducing non-specific binding of the cortisol binding ligands and/or improving stability of the metal nanoparticle in solution.

[007] In various embodiments, the metal nanoparticle comprises or consists of a noble metal, preferably gold, more preferably the metal nanoparticle is a citrate-coated gold nanoparticle.

[008] In various embodiments, the metal nanoparticle has a diameter in the range of 5-50 nm, more preferably a diameter in the range of 10-20 nm, even more preferably a diameter of about 13 nm, so long as the metal nanoparticle has desirable plasmonic properties and/or sensitivity to UV-vis shift in response to differential aggregation.

[009] In various embodiments, the cortisol binding ligand is a cortisol aptamer, preferably a cortisol DNA aptamer, optionally the cortisol aptamer is conjugated directly to the surface of the metal nanoparticle or indirectly through an intervening linker.

[010] In various embodiments, the cortisol aptamer comprises or consists of a nucleotide sequence set forth in **SEQ ID NO:1, 2 or 3**, or derivatives thereof.

[011] In various embodiments, the cortisol aptamer comprises or consists of a nucleotide sequence set forth in **SEQ ID NO:2**, or derivatives thereof.

[012] In various embodiments, the cortisol aptamer is labelled with a thiol group, and the thiol group is covalently bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the surface of the metal nanoparticle, preferably the cortisol aptamer is thiol-terminated at the 5' end.

[013] In various embodiments, the cortisol aptamer is terminated with an amine group (NH<sub>2</sub>), and the amine group is covalently bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the metal nanoparticle, wherein the surface of the metal nanoparticle is functionalized with carboxylic acid groups (COOH).

[014] In various embodiments, the cortisol aptamer is biotinylated with a biotin group, optionally introduced via a linker molecule or by incorporating a biotinylated nucleotide during the aptamer synthesis, and the biotin group is bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the metal nanoparticle, wherein the surface of the metal nanoparticle is functionalized with streptavidin.

[015] In various embodiments, the conjugated peptides are interspersed between conjugated cortisol binding ligands to separate and space apart the cortisol binding ligands from being proximate to each other to improve the stability of the metal nanoparticle.

[016] In various embodiments, the plurality of peptides is a plurality of short peptides, optionally each short peptide comprises or consists of an amino acid sequence CX<sub>n</sub>, CAX<sub>n</sub>, or CALX<sub>n</sub>, wherein X denotes any amino acid residue and n is an integer of 1 or more, and/or optionally each short peptide is labelled with a thiol group.

[017] In various embodiments, the short peptide comprises or consists of an amino acid sequence set forth in **SEQ ID NO: 4** or variants thereof.

[018] In various embodiments, the metal nanoparticle is a gold nanoparticle; the cortisol binding ligand is a cortisol DNA aptamer comprising or consisting of a nucleotide sequence set forth in **SEQ ID NO: 1, 2 or 3**, or derivatives thereof, and is labelled with a thiol group at the 5' end, wherein the thiol group is covalently bound to the surface of the gold nanoparticle; and the peptide comprises or consists of an amino acid sequence set forth in **SEQ ID NO: 4** or variants thereof.

[019] In a second aspect, there is provided a method of preparing a cortisol sensor, comprising or consisting the steps of: a) preparing a first solution comprising a plurality of metal nanoparticles; b) preparing a second solution comprising a plurality of cortisol-binding ligands; c) adding the first and second solutions together to produce a first mixture; d) incubating the first mixture under conditions suitable to conjugate the cortisol-binding ligands onto the surface of the metal nanoparticles; and e) adding a third solution comprising a plurality of peptides to the incubated first mixture under conditions suitable to conjugate the peptides onto the surface of the metal nanoparticles to produce a second mixture comprising one or more of the cortisol sensor, optionally wherein the second mixture is used to prepare a colloidal solution or composition comprising a plurality of the cortisol sensors.

[020] In various embodiments, the method further comprises isolating the cortisol sensors, wherein each cortisol sensor comprises or consists of: a metal nanoparticle; a plurality of cortisol binding ligands conjugated to the surface of the metal nanoparticle; and a plurality of peptides conjugated to the surface of the metal nanoparticle by means of a Cysteine (C) residue for reducing non-specific binding of the cortisol binding ligands and/or improve stability of the metal nanoparticle in solution,

optionally the isolated cortisol sensors are stored at suitable conditions before further use or processing, preferably at a temperature in the range of 1-4°C.

[021] In various embodiments, the metal nanoparticle is a gold nanoparticle, and step a) comprises: adding a sodium citrate dihydrate solution, preferably about 5mL at a concentration of 40 mM, to boiling gold (III) chloride solution, preferably about 50mL at a concentration of 1mM, to provide a mixture, refluxing the mixture for about 20-40 minutes, preferably about 30min, to provide the first solution comprising a plurality of gold nanoparticles; and cooling the first solution to room temperature.

[022] In various embodiments, the cortisol binding ligand is a cortisol aptamer, and step b) comprises: adding the cortisol aptamer in a buffer solution, optionally shaking the buffer solution comprising the cortisol aptamer; centrifuging the buffer solution to produce a supernatant, preferably the buffer solution is transferred to a 3kDa centrifugal filter; and adding water to the supernatant to provide the second solution.

[023] In various embodiments, step c) further comprises adding an acidic solution, preferably hydrochloric acid, and wherein the second solution is added to the first solution and subsequently the acidic solution is added thereafter, optionally the acidic solution is in an amount of about 0.5-50µl, preferably about 10 µl, optionally the concentration of the acidic solution is about 0.01M-1M, preferably 0.1M.

[024] In various embodiments, step e) further comprises adding an alkaline solution, preferably sodium hydroxide, optionally the alkaline solution is in an amount of about 0.5-50µl, preferably about 10 µl, optionally the concentration of the alkaline solution is about 0.01M-1M, preferably 0.1M.

[025] In various embodiments, the production of the cortisol sensor is verified by: i) UV-vis spectroscopy, wherein the UV-vis spectroscopy comprises comparing a UV-vis spectrum of the cortisol sensor to a UV-vis spectrum of the metal nanoparticle in the first solution; and/or, ii) a salt stability test, wherein the salt stability test comprises adding the cortisol sensor to a saline solution, and evaluating the dispersion of the cortisol sensor for aggregation.

[026] In a third aspect, there is provided the use of the cortisol sensor disclosed herein for detecting the presence or absence, and optionally the quantity, of cortisol in a sample.

[027] In a fourth aspect, there is provided a method to detect the presence or absence, and optionally the quantity, of cortisol in a sample, comprising or consisting the steps of: a) providing a cortisol sensor disclosed herein; b) contacting the cortisol sensor to the sample in a saline environment that is suitable for binding the cortisol binding ligand to cortisol, to produce a sample mixture; and c) detecting the presence or absence, and optionally the quantity, of cortisol in the sample mixture by determining the degree of aggregation of the cortisol sensor in the sample mixture, wherein the

degree of aggregation of the sample mixture is compared with the degree of aggregation of a control sample and a differential degree of aggregation is indicative of the presence or absence, and optionally the quantity, of cortisol in the sample.

[028] In various embodiments, a bivalent salt ion is added to the sample mixture in step b), wherein the bivalent salt ion is selected from the group consisting of a calcium chloride ( $\text{CaCl}_2$ ), calcium carbonate ( $\text{CaCO}_3$ ), calcium sulfate ( $\text{CaSO}_4$ ), magnesium chloride ( $\text{MgCl}_2$ ), magnesium sulfate ( $\text{MgSO}_4$ ), magnesium carbonate ( $\text{MgCO}_3$ ), barium chloride ( $\text{BaCl}_2$ ), barium sulfate ( $\text{BaSO}_4$ ), lead acetate ( $\text{Pb}(\text{CH}_3\text{COO})_2$ ), lead nitrate ( $\text{Pb}(\text{NO}_3)_2$ ), ferrous sulfate ( $\text{FeSO}_4$ ), ferrous chloride ( $\text{FeCl}_2$ ) and combinations thereof, preferably the bivalent salt ion is  $\text{MgCl}_2$ .

[029] In various embodiments, stability of the cortisol sensor increases in the presence of cortisol in the sample, such that the degree of aggregation is reduced in the presence of cortisol in the sample compared to the control sample.

[030] In various embodiments, the degree of aggregation is measured by UV-Visible Spectroscopy, microscopy techniques, dynamic light scattering techniques, visual observation of colour, or surface plasmon resonance techniques.

[031] In various embodiments, the degree of aggregation is measured by UV-vis spectroscopy to obtain a UV-vis spectrum of the sample mixture, wherein the UV-vis spectrum of the sample mixture is compared with the UV-vis spectrum of a control sample and a differential UV-vis spectrum is indicative of the presence or absence, and optionally the quantity, of cortisol in the sample.

[032] In various embodiments, the degree of aggregation is determined by measuring the absorbance ratio of the sample mixture, wherein the absorbance ratio of the sample mixture is compared with the absorbance ratio of the control sample, and a differential absorbance ratio is indicative of the presence or absence, and optionally the quantity, of cortisol.

[033] In various embodiments, an absorbance ratio of  $A_{520}/A_{650}$  is measured for the sample mixture and control sample, wherein a differential absorbance ratio of  $A_{520}/A_{650}$  between the sample and control sample is indicative of the presence or absence of cortisol in the sample.

[034] In various embodiments, the method further comprises quantifying the cortisol in the sample mixture based on the absorbance ratio of  $A_{520}/A_{650}$ , wherein the absorbance ratio of the sample mixture is compared with the absorbance ratio of a control sample, wherein a differential absorbance ratio between the sample and control sample is indicative of the presence, and quantity, of cortisol in the sample mixture, and the absorbance ratio of the control sample is subtracted from the absorbance ratio of the sample mixture.

[035] In various embodiments, the differential absorbance ratio is in the range of 0.2-1.2 and corresponds to cortisol concentrations ranging from 0.1-1000 nM.

[036] In various embodiments, after step b), the sample mixture is not further processed, treated, incubated or washed before the detection step c) is carried out, such that the method does not comprise an additional or intermediary step between steps b) and c).

[037] In various embodiments, the detection step is completed in about 10-30 minutes, preferably about 15 minutes, after step b), preferably step c) immediately follows, or is immediately initiated after, step b).

[038] In various embodiments, the sample is a water sample obtained from an aquaculture tank, preferably a fish tank, or the sample is a biological sample, preferably a biological tissue or fluid sample that has been obtained from an animal, even more preferably a fish tissue sample, optionally the fish tissue sample is derived from the fin, scales, mucus, or plasma of the fish and may be in a fluid form or a solid form, optionally wherein the solid form is processed and extracted into a liquid form.

[039] In a fifth aspect, there is provided a method of measuring a stress level in a subject, comprising: a) contacting a biological sample that has been obtained from the subject with a cortisol sensor disclosed herein in a saline environment that is suitable for binding the cortisol binding ligand to cortisol, to produce a biological sample mixture; and b) detecting the quantity of cortisol in the biological sample mixture by determining the degree of aggregation, wherein the degree of aggregation of the biological sample mixture is compared with the degree of aggregation of a control sample and a differential degree of aggregation is indicative of the quantity of cortisol in the biological sample mixture and the stress level of the subject.

[040] In various embodiments, the quantity of cortisol in the biological sample mixture is compared with known reference levels of cortisol associated with certain stress levels in the subject, wherein each known reference level is specific for gender, age, mental condition, and/or predictive of a mental disorder of said subject.

[041] In a sixth aspect, there is provided a method for assisting in determining a subject's risk for developing an autoimmune disease, an inflammatory disease, and/or an allergic disease through measuring the subject's cortisol level with the method to detect the presence or absence, and optionally the quantity, of cortisol in a sample disclosed herein, wherein the sample has been obtained from the subject.

[042] In a seventh aspect, there is provided a method for predicting a subject's response to a steroid treatment for treating a disorder through measuring the subject's cortisol level with the method to

detect the presence or absence, and optionally the quantity, of cortisol in a sample disclosed herein, wherein the sample has been obtained from the subject.

[043] In an eighth aspect, there is provided a method for assisting in determining the presence or absence of a mental disorder in a subject through measuring the subject's cortisol level with the method to detect the presence or absence, and optionally the quantity, of cortisol in a sample disclosed herein, wherein the sample has been obtained from the subject, optionally wherein the mental disorder is one or more of an anxiety disorder, a major depressive disorder (MDD), a post-traumatic stress disorder (PTSD), a traumatic brain injury (TBI), a psychotic disorder, and/or a personality disorder.

[044] In a ninth aspect, there is provided a method for developing or identifying therapeutic agents capable of modifying the stress level of a subject, the method comprising: a) measuring the subject's stress level with the method disclosed herein, b) administering a candidate therapeutic agent to the subject, c) measuring the subject's stress level with the method disclosed herein following the administration of the candidate therapeutic agent, d) characterizing the candidate therapeutic agent as capable of modifying the stress level of the subject, if the subject's measured cortisol level increases or decreases following administration of the candidate therapeutic agent.

[045] In a tenth aspect, there is provided a colloidal solution or composition comprising a plurality of cortisol sensors disclosed herein.

[046] In an eleventh aspect, there is provided a substrate comprising a plurality of cortisol sensors disclosed herein, for use in detecting the presence or absence, and optionally the quantity, of cortisol in a sample.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[047] Various embodiments will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings.

[048] **FIG. 1** shows an absorbance spectrum of AuNP with: **(A)** 15-1a Aptamer, before and after COR binding (1-10000 nM); **(B)** CSS.1 Aptamer before and after COR binding (0.1 – 100 nM); and **(C)** the Abs ratio of A520/A650 of the two cases of (A) and (B) as a function of cortisol concentrations.

[049] **FIG. 2** shows: **(A)** Normalized UV-vis spectrums of AuNP and Au-Apt-CALNN; **(B)** Normalized absorbance spectrum of Au-Apt-CALNN in water, ASW, RP Sea 1 and RP Sea 2 water; and **(C)** Image of RPSea 1 and RP Sea 2 water samples.

[050] **FIG. 3** shows a schematic illustration of the detection principle using Au-Apt-CALNN.

[051] FIG. 4 shows the UV-vis spectra of: (A) Au-Apt-CALNN in the presence of 0 to 1000 nM COR; (B) Plot of absorbance ratio A520/A650 as a function of log COR (nM); and (C) Plot of the change in the absorbance ratio A520/A650 as a function of log COR (nM).

[052] FIG. 5 shows the selectivity test of Au-Apt-CALNN against other interfering molecules.

[053] FIG. 6 shows Cortisol concentrations obtained for 1st chasing water samples at different time periods, where Cortisol concentrations were obtained using: (A) Au-Apt-CALNN nanosensor; (B) HPLC; (C) ELISA; and the correlation between (D) nanosensor versus HPLC, and (E) nanosensor versus ELISA.

[054] FIG. 7 shows Cortisol concentrations obtained for fin samples at different time periods using: (A) Au-Apt-CALNN nanosensor and (B) ELISA; and (C) Correlation between nanosensor versus ELISA.

#### DETAILED DESCRIPTION

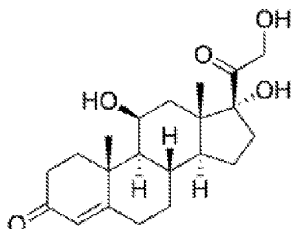
[055] The following detailed description refers to, by way of illustration, specific details and embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention. Other embodiments may be utilized and structural and logical changes may be made without departing from the scope of the invention. Embodiments described below in context of the sensor are analogously valid for the respective methods, uses and *vice versa*. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[056] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "comprises" means "includes." In case of conflict, the present specification, including explanations of terms, will prevail. "About", as used herein in connection with numerical values refers to the referenced numerical value  $\pm 10\%$  or  $\pm 5\%$ .

[057] The present invention provides a sensor, more particularly a nanosensor, for use in the detection of cortisol in a sample. In various embodiments, there is provided a cortisol sensor that comprises or consists of i) a metal nanoparticle, ii) a plurality of cortisol binding ligands conjugated to the surface of the metal nanoparticle; and iii) a plurality of peptides conjugated to the surface of the metal nanoparticle.

[058] As used herein, "cortisol" refers to a steroid hormone, in the glucocorticoid class of hormones and is a stress hormone. In animals, cortisol is secreted from the adrenal cortex in the adrenal gland, but may be produced in other tissues in lower amounts. Cortisol is well known to those skilled in the

art, by other names that may be used interchangeably therewith, such as hydrocortisone and any synthetic or natural analogues thereof. The term "COR" may be used interchangeably herein to refer to cortisol. In particular, cortisol is an organic compound with the chemical formula  $C_{21}H_{30}O_5$  and a chemical structure of:



[059] In various embodiments, the cortisol sensor disclosed herein may also be used to detect synthetic derivatives of cortisol such as prednisone, methylprednisolone, and dexamethasone, or may be used to detect metabolites of cortisol including but not limited to endogenous metabolites (6 $\beta$ -hydroxycortisol, 20 $\alpha$ -dihydrocortisol, 20 $\alpha$ -dihydrocortisone, 20 $\beta$ -dihydrocortisol, 20 $\beta$ -dihydrocortisone, prednisolone, cortisone,  $\alpha$ -cortolone,  $\beta$ -cortolone, allotetrahydrocortisol, 5 $\alpha$ -dihydrocortisol, tetrahydrocortisol, allotetrahydrocortisone, 5 $\beta$ -dihydrocortisol, tetrahydrocortisone).

[060] As used herein, "conjugated" refers to two entities stably bound to one another by any physicochemical means. It is important that the nature of the attachment is such that it does not substantially impair the effectiveness of either entity in their function and purpose. Keeping these parameters in mind, any covalent or non-covalent linkage known to those of ordinary skill in the art is contemplated unless explicitly stated otherwise herein. Non-covalent conjugation may include but are not limited to hydrophobic interactions, ionic interactions, high-affinity interactions such as biotin-avidin and biotin-streptavidin complexation and other affinity interactions. Covalent conjugation may include but are not limited to the use of reactive groups on both the two entities. Common strategies involve using linkers that react with functional groups (such as thiol, amine, or carboxyl groups). The terms "attached" and "attachment", or "linked" may be used interchangeably herein with the terms "conjugated" to refer to moieties that are covalently or noncovalently physically associated and/or joined to one another, either directly or indirectly. Two attached moieties may be referred to as a "conjugate" or a complex. Conjugation can be performed using standard techniques common to those of ordinary skill in the art. In various embodiments, the conjugation of the peptide and cortisol binding ligand to the surface of the metal nanoparticle, may also refer to the conjugation to the peripheral layer of the nanoparticle.

[061] The term "nanoparticle" refers to a particle having a longest straight dimension of 1  $\mu\text{m}$  or less, typically 500 nm or less, more typically 200nm or less. Nanoparticles may have a range of shapes and cross-sections including spheres, oblate spheroids, cylinders, shells, cubes, pyramids, hexagons, tetrapods (particles having four leg-like appendages), triangles, prisms, ellipses, rods (e.g.,

cylinders or elongated structures having a square or rectangular cross-section). Spherical nanoparticles of interest herein generally have a diameter of 200 nm or less, preferably 100 nm or less, and often 50 nm or less. The term "nanoparticle" encompasses atomic clusters, which have a typical diameter of 1 nm or less.

**[062]** The metal nanoparticle disclosed herein, may include any one of an alkali metal, alkaline earth metal, transition metal, post-transition metal, lanthanide metal, actinide metal or any alloy thereof. The metal may be a transition metal or any alloy thereof. The metal may be any one of a group 8 transition metal, group 9 transition metal, group 10 transition metal, group 11 transition metal, group 12 transition metal, group 13 transition metal or any alloy thereof. The metal may be any one of a group 8 transition metal, group 10 transition metal, group 11 transition metal or any alloy thereof. The metal may be resistant to corrosion or oxidation. The metal may be a noble metal. The noble metal may not readily undergo corrosion or oxidation. In various embodiments, the metal may be any one of ruthenium (Ru), rhodium (Rh), palladium (Pd), silver (Ag), osmium (Os), iridium (Ir), platinum (Pt), gold (Au) or any alloy thereof. The metal may be chemically or biologically inert, such that the metal may not initiate a response or undergo chemical reactions when introduced to biological tissue. In various embodiments, the metal may be any one of ruthenium (Ru), platinum (Pt), silver (Ag), gold (Au) or any alloy thereof.

**[063]** In various embodiments, the metal nanoparticle may comprise or consist of a metal selected from the group consisting of gold, silver, ruthenium, rhodium, palladium, osmium, iridium, and platinum.

**[064]** In various embodiments, the metal nanoparticle may comprise or consist of a noble metal, preferably gold, such that the metal nanoparticle is a gold nanoparticle. Gold nanoparticles (AuNP) have unique optical and electronic properties. The interaction of AuNPs with light is strongly dictated by their environment, size and physical dimensions. Oscillating electric fields of a light ray may propagate near a colloidal nanoparticle and may interact with free electrons, causing a concerted oscillation of electron charge that is in resonance with the frequency of light. These resonant oscillations, known as plasmons, may depend on the size of the NP. For small monodisperse (30 nm) AuNPs, the surface plasmon resonance phenomena may cause an absorption of light in the blue-green portion of the spectrum (450 nm) while red light (700 nm) may be reflected. As particle size increases, the wavelength of surface plasmon resonance-related absorption shifts to longer wavelengths. Red light is then absorbed and blue light is reflected. The surface plasmon resonance may be tuned by varying the size or shape of the NP, leading to particles with tailored optical properties for different applications. In various embodiments, the metal nanoparticle is a citrate-coated gold nanoparticle, whereby the citrate-coating may allow for functional conjugation to the cortisol binding ligand and peptides. Preferably, the citrate-coating also imparts additional protection to the nanoparticles from aggregation, for example, 13 nm citrate-coated gold nanoparticles are stable at room temperature.

[065] In various embodiments, the metal nanoparticle may have a diameter in the range of 1-100 nm, preferably a diameter in the range of 10-50 nm, more preferably a diameter in the range of 10-20 nm, even more preferably a diameter of about 13 nm. It will be appreciated that the diameter of the metal nanoparticle may be any size so long as the metal nanoparticle has desirable plasmonic properties and/or sensitivity to UV-vis shift in response to differential aggregation. For example, a metal nanoparticle having a size of less than 5nm would exhibit weak plasmonic properties, and would not be desirable for use for detecting cortisol.

[066] In various embodiments, the metal nanoparticle has a diameter that is greater than 5 nm, preferably between 5-50nm, since a diameter of less than 5nm is expected to provide a weak plasmonic property.

[067] As used herein, the term "cortisol binding ligand" refers to any molecule or compound that has an affinity for binding to cortisol, preferably the cortisol binding ligand specifically and/or selectively binds to cortisol. The cortisol binding ligand may also be termed interchangeably as an "affinity ligand" or "affinity moiety". As used herein, the term "affinity moiety" or "affinity ligand" refers to any molecule that binds to a targeting ligand, in the present case cortisol. In various embodiments, the affinity ligand may bind to the targeting ligand non-covalently. Non-covalent interactions include, but are not limited to, hydrophobic interactions, ionic interactions, hydrogen bonding, van der Waals interactions, dipole-dipole interactions, electrostatic interactions, shape recognition interactions, ionic charge complex formation,  $\pi$ - $\pi$  interactions, and host guest interaction (e.g., cyclodextrin/adamantine). Thus, the affinity ligand can be any molecule able to bind to the targeting moiety non-covalently, specifically and at definite sites on the targeting moiety with high affinity.

[068] In various embodiments, the cortisol binding ligand may include but is not limited to antibodies and antigen-binding fragments thereof, oligonucleotide molecules (such as aptamers), receptor molecules and derivatives which bind specifically and selectively to cortisol, and are also capable of conjugating to the surface of the metal nanoparticle.

[069] In various embodiments, the cortisol binding ligand may be an anti-cortisol antibody, which may be conjugated onto the metal nanoparticles through any conventionally method known to the skilled person. In this regard, The metal nanoparticle conjugated antibody may form a sandwiched assay or competitive assay in combination with another metal nanoparticle conjugated binding ligand.

[070] In various embodiments, the cortisol binding ligand may have a high binding affinity to cortisol, and represents a cortisol-selective binding ligand for the sensitive detection of cortisol. The binding affinity may be determined by any suitable method, such as Surface Plasmon Resonance (SPR), Nuclear Magnetic Resonance (NMR), radiolabel filter-binding assay or a fluorescence bead-based Luminex assay. As used herein, the term "sensitivity" is defined as a statistical measure of

performance of any suitably known assay (e.g., method, test), calculated by dividing the number of true positives by the sum of the true positives and the false negatives.

**[071]** As used herein, the term "high affinity" or "high binding affinity" refers to the cortisol binding ligand binding to the targeting cortisol with a higher affinity than to a reference molecule or ligand. The binding affinity may be expressed in terms of a dissociation constant ( $K_d$ ), whereby the lower the  $K_d$ , the higher the binding affinity of the binding ligand to the target analyte. In various embodiments, the dissociation constant ( $K_d$ ) of a complex in which the cortisol binding ligand and target cortisol are bound may be 50  $\mu\text{M}$  or less, 10  $\mu\text{M}$  or less, 5  $\mu\text{M}$  or less, 3  $\mu\text{M}$  or less, preferably less than 300 nM, 275 nM, 250 nM, 200 nM, 175 nM, 150 nM, or 125 nM. In various embodiments, the dissociation constant ( $K_d$ ) may be less than 1  $\mu\text{M}$ , or less than 0.5  $\mu\text{M}$ , more preferably less than 0.25  $\mu\text{M}$ . In various embodiments, the cortisol binding ligand does not substantially bind to any molecule other than cortisol. For example, the  $K_d$  of the cortisol binding ligand and any other molecule may be at least 10-fold greater, at least 100-fold greater, or at least 1000-fold greater than the  $K_d$  of the cortisol binding ligand and the target cortisol. In various embodiments, the  $K_d$  of the cortisol binding ligand to cortisol may be less than 245 nM, whereby the  $K_d$  of the representative aptamer CSS.1 is  $245 \pm 39$  nM.

**[072]** The conjugation of the cortisol binding ligand (i.e. aptamer) to the metal nanoparticle (i.e. AuNP), causes the nanoparticles to be protected from aggregation in a salt medium (i.e. salt induced aggregation). In particular, the cortisol sensor disclosed herein has high colloidal stability to protect against salt-induced aggregation due to steric stabilization.

**[073]** In various embodiments, the cortisol binding ligand may be an oligonucleotide, preferably a single-stranded oligonucleotide. The terms "nucleic acid" and "oligonucleotide" are used interchangeably herein to refer to a single or double-stranded polymer of nucleotides. The term includes the naturally occurring nucleic acids DNA and RNA. One of ordinary skill in the art will appreciate that nucleic acids can comprise various analogs of the nucleotides found in naturally occurring nucleic acids and that a nucleic acid can contain a variety of modifications to the sugar or backbone structure. The term "nucleic acid sequence" as used herein can refer to the nucleic acid material itself and is not restricted to the sequence information (e.g., the succession of letters chosen among the five base letters A, G, C, T, or U) that biochemically characterizes a specific nucleic acid.

**[074]** In various embodiments, the cortisol binding ligand may be a cortisol aptamer. As used herein, a "cortisol aptamer" may refer to a peptide or oligonucleotide aptamer that selectively binds to cortisol. Preferred aptamers are oligonucleotide aptamers that may be single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA) molecules that can bind to cortisol. In various embodiments, the cortisol aptamer may be a cortisol DNA aptamer.

**[075]** In various embodiments, the cortisol aptamer may be a high-affinity aptamer for cortisol, more particularly the cortisol aptamer may be a single-stranded DNA aptamer.

[076] In various embodiments, the cortisol aptamer may comprise or consist of a nucleotide sequence set forth in **SEQ ID NO:1** (ATGGGCAATGCGGGGTGGAGAATGGTTGCCGCACTTCGG) or derivatives thereof. This aptamer is also termed as 15-1a.

[077] In various embodiments, the cortisol aptamer may comprise or consist of a nucleotide sequence set forth in **SEQ ID NO:2** (GACGACGCCCGCATGTTCCATGGATAGTCTTGACTAGTCGTC) or derivatives thereof. Advantageously, the aptamer termed CSS.1 with the nucleotide sequence set forth in SEQ ID NO: 2, was confirmed to exhibit a high affinity to cortisol, which translated into a higher sensitivity in the metal nanoparticle based cortisol detection sensor and method disclosed herein.

[078] In various embodiments, the cortisol aptamer may comprise or consist of a nucleotide sequence set forth in **SEQ ID NO:3** (GGAATGGATCCACATCCATGGATGGGCAATGCGGGGTGGAGAATGGTTGCCGCACTTCGGCT TCACTGCAGACTTGACGAAGCTT) or derivatives thereof. This aptamer has been confirmed to be capable of binding to cortisol, as detailed in prior publications [6] and [7].

[079] In various embodiments, a plurality of cortisol aptamers may be conjugated to the surface of the metal nanoparticle, wherein the plurality of cortisol aptamers may comprise one or a combination of the nucleotide sequences set forth in **SEQ ID NO:1, 2 and/or 3** or derivatives thereof.

[080] The term "derivative" as used herein relates to nucleotide sequences having one or more substitutions, deletions and/or additions, preferably two, three, four, five or six compared to the reference sequence. The term "derivative" also relates to any functional fragments of the reference nucleotide sequences and in general includes nucleotide sequences derived from the reference nucleotide sequences suitable for realizing the intended use of the present invention, which means that the derivative nucleotide sequences of cortisol aptamers are able to bind to cortisol for use in the methods disclosed herein. In various embodiments, the term "derivative" covers nucleotide sequences that have at least 80%, or at least 90% sequence identity with the sequence set forth in SEQ ID NO:1, 2 or 3 over their entire length, preferably at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % sequence identity. The identity of nucleic acid sequences is generally determined by means of a sequence comparison. This sequence comparison is based on the BLAST algorithm that is established in the existing art and commonly used (cf. e.g. Altschul et al. (1990) "Basic local alignment search tool", J. Mol. Biol. 215:403-410, and Altschul et al. (1997): "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs"; Nucleic Acids Res., 25, p. 3389-3402) and is effected in principle by mutually associating similar successions of nucleotides in the nucleic acid sequences, respectively. A tabular association of the relevant positions is referred to as an "alignment." Sequence comparisons (alignments), in particular multiple sequence comparisons, are commonly prepared using computer programs which are available and known to those skilled in the art.

[081] In various embodiments, the cortisol DNA aptamer may be modified at the 3'-end or at the 5'-end with a chemically reactive functional group. The chemically reactive functional group may be any one of a thiol, alcohol, amine, azide, alkyne, or biotin. The chemically reactive functional group may be reacted with the metal nanoparticle to form a chemical bond. Thus, the cortisol aptamer may be chemically bonded, and thus conjugated, onto the surface of the metal nanoparticle. The chemical bond may be a covalent bond. The cortisol aptamer may be covalently bonded onto the metal nanoparticle. The covalent bond may be selected from the group consisting of any one of thiol, cyclic disulfide,  $\text{---S---}$ ,  $\text{---S(O)---}$ ,  $\text{---S(O)}_2\text{---}$ ,  $\text{---S---S---}$ ,  $\text{---O---}$ ,  $\text{---NH}_2\text{---}$ ,  $\text{---CH}_2\text{---}$ ,  $\text{---CO}_2\text{---}$ ,  $\text{---N(Me)---}$ ,  $\text{---N(Ac)---}$ ,  $\text{---CONH---}$ ,  $\text{---NHCO---}$ ,  $\text{---NHCONH---}$ ,  $\text{---S(O)}_2\text{NH---}$ ,  $\text{---NHS(O)}_2\text{---}$  and  $\text{---NHS(O)}_2\text{NH---}$ . The cortisol aptamer may be covalently bonded onto the metal nanoparticle through one or more sulfur groups. The covalent bond may be any one of an alkanethiol, acyclic disulphide, cyclic disulphide or other types of multidentate thiolated anchor groups. The covalent bond may be a thiolated-gold ( $\text{S---Au}$ ) bond.

[082] In various embodiments, the cortisol aptamer may be conjugated directly to the surface of the metal nanoparticle or indirectly through an intervening linker. In various embodiments, the 5' end or 3' end of the cortisol aptamer may be conjugated directly to the surface of the metal nanoparticle or indirectly through an intervening linker.

[083] As used herein, a "linker" may refer to any type of molecule that can function in conjugating the aptamer to the surface of the metal nanoparticle. For example, a linker can be an aliphatic chain including at least two carbon atoms (e.g., 3, 4, 5, 6, 7, 8, 9, 10 or more carbon atoms), and can be substituted with one or more functional groups including ketone, ether, ester, amide, alcohol, amine, urea, thiourea, sulfoxide, sulfone, sulfonamide, and disulfide functionalities. Linker design and synthesis are well-known in the art.

[084] In various embodiments, the linker may be any thiol-containing molecule. In various embodiments, the cortisol aptamer may be modified and labelled with a thiol group, and the thiol group is covalently bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the surface of the metal nanoparticle. In various embodiments, the cortisol aptamer is thiol-terminated at the 5' end, and the thiol-terminated 5' end is covalently bound to the surface of the metal nanoparticle. In various embodiments, where the metal nanoparticle is a gold nanoparticle, a reaction of a thiol group of a cortisol aptamer with the gold results in a covalent sulfide ( $\text{---S---}$ ) bond.

[085] In various embodiments, the linker may be any amine group ( $\text{NH}_2$ )-containing molecule. In various embodiments, the cortisol aptamer may be terminated with an amine group ( $\text{NH}_2$ ), and the amine group is covalently bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the metal nanoparticle. In various embodiments, the cortisol aptamer is amine-terminated at the 5' end, and the amine-terminated 5' end is covalently bound to the surface of the metal nanoparticle. In this regard, the surface of the metal nanoparticle may be functionalized with carboxylic acid groups ( $\text{COOH}$ ). Accordingly, the cortisol aptamer may be conjugated to the metal

nanoparticle by the amine coupling of NH<sub>2</sub>-terminated aptamer on COOH functionalized metal nanoparticle.

[086] In various embodiments, the cortisol aptamer may be biotinylated with a biotin group, optionally introduced via a linker molecule or by incorporating a biotinylated nucleotide during the aptamer synthesis, and the biotin group may be bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the metal nanoparticle. In various embodiments, the surface of the metal nanoparticle may be functionalized with streptavidin. Accordingly, the cortisol aptamer may be conjugated to the metal nanoparticle by the coupling of biotinylated aptamer on streptavidin-conjugated metal nanoparticle.

[087] The plurality of peptides conjugated to the metal nanoparticle surface are capable of reducing non-specific binding of the cortisol binding ligands and/or improving the stability of the metal nanoparticle in solution. In particular, the peptide is co-conjugated, along with the cortisol binding ligand, to the metal nanoparticle in order to function as a spacer for stabilizing the cortisol sensor and limiting the non-specific binding of the analyte (i.e. cortisol) to the surface of the metal nanoparticle.

[088] In various embodiments, the plurality of peptides is a plurality of short peptides. As used herein, the term "short peptide" refers to a short chain polypeptide having a total number of amino acid residues of no more than 40, for example, between 2 and 40 amino acids in length, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids in length. In various embodiments, the short peptide may refer to a pentapeptide.

[089] Peptides can conveniently be attached to metal nanoparticles via amine or thiol groups in lysine or cysteine side chains respectively, or by an N-terminal amino group. If a peptide lacks a lysine or cysteine residue, a variant containing one or more of these residues, e.g., at the N- or C-terminus, can easily be prepared. Examples of methods by which peptides can be conjugated to a metal nanoparticle or functionalized nanoparticle include maleimide-sulfhydryl coupling chemistries (e.g., the maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) method). In various embodiments, methods of conjugating the peptides to the metal nanoparticle may include click chemistry or via EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) / sulfo-NHS (N-hydroxy sulfosuccinimide) coupling. In various embodiments, the peptides may be thiol-labelled (i.e. thiolated) for binding and conjugating to the surface of the metal nanoparticle.

[090] In various embodiments, the peptide may have a terminal cysteine residue, for example, at the N-terminus. Other features typically include the presence of hydrophobic amino acids at the second and third positions following the cysteine. In various embodiments, the peptide may be amidated at its C-terminus. The peptides may interact with the surface of the metal nanoparticles via the thiol group in the side chain of an N-terminal cysteine.

[091] In various embodiments, each peptide may be conjugated to the surface of the metal nanoparticle by means of a cysteine (C) residue, whereby the C residue is at the N-terminus of the peptide.

[092] The term "amino acid", as used herein refers to natural and/or unnatural or synthetic amino acids, including both the D and L optical isomers, amino acid analogs (for example norleucine is an analog of leucine) and derivatives known in the art. The term "natural amino acid", as used herein, relates to the 20 naturally occurring L-amino acids, namely Gly (G), Ala (A), Val (V), Leu (L), Ile (I), Phe (F), Cys (C), Met (M), Pro (P), Thr (T), Ser (S), Glu (E), Gln (Q), Asp (D), Asn (N), His (H), Lys (K), Arg (R), Tyr (Y), and Trp (W). Generally, in the context of the present invention, the peptides are shown in the N- to C-terminal orientation. All amino acid residues are generally referred to herein by reference to their one letter code and, in some instances, their three letter code. This nomenclature is well known to those skilled in the art and used herein as understood in the field. As a person skilled in the art would appreciate, amino acids can be categorized in different classes depending upon the chemical and physical properties of the amino acid residue. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala, Val, Leu, Ile, Pro, Phe, Trp, Met; (2) uncharged polar: Gly, Ser, Thr, Cys, Tyr, Asn, Gln; (3) acidic: Asp, Glu; and (4) basic: Lys, Arg, His. Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties: (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe.

[093] In various embodiments, the peptide may comprise or consist of an amino acid sequence CX<sub>n</sub>, CAX<sub>n</sub>, CALX<sub>n</sub>, or variants thereof so long as a Cysteine (C) residue at the N-terminal of the peptide conjugates to the surface of the metal nanoparticle. X denotes any amino acid residue and n is an integer of 1 or more, preferably 1 to 50, 40 or 30, more preferably 1 to 4.

[094] In various embodiments, the peptide of the plurality of peptides may be a short peptide comprising or consisting of an amino acid sequence set forth in **SEQ ID NO: 4** (CALNN) or variants thereof.

[095] As used herein, the term "variant" refers to polymorphisms, i.e. the substitution, deletion, or insertion of one or more amino acids compared to the respectively indicated (referenced) amino acid sequence. Particularly, protein homologues, i.e. those having at least 20%, 40%, 60%, 80%, 90%, 95%, or 99% sequence similarity or identity to the respectively indicated amino acid sequences, as determined by the BLAST algorithm or other well-known techniques, are also encompassed in this concept. The variants are capable of conjugating to the surface of the metal nanoparticle by means of a Cysteine (C) residue contained in the peptide.

[096] In various embodiments, each peptide of the plurality of peptides may be a short peptide consisting of an amino acid sequence set forth in **SEQ ID NO: 4**.

[097] In various embodiments, the plurality of peptides may comprise identical peptides or a combination of different peptides as disclosed herein. In various embodiments, each peptide in the plurality of peptides comprises or consists of an amino acid sequence  $CX_n$ ,  $CAX_n$ ,  $CALX_n$ , or variants thereof. In various embodiments, each peptide in the plurality of peptides comprises or consists of an amino acid sequence set forth in **SEQ ID NO: 4** (CALNN) or variants thereof.

[098] In various embodiments, the plurality of peptides are conjugated to the surface of the metal nanoparticle in order to separate the cortisol binding ligands from being close and proximate to each other which may inhibit or interfere with the binding of cortisol to the cortisol binding ligand. Thus, the peptides space apart cortisol binding ligands conjugated on the surface of the metal nanoparticle in order to facilitate the binding of cortisol to each conjugated cortisol binding ligand. In this regard, the peptides are interspersed between conjugated cortisol binding ligands. Advantageously, the plurality of conjugated peptides spaces apart the conjugated cortisol binding ligands such that the conjugated cortisol binding ligand do not horizontally lay on the surface of metal nanoparticles and/or improve the stability of the metal nanoparticle.

[099] In various embodiments, the cortisol sensor comprises or consists of i) a gold nanoparticle; ii) a plurality of cortisol DNA (i.e. ssDNA) aptamers comprising or consisting of a nucleotide sequence set forth in **SEQ ID NO: 1, 2** and/or **3**, or derivatives thereof, and each aptamer is labelled with a thiol group at the 5' end, wherein the thiol group is covalently bound to the surface of the gold nanoparticle; and iii) a plurality of short peptides comprising or consisting of an amino acid sequence set forth in **SEQ ID NO: 4** or variants thereof, wherein each short peptide is conjugated to the surface of the gold nanoparticle by means of the Cysteine (C) residue in **SEQ ID NO:4**. The cortisol sensor disclosed herein, and more particularly the cortisol sensor in the examples herein included as a proof-of-concept, utilise gold nanoparticles (AuNPs) conjugated with high-affinity cortisol-selective aptamers (Apt) and short peptides (CALNN), to form the cortisol sensor Au-Apt-CALNN. In particular, a thiol-labelled cortisol aptamer was conjugated to AuNPs for selective and sensitive detection of cortisol, and the CALNN peptide was co-conjugated as a spacer to Au-Apt for stabilizing the conjugate and limiting non-specific binding of the analyte (cortisol) to the surface of the nanoparticle. In the presence of cortisol, the cortisol binds to the aptamer and the cortisol-bound aptamer complex folds into a tertiary structure and exerts a higher protection to Au-Apt-CALNN nanoparticle against salt-induced aggregation due to steric stabilization. The differential aggregation profile between Au-Apt-CALNN and cortisol-bound Au-Apt-CALNN thereby allows for cortisol detection, and optionally quantification. The co-conjugation of both the cortisol aptamer and CALNN to the gold nanoparticle (AuNP-Apt-CALNN) results in the cortisol sensor exhibiting high colloidal stability against particle aggregation in complex matrices, such as fish tank water.

[100] There is also provided a method of producing the cortisol sensors disclosed herein. Embodiments described above in the context of the cortisol sensor are analogously valid for the respective methods of production, and *vice versa*.

[101] In various embodiments, the method of producing the cortisol sensors disclosed herein, comprises or consists of the steps of: a) preparing a first solution comprising a plurality of metal nanoparticles; b) preparing a second solution comprising a plurality of cortisol-binding ligands; c) adding the first and second solutions together to produce a first mixture; d) incubating the first mixture under conditions suitable to conjugate the cortisol-binding ligands onto the surface of the metal nanoparticles; and e) adding a third solution comprising a plurality of peptides to the incubated first mixture under conditions suitable to conjugate the peptides onto the surface of the metal nanoparticles to produce a second mixture comprising one or more of the cortisol sensors.

[102] In various embodiments, the first, second and/or third solution may be an aqueous solution. The aqueous solution may be a solution that has a solvent that is water. The aqueous solution may be water or a buffer solution. The buffer solution may be a biological buffer solution. The buffer may be any one of a PBS buffer, Tris buffer, Tricine buffer, HEPES buffer, TES buffer, MOPS buffer, PIPES buffer, cacodylate buffer, SSC buffer, MES buffer, Bicine buffer, TAPS buffer or any mixture thereof. The buffer solution may be a HEPES buffer solution. In various embodiments, the buffer solution may comprise Tris (2-carboxyethyl) phosphine (TCEP), preferably the buffer solution is a HEPES buffer solution.

[103] In various embodiments, the first solution may be any suitable solution containing the metal nanoparticles. In various embodiments, where the metal nanoparticle is a gold nanoparticle, the step of preparing the first solution may comprise, adding a sodium citrate dihydrate solution to boiling (at 110-150°C) gold (III) chloride solution to provide a mixture, and then refluxing the mixture for about 20-40 minutes, preferably about 30min, to provide the first solution comprising a plurality of gold nanoparticles; and cooling the first solution to room temperature (i.e. about 20-25°C). In various embodiments, the sodium citrate dihydrate solution may be added in an amount of about 5mL at a concentration of 40 mM, and the boiling gold (III) chloride solution may be in an amount of about 50mL at a concentration of 1mM.

[104] In various embodiments, the second solution may be any suitable solution containing the cortisol binding ligands. In various embodiments, where the cortisol binding ligand is a cortisol DNA aptamer, the step of preparing the second solution may comprise, adding the cortisol aptamer in a buffer solution, optionally shaking the buffer solution comprising the cortisol aptamer, followed by centrifuging the buffer solution to produce a supernatant, preferably the buffer solution is transferred to a 3kDa centrifugal filter, and subsequently adding water to the supernatant to provide the second solution.

[105] In various embodiments, the step of producing a first mixture, may comprise an amount of about 100-1200 $\mu$ l, preferably about 850-950 $\mu$ l, more preferably about 908  $\mu$ l, of the first solution, and an amount of about 10-100 $\mu$ l, preferably about 50-60 $\mu$ l, more preferably about 56  $\mu$ l, of the second solution.

[106] In various embodiments, the step of producing a first mixture, may further comprise adding an acidic solution (i.e. a solution with a pH less than 7, which contains excess hydrogen ions (H<sup>+</sup>)). In various embodiments, the second solution may be added to the first solution and subsequently, the acidic solution may be added thereafter. The acidic solution may be in an amount of about 0.5-50 $\mu$ l, preferably about 10  $\mu$ l, and the concentration of the acidic solution may be about 0.01M-1M, preferably 0.1M. In various embodiments, the acidic solution may be a hydrochloric acid solution. In various embodiments, the step of producing a first mixture, may comprise adding hydrochloric acid solution to the first solution to adjust the pH to about 3 prior to addition of the second solution.

[107] In various embodiments, the first mixture may be shaken and mixed at about 250-750rpm, preferably 500rpm, for about 10-20 minutes, preferably about 15 minutes. In this context, the term "shaken" refers to the agitation or rotation of the mixture at the defined rate (rpm) in order to ensure adequate mixing and uniformity of the solutions. Suitable devices and equipment to carry out the shaking are well-known in the art of selecting. In various embodiments, the first mixture is shaken at room temperature (about 25°C).

[108] In various embodiments, the first mixture may be incubated for about 12-24 hours at room temperature (25°C) or refrigerated at 4 °C.

[109] In various embodiments, the third solution may be any suitable solution containing the plurality of peptides. The concentration of the peptides in the third solution may be about 0.5M-3M, preferably about 1M to 2M, more preferably about 1.5M to 2M. In various embodiments, the step of producing a second mixture comprising one or more of the cortisol sensors, may comprise adding an amount of about 20-1000 $\mu$ l, preferably about 20-500 $\mu$ l, more preferably about 20-100  $\mu$ l, of the third solution, to the first mixture.

[110] In various embodiments, the step of producing the second mixture may further comprise adding an alkaline solution (i.e. a solution with a pH greater than 7, which means it has a higher concentration of hydroxide ions (OH<sup>-</sup>) compared to hydrogen ions (H<sup>+</sup>)). The alkaline solution may be in an amount of about 0.5-50 $\mu$ l, preferably about 10  $\mu$ l, and the concentration of the alkaline solution may be about 0.01M-1M, preferably 0.1M. In various embodiments, the alkaline solution may be sodium hydroxide (NaOH). The addition of alkaline solution adjusts the pH of the mixture back to neutral conditions by adding NaOH. In various embodiments, the alkaline solution is added to the second mixture (i.e. after the third solution has been added to the first mixture).

[111] In various embodiments, the second mixture may be shaken at about 250-750rpm, preferably 500rpm, for about 10-20 minutes, preferably 15 minutes. In various embodiments, the second mixture is shaken at room temperature (about 25°C).

[112] In various embodiments, the method may further comprise isolating the cortisol sensors in the second mixture. In various embodiments, the second mixture may be centrifuged at about 9000-12000rpm, preferably 10000 rpm, for about 30-60 minutes, preferably about 45 minutes. Following centrifugation, the supernatant may be discarded and subsequently the plurality of cortisol sensors may be redispersed in water or other suitable solutions.

[113] In various embodiments, the isolated cortisol sensors may be stored at suitable conditions before further processing and/or use in one of the methods disclosed herein. The conditions may include storing the cortisol sensor at a temperature in the range of 1-4°C for a desired time frame.

[114] In various embodiments, the second mixture or the isolated cortisol sensors may be used to prepare a colloidal solution or composition comprising a plurality of the cortisol sensors disclosed herein. Accordingly, there may be provided a colloidal solution or composition comprising a plurality of cortisol sensors disclosed herein. It will be appreciated that the colloidal solution or composition may be used in any one of the methods disclosed herein for detecting cortisol and measuring stress levels and the like. The term "colloidal solution" as used herein refers to the cortisol sensors being dispersed throughout a solution without dissolving, settling out or separating on its own. This mixture, where one substance (the dispersed phase) is evenly distributed throughout another substance (the continuous phase), is typically known as a colloid. The colloidal solution may be a hydrocolloid solution.

[115] In various embodiments, the production of the cortisol sensor may be verified by UV-vis spectroscopy and/or a salt stability test. The UV-vis spectrum of the cortisol sensor may be compared to the UV-vis spectrum of the metal nanoparticle in the first solution, for example, a peak absorbance shift from 520 nm to 525 nm may be an indication of successful co-conjugation. The salt stability test may comprise adding the cortisol sensor to a saline solution (e.g. sodium chloride solution or various types of seawater) and evaluating the dispersion of the cortisol sensor for aggregation, whereby the saline solution is observed for changes such as precipitation using suitable analytical techniques like spectroscopy (UV-visible, fluorescence), dynamic light scattering, or centrifugation. The saline solution may be water comprising one or more of NaCl, MgCl<sub>2</sub>, MgSO<sub>4</sub>, CaCl<sub>2</sub>, KCl, or NaHCO<sub>3</sub> dissolved in the water.

[116] There is also provided a method of detecting the presence or absence, and optionally the quantity, of cortisol in a sample, using the cortisol sensor disclosed herein. Accordingly, there is also provided the use of the cortisol sensor disclosed herein for detecting the presence or absence, and optionally the quantity, of cortisol in a sample. The methods disclosed herein may be *in vitro* or *ex vivo* methods

[117] Embodiments described above in the context of the cortisol sensor are analogously valid for the respective methods of detecting cortisol, and *vice versa*.

[118] In various embodiments, the method of detecting the presence or absence, and optionally the quantity, of cortisol in a sample, may comprise measuring colour differences wherein a colour difference compared to the initial colour of the mixture is due to aggregation of the cortisol sensor. Aggregation of the cortisol sensors influences the resonant frequencies of the cortisol sensor, and thus results in a shift in the colorimetric properties of the cortisol sensor. The aggregated state of the cortisol sensor may be readily detected using, for example, one or more visualization methods, comprising but not limited to one or more of microscopy techniques, dynamic light scattering techniques, visual observation of colour, UV-vis absorption techniques, or surface plasmon resonance techniques. For example, the aggregation state or profile of the cortisol sensors may be observed using microscopic examination or dynamic light scattering to determine whether the particles are clumped together or are evenly dispersed in the sample mixture. More rapid detection may be achieved by observation of the colour of the sample mixture by visual inspection, measurement of UV/Vis absorbance, or measurement of localized surface plasmon resonance. Accordingly, cortisol may be detected, and quantified, based on measuring and determining the degree of aggregation of the sample-sensor mixture, whereby a decrease in the degree of aggregation relative to a control is indicative of the presence, and optionally the quantity, of cortisol.

[119] The term "aggregation" as used herein in the context of the cortisol sensor refers to the formation and growth of clusters of the cortisol sensor in aqueous dispersions. Such aggregates or clusters comprise a tightly bound collection of the cortisol sensors which are difficult to break up into primary particles using mechanical forces. Spectral peaks can shift due to changes in the aggregation state of the cortisol sensor, where "Blue-shifting" refers to an electromagnetic response that is shifted towards shorter wavelengths (i.e. higher frequencies, higher energies), and Red-shifting refers to a peak that shifts to longer wavelengths. As such, the "degree of aggregation" refers to the extent to which the cortisol sensor aggregates when bound to cortisol, and is a numerical or quantitative description of the aggregation process, which can be measured in various ways known to those skilled in the art.

[120] As used herein, the term "aggregation state" refers to the physical or structural condition of the cortisol sensor when it binds to the cortisol, for example a monomeric state, oligomeric state or aggregated state. The term "aggregation profile" refers to how the cortisol sensors aggregate over time or under specific conditions when binding to the cortisol, including factors affecting aggregation, kinetics, and the impact on nanosensor performance. In this regard, the degree of aggregation provides a more precise and quantifiable aspect of the aggregation state, and the aggregation profile refers to the degree of aggregation relative to the amount (absence or presence) of cortisol in the sample.

[121] In various embodiments, the methods of detecting, and optionally quantifying, cortisol, may be considered as colorimetric methods or analysis comprising contacting a sample suspected to contain cortisol with a solution that contains the cortisol sensors disclosed herein to obtain a sample-sensor mixture. As used herein the term “colorimetric” and grammatical variants thereof refer to the physical description and quantification of the color spectrum including the human color perception spectrum (i.e. visible spectrum). In various embodiments, a colorimetric method, or colorimetric assay, or colorimetric analysis, quantifies and determines the wavelength and/or absorbance of a solution under analysis (i.e. sample-sensor mixture), as is known in the art. This can be done by using instruments including, but not limited to, UV-visible spectrophotometers, Localized Surface Plasmon Resonance spectrometers and multi-well plate readers. In various embodiments, the colorimetric method or colorimetric assay may include using the naked eye and the endpoint will be determined.

[122] In this regard, the method disclosed herein may allow the colorimetric detection, and optional quantification, of cortisol by using simple instrumentation (e.g., UV-Vis spectroscopy) and/or the naked eye observation owing to the degree of aggregation and optionally the interparticle distance-dependent Localized Surface Plasmon Resonance (LSPR) properties of the cortisol sensor in the presence and/or absence of cortisol.

[123] In various embodiments, the surface plasmon resonance technique is a localized surface plasmon resonance technique (LSPR). LSPR is a characteristic of noble metal particles, including noble metal nanoparticles, that is used for biosensing. The LSPR of metal nanoparticles arises from the electromagnetic waves that propagate along the surface of the conductive metal. When excited with an electromagnetic wave, such as light, most noble metal particles produce an intense absorption and scattering due to the collective oscillation of the conduction electrons located at the particles' surface. For example, in gold and silver particles, the LSPR yields exceptionally high absorption coefficients and scattering properties within the UV/visible wavelength range that allows them to have a higher sensitivity in optical detection methods than conventional organic dyes. Moreover, their LSPR properties can be easily modulated according to their size, shape and composition. The LSPR absorption band of gold/silver alloy particles increases to longer wavelengths with increasing amounts of gold. Typically, colloidal solutions of spherical gold nanoparticles (<40 nm) present a red color with their LSPR band centered at about 520 nm, while spherical silver NPs present a yellow color with their LSPR band centered at about 420 nm.

[124] In various embodiments, the methods provided herein include determining the degree of aggregation of the cortisol sensors in order to detect whether any cortisol is present in the sample (and optionally how much), using visual, colorimetric or other techniques/instrumentation capable of determining the state, profile and/or degree of aggregation of the cortisol sensor in the sample mixture. The results of such detection may be compared to the aggregated state, profile and/or degree of cortisol sensors in a control/reference sample in the absence of cortisol, and optionally plotted on a suitable calibration curve for quantifying the cortisol.

[125] In various embodiments, in the presence of cortisol, the cortisol binds to the cortisol binding ligands of the cortisol sensor and may exert a higher protection to the cortisol sensor against aggregation due to steric stabilization compared to in the absence of cortisol. The differential degree of aggregation between the unbound cortisol sensor and bound cortisol sensor thereby allows for cortisol detection (and optional quantification), whereby a relative decrease in the degree of aggregation compared to a control (unbound cortisol sensor) indicates the presence, and amount, of cortisol.

[126] In various embodiments, the detection may be measured by a qualitative colorimetric method of detecting cortisol and/or a quantitative colorimetric method of detecting cortisol.

[127] In various embodiments, the method of detecting the presence or absence, and optionally the quantity, of cortisol in a sample, may comprise or consist of: a) providing a cortisol sensor disclosed herein; b) contacting the cortisol sensor to the sample in a saline environment (i.e. an aqueous solution containing salt) that is suitable for binding the cortisol binding ligand to cortisol, to produce a sample mixture; and c) detecting the presence or absence, and optionally the quantity, of cortisol in the sample mixture by determining the degree of aggregation of the cortisol sensor in the sample mixture, wherein the degree of aggregation of the sample mixture is compared with the degree of aggregation of a control sample and a differential degree of aggregation is indicative of the presence or absence, and optionally the quantity, of cortisol in the sample.

[128] In various embodiments, the degree of aggregation is proportional to the quantity of cortisol in the sample, when compared with a control sample. In various embodiments, the degree of aggregation decreases relative to the amount of cortisol, wherein a decrease in the degree of aggregation of the sample mixture compared with a control sample is indicative of the presence, and optionally quantity, of cortisol in the sample.

[129] In various embodiments, the degree of aggregation may be determined/detected/measured by using a colorimetric method, including but not limited to UV-Visible Spectroscopy, microscopy techniques, dynamic light scattering techniques, visual observation of colour, or surface plasmon resonance techniques. In various embodiments, the degree of aggregation may be determined/detected/measured by using a UV-Visible Spectroscopy, wherein the UV-vis spectrum of the sample mixture is compared with the UV-vis spectrum of a control sample and a differential UV-vis spectrum is indicative of the presence or absence, and optionally the quantity, of cortisol in the sample. In various embodiments, the degree of aggregation may be determined by measuring the absorbance intensity and/or absorbance ratio of the sample mixture.

[130] The term "sample" is used herein in its broadest sense, and the sample may include a specimen or culture, and may also include both biological and environmental samples, such as soil or water samples from a variety of water bodies such as lakes, rivers, ponds, tanks etc. A sample may also include a specimen of synthetic origin. Biological samples may be animal, including human, fluid,

solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients (e.g. samples from food intended for human or animal consumption such as processed foods, raw food material, produce, legumes, meats, fish, seafood, nuts, beverages, drinks, fermentation broths, and/or a selectively enriched food matrix comprising any of the above-listed foods, infant formulas, infant food, etc). Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, lagamorphs, rodents, etc. A biological sample may contain any biological material suitable for detecting cortisol, and may comprise cellular and/or non-cellular material from a subject. The sample can be isolated from any suitable biological tissue or fluid such as, for example, prostate tissue, blood, serum, blood plasma, sweat, pleural fluid, tears, lactal duct fluid, lymph, sputum, urine, amniotic fluid, semen, or cerebral spinal fluid (CSF).

[131] In various embodiments, the sample may be a water sample. The water sample may have been obtained from an aquaculture tank, for example a fish tank.

[132] In various embodiments, the sample may be a biological sample. The biological sample may be a biological tissue or fluid sample that has been obtained from an animal. In various embodiments, the biological sample may be a fish tissue sample. The fish tissue sample may be derived from the fin, scales, mucus, or plasma of the fish and may be in a fluid form or a solid form, wherein the solid form may be processed and extracted into a liquid form.

[133] In various embodiments, the method of detecting the presence or absence, and optionally the quantity, of cortisol in a sample, may comprise or consist of: a) providing a cortisol sensor disclosed herein; b) contacting the cortisol sensor to the sample in a saline environment (i.e. an aqueous solution containing salt) that is suitable for binding the cortisol binding ligand to cortisol, to produce a sample mixture; and c) detecting the presence or absence, and optionally the quantity, of cortisol in the sample mixture by determining the degree of aggregation of the cortisol sensor in the sample mixture by measuring the absorbance ratio of the sample mixture, wherein the absorbance ratio of the sample mixture is compared with the absorbance ratio of a control sample, and a differential absorbance ratio is indicative of the presence or absence, and optionally the quantity, of cortisol.

[134] The term "absorbance ratio" used herein, refers to the ratio of absorbance measurements taken at two different wavelengths using a spectrophotometer. The absorbance ratio is particularly useful for identifying or quantifying specific components in a mixture. By selecting appropriate wavelengths that correspond to the absorbance peaks of different substances, the ratio can highlight the presence or concentration of these substances relative to others. The absorbance ratio can also be used to correct for background absorbance in a sample, whereby if one wavelength is affected by an interfering substance and another is not, the ratio can help adjust the measurements to more accurately reflect the concentration of the target analyte. In this regard, measuring the absorbance

ratio of the sample, both qualitative and quantitative information can be obtained to detect and infer the presence of, and quantity of, the analyte (i.e. cortisol).

**[135]** As used, herein, the term "control sample" refers to a suitable sample (i.e. solution) that is known not to contain any cortisol. The term control sample may be interchangeably used with "reference sample", so long as these samples do not contain any cortisol. For example, the control sample may comprise phosphate buffered saline (PBS) solution. A control/reference sample, may also be referred to as the "blank sample", and may comprise a similar solution or solvent used to prepare the sample to be analysed for use in a microplate or cuvette. The control sample may also contain a salt ion (i.e. bivalent salt ion) for inducing aggregation of the cortisol sensor in the absence of cortisol, in other words the control sample may be a salt solution capable of inducing aggregation of the cortisol sensor disclosed herein. In various embodiments, the control sample comprises the cortisol sensor. In various embodiments, the control sample comprises the cortisol sensor in a background sample matrix. The term "sample matrix" may refer to the content present in the test sample except for the analyte of interest (i.e. cortisol).

**[136]** The "contacting" step in the methods generally refers to any suitable means for delivering, exposing or bringing the sample and the cortisol sensor together in a controlled experimental setting under suitable conditions. As will be appreciated by the skilled person, the "contacting" step can vary depending on the nature of the sample involved, whereby the contacting may involve physically mixing the sample with the cortisol sensor in a test tube or other container, or it could involve applying the cortisol sensor to the sample through techniques such as pipetting, or incubation. The purpose of the "contacting" step is to allow the cortisol sensor to interact with the sample. In various embodiments, contacting occurs in a solution in which the sample and cortisol sensor are mixed in a common solution and are allowed to freely associate.

**[137]** In various embodiments, the contacting step may be carried out under conditions suitable for binding the cortisol binding ligand to cortisol. In particular, the contacting step may comprise incubating the sample mixture for a time sufficient to allow binding of the cortisol sensor (i.e. cortisol binding ligand) to cortisol. The time sufficient to allow binding may be about 15 seconds, 30 seconds, 45 seconds, 60 seconds, 90 seconds, 120 seconds, 200 seconds, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, 120 minutes, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours or 24 hours. In various embodiments, the contacting step may be carried out at room temperature (about 25°C) for a time sufficient to allow binding in the range of 20 minutes to 24 hours.

**[138]** In various embodiments, the cortisol sensor may be provided in a solution, and the step of contacting may comprise an amount of about 30-100µl, preferably about 60µl, of the solution. The solution that contains one or more cortisol sensors disclosed herein may be a colloidal solution or composition disclosed herein. The solution may be contained within a cuvette or microplate configured for subsequent analysis with a colorimetric assay or a UV Visible (UV VIS) Spectrometer

or similar other well-known devices capable of measuring absorption or transmission of UV and visible light in the sample mixture.

**[139]** In various embodiments, the absorbance ratio of the sample mixture may be measured by UV-vis spectroscopy. UV-vis spectroscopy refers to an analytical chemistry technique used to measure the absorbance or transmittance of UV and visible light by a sample. This absorbance or transmittance may then be used to determine the presence, and optionally quantity of cortisol in the sample. UV-Vis spectroscopy information may be presented as a graph of absorbance, optical density or transmittance as a function of wavelength. The information may be presented as a graph of absorbance on the vertical y axis and wavelength on the horizontal x axis, whereby this graph is typically referred to as an absorption spectrum. Recording a baseline spectrum using a "blank" reference sample is necessary, and the small absorbance values in the reference sample may be automatically subtracted from the sample absorbance values for each wavelength of light to obtain the true absorbance values.

**[140]** In various embodiments, an absorbance intensity ratio selected from any one of  $A_{525}/A_{700}$ ,  $A_{520}/A_{700}$ , or  $A_{525}/A_{650}$  may be used as a qualitative and quantitative measure of the spectrum difference in the absence and presence of cortisol. In various embodiments, an absorbance intensity ratio of 520 and 650 nm ( $A_{520}/A_{650}$ ) may be used as a qualitative and quantitative measure of the spectrum difference in the absence and presence of cortisol, which is proportionally related to cortisol concentration. The absorbance ratio ( $A_{520}/A_{650}$ ) refers to a calculation of the absorbance of light by a sample at 520 nm divided by the absorbance at 650 nm, and the provision of information about the state of the cortisol sensor disclosed herein in a solution. For instance, if the absorbance at 520 nm would be higher relative to 650 nm, a higher  $A_{520}/A_{650}$  ratio is given. Conversely, if the peak may shift towards 650 nm, then the  $A_{520}/A_{650}$  ratio is reduced.

**[141]** In various embodiments, the  $A_{520}/A_{650}$  absorbance ratio may be from about 0.1 to about 5, as measured by a spectrophotometer. In various embodiments, the  $A_{520}/A_{650}$  absorbance ratio may be about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0 or any number therebetween. In various embodiments, the obtained  $A_{520}/A_{650}$  absorbance ratio may be further processed by subtracting the absorbance ratio of the control sample for quantifying cortisol levels.

**[142]** In the presence of cortisol in the sample and upon formation of cortisol sensor-cortisol complex, the protection effect leads to a degree of aggregation, which may be quantified by the absorbance ratio of  $A_{520}/A_{650}$ , and compared with unbound cortisol sensors in a suitable control sample. Accordingly, the degree of aggregation of the cortisol sensor is able to indicate differences in cortisol concentrations in the sample. In various embodiments, the degree of aggregation of the cortisol sensor is capable of indicating a difference in cortisol concentrations of between 0.1-10,000 nM in a sample.

[143] Accordingly, in various embodiments, the step of detecting the presence or absence, and optionally the quantity, of cortisol in the sample mixture may comprise measuring the absorbance ratio of  $A_{520}/A_{650}$  in the sample mixture, wherein the absorbance ratio is indicative of the degree of aggregation of the cortisol sensor. A differential absorbance ratio between the sample and control sample (e.g. unbound cortisol sensor) indicates the presence and/or the quantity of cortisol in the sample mixture, optionally the absorbance ratio of the control sample is subtracted from the absorbance ratio of the sample mixture.

[144] In various embodiments, the method may quantify cortisol in the sample mixture, and quantifying the cortisol in the sample mixture may be based on the absorbance ratio of  $A_{520}/A_{650}$ , wherein the absorbance ratio of the sample mixture is compared with the absorbance ratio of a control sample. In particular, the absorbance ratio of the sample mixture may be plotted as a function of logarithmic cortisol concentrations for quantitative evaluation, whereby the absorbance ratio increases linearly with increasing cortisol concentrations. For example, a differential absorbance ratio in the range of 0.2-1.2 may correspond to cortisol concentrations ranging from 0.1-1000 nM.

[145] In various embodiments, the method has a limit of detection (LOD) of about 0.1 nM (LOD 100 pM) for quantification of the cortisol in the sample. As will be appreciated, the cortisol sensor may have a LOD of 100 pM which can be improved by further exploration of other sensing methodology.

[146] In various embodiments, the cortisol in the sample may be quantified based on a calibration curve of the absorbance ratio of the cortisol sensor with known concentrations of cortisol, and subtracting the absorbance intensity in the absence of cortisol. In various embodiments, the cortisol in the sample may be quantified by first preparing a calibration curve by plotting the absorbance ratio versus known concentrations of cortisol (i.e. involves measuring the absorbance ratio of a series of standard solutions with known concentrations of cortisol containing the cortisol sensor, and calculating the absorbance ratios), and then using the calibration curve to determine the concentration of cortisol in the sample by matching the measured absorbance ratio to the corresponding concentration on the calibration curve.

[147] In various embodiments, the saline environment in the contacting step may comprise an aqueous solution containing salt or a salt medium or solution that is suitable for allowing the binding of the cortisol binding ligand to cortisol. In this regard, a bivalent ion may be used to differentiate the colloidal steric stability of cortisol bound and unbound to the cortisol sensor, thereby allowing for cortisol detection. Accordingly, in various embodiments, a bivalent salt ion may be added to the sample mixture in the contacting step.

[148] In various embodiments, the saline environment and salt solution may refer to the presence and use of a bivalent salt ion or bivalent salt ion solution. As used herein, a "bivalent salt ion" refers to a salt that contains ions with a valency of two. This means that the ions in the salt either have a charge of +2 or -2. In simpler terms, a bivalent ion can form two bonds with oppositely charged ions.

Bivalent salts are a type of polyvalent or multivalent salt, which includes any salt with ions having a valency greater than one. A bivalent salt, therefore, could be composed of a bivalent cation and a monovalent anion, a monovalent cation and a bivalent anion, or both ions being bivalent. As such, a "bivalent salt ion solution" may be referred to as a solution in which a bivalent salt is dissolved in a solvent, typically water. These solutions are common in various scientific and industrial applications, and may be appropriately selected by those skilled in the art.

[149] In various embodiments, the bivalent salt ion may be selected from the group consisting of a calcium chloride ( $\text{CaCl}_2$ ), calcium carbonate ( $\text{CaCO}_3$ ), calcium sulfate ( $\text{CaSO}_4$ ), magnesium chloride ( $\text{MgCl}_2$ ), magnesium sulfate ( $\text{MgSO}_4$ ), magnesium carbonate ( $\text{MgCO}_3$ ), barium chloride ( $\text{BaCl}_2$ ), barium sulfate ( $\text{BaSO}_4$ ), lead acetate ( $\text{Pb}(\text{CH}_3\text{COO})_2$ ), lead nitrate ( $\text{Pb}(\text{NO}_3)_2$ ), ferrous sulfate ( $\text{FeSO}_4$ ), ferrous chloride ( $\text{FeCl}_2$ ) and combinations thereof. In various embodiments, the bivalent salt ion may be  $\text{MgCl}_2$ .

[150] In various embodiments, the bivalent salt ion is in a solution (i.e. bivalent salt ion solution) at a concentration of about 0.01M-0.1M, preferably 0.05M. In this regard, the bivalent salt ion is comprised in the solution that is then subsequently added to the sample mixture.

[151] In various embodiments, the bivalent salt ion solution may be added in an amount of about 1-20 $\mu\text{l}$ , preferably about 10  $\mu\text{l}$  to the sample mixture.

[152] The method of detecting cortisol disclosed herein may represent and function as a one step "mix and measure" assay, which may be completed within 15 minutes from sample-to-result. Accordingly, the method disclosed herein reflects the development of a "mix and measure" cortisol detection protocol exploiting the metal nanoparticles aggregation profile in a saline environment. Such saline/salt conditions are employed to differentiate the steric stability of the nanoparticles with folded or unfolded (bound and unbound) cortisol binding ligand, thereby allowing for cortisol detection. Thus, the disclosed method may be a "mix-and-measure" assay, which may be simple, rapid and sensitive. Advantageously, the method may not require any bulky and expensive equipment or consumables, and may circumvent the conventional potentially hazardous, labour- and time-intensive steps of labelling with radio-isotopes or dyes. Thus, fewer reagents may be needed. In various embodiments, the one step "mix and measure" method disclosed herein may be carried out with only small volumes of chemical reagents (about 60  $\mu\text{L}$ ) and the sample (about 20  $\mu\text{L}$ ) to be tested.

[153] In various embodiments, after step b), the sample mixture may not be further processed, treated, incubated or washed before the detection step c) is carried out, such that the method does not comprise an additional or intermediary step between steps b) and c). In particular, the method disclosed herein does not require the incubation and washing steps of the sample mixture as used in ELISA.

[154] In various embodiments, the time taken from the contacting (i.e. mixing) step to obtaining the results of the detection step (i.e. sample to result) may be in about 10-45 minutes, 10-35 minutes, 10-30 minutes, 10-20 minutes, preferably about 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 minutes after mixing the cortisol sensor with the sample.

[155] In various embodiments, the detection step may be completed (sample to result) in about 10-45 minutes, 10-35 minutes, 10-30 minutes, 10-20 minutes, preferably about 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 minutes after step b). In various embodiments, the detection step may be completed in about 15 minutes after step b). In various embodiments, step c) may be initiated (i.e. within 1 second to 1 minute) following, or is immediately initiated after, step b).

[156] As will be appreciated, the method of detecting the presence or absence, and optionally the quantity, of cortisol in a sample, may be further used and deployed for stress monitoring in the context of aquaculture fish tanks, animals (i.e. farms), human health and behavioural science studies. Thereby such methods disclosed herein may be adapted for use in the on-site monitoring and detection of stress levels in aquaculture tanks, animal farms, livestock industries, or in a clinical setting, whereby advantageously the method disclosed herein is capable of rapid and sensitive detection and quantification of cortisol in an applicable sample. This has benefits in the timely identification of abnormal stress levels in such samples, thus assisting in the subsequent expedition of an appropriate intervention and course of action.

[157] In this regard, the cortisol concentration and level in a sample obtained from a subject may be correlated with a stress level.

[158] Accordingly, there is provided a method of measuring and monitoring a stress level in a subject, comprising: contacting a biological sample that has been obtained from the subject with a cortisol sensor disclosed herein in a saline environment that is suitable for binding the cortisol binding ligand to cortisol, to produce a biological sample mixture; and detecting the quantity of cortisol in the biological sample mixture by determining the degree of aggregation of the biological sample mixture, wherein the degree of aggregation of the biological sample mixture is compared with the degree of aggregation of a control sample and a differential degree of aggregation is indicative of the quantity of cortisol in the biological sample mixture and the stress level of the subject.

[159] Embodiments described above in the context of the cortisol sensor, and method of detecting cortisol, are analogously valid for the respective methods of measuring a stress level, and *vice versa*.

[160] In various embodiments, the biological sample may be derived/obtained from the subject, and the subject may be suffering from or suspected of suffering from a relevant condition or disease associated with abnormal cortisol levels, the subject preferably being a mammal, for example a human. Alternatively, the subject may also be an animal such as a fish. The term "abnormal cortisol level" as used herein refers to cortisol concentrations in the sample (e.g. blood, urine, sweat or saliva)

that are either higher or lower than the normal range of cortisol expected in the subject or environment the sample is derived from. The "normal cortisol levels" are readily available in environmental or medical charts or known/deducible by veterinary/medical practitioners for making an assessment as to what constitutes abnormally high or low cortisol levels in the context of the subject and/or environment. As appreciated by the skilled artisan, cortisol levels in a subject (i.e. human) vary dependent on the time of day, age of subject and type of sample obtained itself.

[161] In various embodiments, the quantity of cortisol in the biological sample mixture is compared with known reference levels of cortisol associated with certain stress levels in the subject.

[162] In various embodiments, the cortisol sensor disclosed herein may first be calibrated with known concentrations of cortisol in the relevant sample matrix. Following which, a suitable biological sample can be obtained from the subject and incubated with the cortisol sensor. The differential absorbance ratio between the biological sample and a control can be used to quantify cortisol levels for comparison with known reference levels of cortisol.

[163] A "reference level" of cortisol means a level of the cortisol that is indicative of a particular disease state, stress level, phenotype, or lack thereof, as well as combinations of disease states, phenotypes, or lack thereof. A "positive" reference level of cortisol means a level that is indicative of a particular disease state or phenotype. A "negative" reference level of cortisol means a level that is indicative of a lack of a particular disease state or phenotype. A "reference level" of cortisol may be an absolute or relative amount or concentration of cortisol, a presence or absence of cortisol, a range of amount or concentration of cortisol, a minimum and/or maximum amount or concentration of cortisol, a mean amount or concentration of cortisol, and/or a median amount or concentration of cortisol. Reference levels of cortisol in suitable samples (i.e. blood, urine, sweat, saliva) indicative of a particular condition or disease state are readily available in medical charts or known/deducible by medical practitioners that is predictive or indicative of a particular disease state, stress level, phenotype, or lack thereof. For example, high cortisol levels may be typically associated with Cushing's syndrome or stress, whereas low cortisol levels may be typically associated with Addison's disease or adrenal insufficiency.

[164] Appropriate positive and negative reference levels of cortisol for a particular disease state, phenotype, or lack thereof may be determined by measuring levels of cortisol in one or more appropriate subjects, and such reference levels may be tailored to specific populations of subjects (e.g., a reference level may be age-matched so that comparisons may be made between cortisol levels in samples from subjects of a certain age and reference levels for a particular disease state, phenotype, or lack thereof in a certain age group). Such reference levels may also be tailored to specific techniques that are used to measure levels of cortisol in biological samples, where the levels of cortisol may differ based on the specific technique that is used.

[165] In various embodiments, each known reference level is specific for gender, age, mental condition, and/or predictive of a mental disorder of said subject. In various embodiments, the subjects may be human and specific to a particular psychiatric profile (e.g., no history of mental illness; history of mental illness; no recent history of mental illness; currently experiencing mental illness). In various embodiments, the subject may be experiencing a mental disorder. As used herein, the term "mental disorder" may be one or more of an anxiety disorder, a major depressive disorder (MDD), a post-traumatic stress disorder (PTSD), a traumatic brain injury (TBI), a psychotic disorder, and/or a personality disorder. Accordingly, through measuring the subject's stress level, the method disclosed herein may also be used for assisting in determining the presence or absence of a mental disorder in a subject.

[166] In various embodiments, the subject may be experiencing any disorder recognized in a relevant field medicine (e.g., psychiatry) that is associated with abnormal cortisol levels.

[167] In various embodiments, such methods of measuring a stress level in a subject are used to characterize stress in a subject either not having a stress-related disorder or having stress-related disorders, and their risk for developing conditions, including but not limited to diabetes, obesity, prolonged wound healing, certain cancers, increased susceptibility to and severity of infectious diseases (viral, bacterial), and poor vaccine/immunization take-rate.

[168] In various embodiments, the stress to be measured may be characterised as, or related to, a quasi-social stress, a physical stress, a health stress, a stress caused by an infection and/or an environmental condition, optionally wherein the stress is characterized by at least one of an altered well-being of the subject, an altered aggressiveness of the subject, a lack of appetite of the subject, and an enhanced susceptibility of the subject for a disease.

[169] In various embodiments, the absorbance ratio of the biological sample mixture may be measured by UV-vis spectroscopy, and the absorbance ratio is  $A_{520}/A_{650}$ .

[170] In various embodiments, the biological sample may be a fluid sample. In various embodiments, the subject is human and the biological sample is a sweat sample or blood sample, or urine sample.

[171] In various embodiments, the sample may be derived from a fish and/or fish culture such as an aquaculture water sample. As will be appreciated, a method to monitor and measure stress levels in fish and/or a fish culture enables to detect stress, in time to initiate countermeasures. This may be important to enhance the well-being of fish, and, thereby, reduces the cost of fish farming and allows to provide a more reliable yield of farmed fish.

[172] In various embodiments, any kind of stress can be analysed in fish and/or a fish culture. In various embodiments, the stress is a quasi-social stress, a physical stress, a health stress, a stress

caused by an infection and/or an environmental condition, optionally wherein the stress is characterized by at least one of an altered well-being of the fish and/or the fish culture, an altered aggressiveness of the fish and/or the fish culture, a lack of appetite of the fish and/or the fish culture, a reduced food intake of the fish and/or the fish culture, and an enhanced susceptibility of the fish and/or the fish culture for a disease, such as an enhanced susceptibility for a fungal infestation or a worm attack. In various embodiments, the stress is characterized by a stocking density, a water quality, a system design and handling processes such as grading, sampling and transportation. As such, a high stocking density, a poor water quality, a poor system design and/or poor handling processes can induce stress in fish. In various embodiments, the method may be used to detect and measure stress-levels in any kind of fish.

[173] There is also provided a method for assisting in determining a subject's risk for developing an autoimmune disease, an inflammatory disease, and/or an allergic disease through measuring the subject's cortisol level with the method disclosed herein. As readily appreciated by those skilled in the art, cortisol plays a critical role in regulating inflammation, metabolism, and the immune response, thus the cortisol levels detecting in a subject may be indicative of an autoimmune disease, an inflammatory disease, and/or an allergic disease.

[174] There is also provided a method for predicting a subject's response to a steroid treatment for treating a disorder through measuring the subject's cortisol level with the method disclosed herein. In particular, the steroid treatment may be with glucocorticoids such as prednisone and dexamethasone, which are synthetic steroids that mimic the effects of cortisol. When glucocorticoids are administered as medication, they can suppress the subject's own production of cortisol. This happens because of feedback mechanisms that recognize the high levels of glucocorticoids as sufficient, leading to a decrease in the production of cortisol by the adrenal glands. This phenomenon is part of the hypothalamic-pituitary-adrenal (HPA) axis feedback loop. Thus, by measuring cortisol levels the response to steroid treatment can be monitored. For example, in conditions like Addison's disease (where the body doesn't produce enough cortisol), the effectiveness of glucocorticoid treatment might be gauged through the normalization of cortisol levels. However, in other conditions where steroids are used to suppress immune response or inflammation, the focus might not be directly on cortisol levels.

[175] There is also provided a method for developing or identifying therapeutic agents capable of modifying the stress level of a subject, the method comprising: a) measuring the subject's stress level with the method disclosed herein, b) administering a candidate therapeutic agent to the subject, c) measuring the subject's stress level with the method disclosed herein following the administration of the candidate therapeutic agent, d) characterizing the candidate therapeutic agent as capable of modifying the stress level of the subject, if the subject's measured cortisol level increases or decreases following administration of the candidate therapeutic agent.

[176] The term “administering” and variations of that term including “administer” and “administration”, includes contacting, applying, delivering or providing a compound or composition (i.e. candidate therapeutic agent) to a subject by any appropriate means.

[177] The term “candidate therapeutic agent” refers to an agent for which there is some evidence of potential therapeutic efficacy. The term candidate compound refers to a compound that may or may not have been previously evaluated for any therapeutic application, particularly in the context of a disease or condition involving a nucleotide repeat sequence. Any candidate therapeutic agent or candidate compound may be implemented as the “agent” for achieving a particular physiological effect. In various embodiments, a candidate therapeutic agent is a small molecule, protein (peptide or antibody), or nucleic acid. Consequently, candidate therapeutic agents may also be a small molecule, protein, or nucleic acid. Such agents and compounds may be in a pharmaceutically acceptable formulation. As will be appreciated, the increases or decrease of the measured stress level (i.e. cortisol level) following administration of the candidate therapeutic agent will characterize the candidate therapeutic agent as being an agonistic or antagonistic of cortisol expression and activity in the subject.

[178] The cortisol sensor disclosed herein may be comprised or integrated into a substrate, whereby once a sample is applied to the substrate the binding of cortisol to the cortisol sensors in the substrate is converted into a measurable signal indicative of the presence, and optionally quantity, of cortisol in the sample. The measurable signal (i.e. change of colour or degree of aggregation) may be detected and analysed by one or more colorimetric methods or analysis as disclosed above. Accordingly, the substrates may be used for non-invasive, real-time monitoring of cortisol levels (i.e. stress levels) in subjects and for managing their health and well-being.

[179] All embodiments disclosed above in relation to the cortisol sensor and methods similarly apply to the substrate and *vice versa*.

[180] In various embodiments, the substrate may be any suitable substrate capable of integrating the cortisol sensor disclosed herein effectively for use in detecting cortisol in a sample. The substrate may be prepared by adsorbing, immobilizing, embedding or attaching the cortisol sensor, e.g. metal nanoparticles, to the substrate.

[181] In various embodiments, the substrate may be fabricated using a variety of materials, including, but not limited to, glass, fused-silica, silicon, ceramic, metal, or a polymer material or combinations thereof. In various embodiments, the substrate material may be optically transparent so that the surface may be illuminated from the back side. In other embodiments, the surface may be illuminated from the front side.

[182] In various embodiments, the substrates may include but are not limited to polydimethylsiloxane (PDMS), polyethylene terephthalate (PET), polyimide (PI), polyurethane (PU),

ecoflex, and cellulose-based films, or combinations thereof. The substrates may be selected to be flexible and/or biocompatible and/or biodegradable. For example, a suitable substrate may be selected for skin-contact applications, and/or flexible enough to conform to the movement of the subject's body if the substrate is incorporated into a wearable device. In various embodiments, the substrate may be configured in the form of a test strip.

[183] In various embodiments, the cortisol sensors or substrate may be incorporated into a sensor device (i.e. cortisol sensor device) for detecting, and optionally quantifying, cortisol levels. The cortisol sensor device may be a detector or converter, whereby the device may detect, and optionally quantify, cortisol levels and convert it to a signal that can be read by an observer or an instrument. The sensor device may respond to an input cortisol detection/quantity by generating a functionally related output in the form of an electrical or optical signal.

[184] All embodiments disclosed above in relation to the cortisol sensor, substrate and methods similarly apply to the cortisol sensor device and *vice versa*.

[185] The sensor device may include but is not limited to wearable patches (i.e. flexible, skin-adherent patches to detect cortisol levels from sweat); flexible electronic tattoos (i.e. ultra-thin, flexible electronic tattoos to detect cortisol levels through skin contact); smart bandages (i.e. bandages that can detect cortisol in interstitial fluid); microfluidic devices (i.e. flexible microfluidic devices to analyze small amounts of bodily fluids, such as blood, saliva or sweat, for cortisol levels, using capillary action to draw fluids into a sensing area); electronic textiles (i.e. clothing or accessories to monitor cortisol levels from sweat or skin contact); smartwatches and fitness bands (i.e. these devices can be equipped with flexible substrates to monitor cortisol levels from sweat or skin contact) or flexible electronic devices (i.e. flexible electronic platforms that can be worn or attached to the body for detecting cortisol levels in various body fluids).

[186] The cortisol sensor, substrates and methods disclosed herein may be useful in developing and providing kits (i.e. assay kits). Accordingly, there is also provided kits for use in the methods disclosed herein, comprising the cortisol sensor and/or substrate disclosed herein and optionally other reagents and appropriate controls.

[187] In various embodiments, there may be provided a kit for preparing/producing the cortisol sensor disclosed herein comprising the metal nanoparticles, and the cortisol binding ligands and peptides to be conjugated to the metal nanoparticle, along with instructions for use. In particular, the kit may comprise a first solution comprising a plurality of metal nanoparticles, a second solution comprising a plurality of cortisol-binding ligands, and a third solution comprising a plurality of peptides.

[188] In various embodiments, there may be provided a kit for detecting the presence or absence, and optionally the quantity, of cortisol in a sample, comprising the cortisol sensor disclosed herein, and/or the substrate disclosed herein, and optionally other reagents and appropriate controls..

[189] In various embodiments, there may be provided a kit for measuring a stress level in a subject, comprising the cortisol sensor disclosed herein, and/or the substrate disclosed herein, and optionally other reagents and appropriate controls.

[190] All embodiments disclosed above in relation to the cortisol sensor and methods similarly apply to the kit and *vice versa*.

### WORKING EXAMPLES

[191] Non-limiting examples of the invention will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention. Based on the foregoing disclosure, it should be clear that by the method, the objectives set forth herein can be fulfilled.

#### 1. Identification of aptamers for cortisol

[192] Two cortisol aptamers were tested for their binding with cortisol, as stated in **Table 1** below.

**Table 1:** List of cortisol aptamers, sequence and K<sub>d</sub> values

| COR Aptamer            | Nucleotide Sequence (5'-3')                    | K <sub>d</sub>                   |
|------------------------|------------------------------------------------|----------------------------------|
| 15-1a<br>(SEQ ID NO:1) | ATGGGCAATGCGGGGTGGAGAATGGTTGCCGCACTTCG<br>G    | 6.9 ± 2.8 μM to<br>16.1 ± 0.6 μM |
| CSS.1<br>(SEQ ID NO:2) | GACGACGCCCGCATGTTCCATGGATAGTCTTGACTAGTC<br>GTC | 245 ± 39 μM                      |

[193] Using AuNP's aggregation behaviour under the notion that single-stranded DNA aptamers can protect citrate ions - coated AuNP from salt induced aggregation; but such protection is retarded when the aptamer is complexed with the cortisol.

[194] 10 μM of 15-1a or CSS.1 aptamer (10 μl) was added to AuNP (11.6 nM, 70 μl) to protect them from aggregation in the 1:1 mixture of respective cortisol binding buffer (50 mM Tris, 137 mM NaCl, 5 mM MgCl<sub>2</sub> for 15-1a aptamer and 20 mM sodium phosphate monobasic monohydrate, pH 7.5, 2 mM MgCl<sub>2</sub> for CSS.1 aptamer) and PBS. After COR is added, upon formation of aptamer-COR complex, the loss of the protection effect leads to aggregation of AuNP, which is quantified by the absorbance ratio of A<sub>520</sub>/A<sub>650</sub> (**Figure. 1**).

[195] As shown in **Figure. 1**, with increasing cortisol concentrations, a higher degree of aggregation (i.e. smaller  $A_{520}/A_{650}$  ratio) was observed in both cases. In the case of the 15-1a aptamer, AuNPs' aggregation profile was able to show a difference in cortisol concentrations of 1-10,000 nM (**Figure. 1A**). In comparison, in the case of CSS.1 aptamer, a much larger difference can be seen in a lower cortisol concentration range of 0.1-100 nM (**Figures. 1B-C**). To use such a principle as a measure of COR, the LOD obtained using the CSS.1 aptamer (0.1 nM) is lower than the 15-1a aptamer (1 nM), which confirms the former's higher binding affinity (lower  $K_d$  value) to cortisol (**Table 1**).

## 2. Preparation of Au-Apt-CALNN nanoparticles

[196] When aptamers are conjugated to AuNP, the nanoparticles are protected from aggregation in a salt medium. The co-conjugation of CALNN peptide to Au-Apt limits non-specific binding of the aptamer and increases the stability of nanoparticles towards centrifugation and when in salt medium.

[197] AuNP were first conjugated to thiol-labelled CSS.1 aptamer and CALNN peptide (Au-Apt-CALNN). In brief, the thiol-labelled CSS.1 aptamer (100  $\mu$ M) was first reduced using Tris (2-carboxyethyl) phosphine (TCEP, 5 mM) in HEPES buffer (5 mM). 76  $\mu$ L of HEPES, 20  $\mu$ L of aptamer and 4  $\mu$ L of TCEP were first shaken at 700 rpm for 10 mins. The mixture was then transferred into a 3 kDa spin filter (PALL) and centrifuged at 12500 rpm, 7 mins for 3 times. The resulting supernatant was then topped up to 100  $\mu$ L using DI water, yielding a 20  $\mu$ M activated aptamer solution. To 908  $\mu$ L of AuNP, 56  $\mu$ L of the activated aptamer solution and 10  $\mu$ L of hydrochloric acid (0.1 M) were sequentially added. The mixture was shaken at 500 rpm for 15 mins and left to incubate overnight. Then 60  $\mu$ L of CALNN peptide (1.8 mM) was added to the solution and further shaken at 500 rpm for 15 mins. Following which, 10  $\mu$ L of sodium hydroxide (0.1 M) was added to the solution and briefly vortexed. The mixture was then centrifuged at 10000 rpm for 45 mins and redispersed in water.

[198] To confirm the developed conjugation protocol, the UV-vis spectrums of AuNP and Au-Apt-CALNN were obtained. The peak absorbance shift from 520 nm (AuNP) to 525 nm (Au-Apt-CALNN) is an indication of successful conjugation (**Figure. 2A**).

[199] To further confirm the successful conjugation, the stability of Au-Apt-CALNN was evaluated in different types of seawater, such as artificial seawater (ASW, 14.16 g NaCl, 2.74 g  $MgCl_2 \cdot 6H_2O$ , 3.7 g  $MgSO_4 \cdot 7H_2O$ , 0.55 g  $CaCl_2 \cdot 2H_2O$ , 0.39 g KCl, 0.10 g  $NaHCO_3$  dissolved in 500 mL water), fish tank sea water from Republic Polytechnic (labelled as RP Sea 1 and RP Sea 2, **Figure. 2B and C**). As shown by **Figure. 2B**, the Au-Apt-CALNN only undergoes a minor aggregation in RP Sea 1 and RP Sea 2 water; in ASW, the aggregation is more obvious, indicating a higher ionic strength of the ASW than the RP sea water samples.

[200] All these results further confirmed the successful conjugation of the aptamer and the CALNN on the AuNPs surface. The well retained stability in most of these tank waters are an important basis for the Au-Apt-CALNN being used as sensors for non-invasive COR detection in the tank water.

### 3. Procedure for detection of cortisol by Au-Apt-CALNN nanoparticles

[201] The Au-Apt-CALNN NPs were used to detect cortisol, exploiting the difference in colloidal stability of the nanoparticles in a stronger salt condition ( $\text{MgCl}_2$  added) when the attached aptamer is in folded (due to COR binding) or unfolded state (**Figure. 3**). Without COR, the unfolded aptamer adopts a more flexible conformation. Its protection to AuNPs against additional aggregation force by  $\text{MgCl}_2$  is lesser than that with the analyte-aptamer complex. That is to say, when COR binds to the aptamer, the aptamer strains become folded and then are more extended from the surface of the nanoparticles, leading to enhanced steric stabilization than that by unfolded flexible aptamer without COR. In the presence of 0.0056M  $\text{MgCl}_2$  (final concentration due to the addition of 10  $\mu\text{L}$  0.05 M  $\text{MgCl}_2$  into the reaction solution), the differential aggregation of Au-Apt-CALNN with and without COR (measured by absorbance ratio of  $A_{520}/A_{650}$ ) were used for quantitative detection of cortisol. Such detection procedure involves a single “mixing” step before measuring the absorbance (denoted as “mix-and-measure”), but not tedious incubation, washing etc as used in ELISA. Advantageously, this single mixing step is more suitable for on-site detection.

[202] Firstly, cortisol dissolved in PBS buffer has been used in this assay. A cortisol stock solution was prepared by dissolving cortisol solid (Sigma) in absolute ethanol (1 mM) through sonication for 20 minutes. The stock solution was sequentially diluted in 1xPBS to obtain lower cortisol concentrations. For each dilution step, the cortisol solution was thoroughly mixed, vortexed and spun down to prevent adhesion on the side walls of tubes.

[203] For cortisol detection, 60  $\mu\text{L}$  of Au-Apt-CALNN and 20  $\mu\text{L}$  of cortisol solution (0.1-1000 nM) were pipetted into a 96 microwell plate. In the control well, the cortisol solution was replaced with 1xPBS. The sample mixture was mixed and left to incubate at room temperature for 20 minutes. 10  $\mu\text{L}$  of magnesium chloride (0.05 M) was then added to each well and gently mixed. An optimal 15 min is needed to obtain the largest differentiation in the aggregation profile for the Au-Apt-CALNN with and without COR. The UV-vis spectrums of the reaction mixtures were measured after 15 minutes. The differential UV-vis spectra were obtained (**Figure. 4A**).

[204] The absorbance ratio of  $A_{520}/A_{650}$  was plotted as a function of logarithmic cortisol concentrations for quantitative evaluation (**Figure 4B**). With increasing cortisol concentrations, the absorbance ratio of 520/650 nm also increased linearly. Furthermore, the  $A_{520}/A_{650}$  ratio obtained in the presence of cortisol were used to subtract the control sample (no added cortisol). The change in abs ratio of  $A_{520}/A_{650}$  were plotted as a function of logarithmic cortisol concentrations (**Figure 4C**),

yielding a  $r^2$  value of 0.940. The LOD obtained for the sensing assay is 0.1 nM (100 pM) and can be completed as fast as 15 minutes. The  $r^2$  value is a measure of how well a linear regression model fits the data. In this case, the  $r^2$  value of 0.940 is close to 1, showing that the predictor variables are able to predict the value of the response variable.

[205] The cross-selectivity of the nanosensor was evaluated against other hormones and amino acids [5] in fish tank water, such as  $\beta$ -estradiol-6-(O-carboxy-methyl) oxime, cortisol BSA, adenosine and lysine at 1 ng/mL (Figure 5). The specificity of the nanosensor was confirmed as the change in abs ratio of  $A_{520}/A_{650}$  of interfering species is lower than at 10x lower cortisol concentrations (0.1 ng/mL).

#### 4. Real Sample Test

[206] Fish tank water samples and fish tissues (fin etc) were tested as real samples to validate the Au-Apt-CALNN nanosensor, in comparison of the results obtained by HPLC/ELISA.

[207] Firstly, the water samples are from fish tanks, where the fishes were stressed by chasing actions (1<sup>st</sup> chasing experiment). The fish tank water was collected at different chasing time points for measuring cortisol levels by the nanosensor, ELISA and HPLC.

[208] For the 1<sup>st</sup> chasing experiment (Figures. 6A-C), the cortisol concentrations increase significantly from 5 to 15 mins, agreeable by all three methods (especially taken into consideration of the very high standard deviation for ELISA at 15 mins). As detected by all three methods, the cortisol levels decrease in postchase as compared to chase experiments. Quantitative comparison shows a reasonable correlation factor of  $r^2 = 0.7305$  between the nanosensor versus HPLC (Figure. 6D). In comparison, the correlation factor for nanosensor versus ELISA is lower at  $r^2 = 0.4194$  (Figure. 6E). The higher correlation between nanosensor and HPLC maybe attributable to the same cortisol standard used (from Sigma-Aldrich); while the cortisol standard for ELISA was obtained from the provided fish ELISA kit (Cusabio). Visually, the cortisol from Sigma-Aldrich was powder-like while those obtained from ELISA resembled a pellet/cotton puff. This indicated the cortisol sources maybe of different quality, possibly due to sample processing/treatment.

[209] The Au-Apt-CALNN nanosensor was further employed for testing cortisol levels in fish fins during chasing experiments (Figure. 7). The trend obtained for nanosensor is comparable to ELISA. By both methods, the cortisol levels are found to increase from chase 5 mins to chase 15 mins. In post-chase experiments, the cortisol levels increase from after chase 30 mins and peaks at 2 h, before progressively decreasing in after chase 4h and post-chase samples (Figures. 7A-B). The lower cortisol concentrations obtained for the nanosensor could be attributed to the prolonged storage time of the samples prior to testing. Overall, a correlation factor of  $r^2 = 0.642$  was obtained between the two methods (Figure. 7C).

[210] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. Other embodiments are within the following claims.

[211] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Further, it will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The cortisol sensors, methods, and uses described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims. The listing or discussion of a previously published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

[212] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, it should be understood that although the present invention has been specifically disclosed by exemplary embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[213] The content of all documents and patent documents cited herein is incorporated by reference in their entirety.

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## CLAIMS

What is claimed is:

1. A cortisol sensor comprising or consisting of:
  - i) a metal nanoparticle;
  - ii) a plurality of cortisol binding ligands conjugated to the surface of the metal nanoparticle;and
  - iii) a plurality of peptides conjugated to the surface of the metal nanoparticle by means of a Cysteine (C) residue for reducing non-specific binding of the cortisol binding ligands and/or improving stability of the metal nanoparticle in solution.
2. The cortisol sensor of claim 1, wherein the metal nanoparticle comprises or consists of a noble metal, preferably gold, more preferably the metal nanoparticle is a citrate-coated gold nanoparticle.
3. The cortisol sensor of claim 1 or 2, wherein the metal nanoparticle has a diameter in the range of 5-50 nm, more preferably a diameter in the range of 10-20 nm, even more preferably a diameter of about 13 nm, so long as the metal nanoparticle has desirable plasmonic properties and/or sensitivity to UV-vis shift in response to differential aggregation.
4. The cortisol sensor of any one of claims 1-3, wherein the cortisol binding ligand is a cortisol aptamer, preferably a cortisol DNA aptamer, optionally the cortisol aptamer is conjugated directly to the surface of the metal nanoparticle or indirectly through an intervening linker.
5. The cortisol sensor of claim 4, wherein the cortisol aptamer comprises or consists of a nucleotide sequence set forth in **SEQ ID NO:1, 2 or 3**, or derivatives thereof.
6. The cortisol sensor of claim 5, wherein the cortisol aptamer comprises or consists of a nucleotide sequence set forth in **SEQ ID NO:2**, or derivatives thereof.
7. The cortisol sensor of any one of claims 4-6, wherein the cortisol aptamer is labelled with a thiol group, and the thiol group is covalently bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the surface of the metal nanoparticle, preferably the cortisol aptamer is thiol-terminated at the 5' end.
8. The cortisol sensor of any one of claim 4-6, wherein the cortisol aptamer is terminated with an amine group (NH<sub>2</sub>), and the amine group is covalently bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the metal nanoparticle, wherein the surface of the metal nanoparticle is functionalized with carboxylic acid groups (COOH).

9. The cortisol sensor of any one of claims 4-6, wherein the cortisol aptamer is biotinylated with a biotin group, optionally introduced via a linker molecule or by incorporating a biotinylated nucleotide during the aptamer synthesis, and the biotin group is bound to the surface of the metal nanoparticle, such that the cortisol binding ligand is conjugated to the metal nanoparticle, wherein the surface of the metal nanoparticle is functionalized with streptavidin.
10. The cortisol sensor of any one of claims 1-9, wherein the conjugated peptides are interspersed between conjugated cortisol binding ligands to separate and space apart the cortisol binding ligands from being proximate to each other to improve the stability of the metal nanoparticle.
11. The cortisol sensor of any one of claims 1-10, wherein the plurality of peptides is a plurality of short peptides, optionally each short peptide comprises or consists of an amino acid sequence  $CX_n$ ,  $CAX_n$ , or  $CALX_n$ , wherein X denotes any amino acid residue and n is an integer of 1 or more, and/or optionally each short peptide is labelled with a thiol group.
12. The cortisol sensor of claim 11, wherein the short peptide comprises or consists of an amino acid sequence set forth in **SEQ ID NO: 4** or variants thereof.
13. The cortisol sensor of any one of claims 1-12, wherein:
- the metal nanoparticle is a gold nanoparticle;
  - the cortisol binding ligand is a cortisol DNA aptamer comprising or consisting of a nucleotide sequence set forth in **SEQ ID NO: 1, 2 or 3**, or derivatives thereof, and is labelled with a thiol group at the 5' end, wherein the thiol group is covalently bound to the surface of the gold nanoparticle; and
  - the peptide comprises or consists of an amino acid sequence set forth in **SEQ ID NO: 4** or variants thereof.
14. A method of preparing a cortisol sensor, comprising or consisting the steps of:
- preparing a first solution comprising a plurality of metal nanoparticles;
  - preparing a second solution comprising a plurality of cortisol-binding ligands;
  - adding the first and second solutions together to produce a first mixture;
  - incubating the first mixture under conditions suitable to conjugate the cortisol-binding ligands onto the surface of the metal nanoparticles; and
  - adding a third solution comprising a plurality of peptides to the incubated first mixture under conditions suitable to conjugate the peptides onto the surface of the metal nanoparticles to produce a second mixture comprising one or more of the cortisol sensor, optionally wherein the second mixture is used to prepare a colloidal solution or composition comprising a plurality of the cortisol sensors.

15. The method of claim 14, further comprising isolating the cortisol sensors, wherein each cortisol sensor comprises or consists of: a metal nanoparticle; a plurality of cortisol binding ligands conjugated to the surface of the metal nanoparticle; and a plurality of peptides conjugated to the surface of the metal nanoparticle by means of a Cysteine (C) residue for reducing non-specific binding of the cortisol binding ligands and/or improve stability of the metal nanoparticle in solution, optionally the isolated cortisol sensors are stored at suitable conditions before further use or processing, preferably at a temperature in the range of 1-4°C.

16. The method of claim 14 or 15, wherein the metal nanoparticle is a gold nanoparticle, and step a) comprises: adding a sodium citrate dihydrate solution, preferably about 5mL at a concentration of 40 mM, to boiling gold (III) chloride solution, preferably about 50mL at a concentration of 1mM, to provide a mixture, refluxing the mixture for about 20-40 minutes, preferably about 30min, to provide the first solution comprising a plurality of gold nanoparticles; and cooling the first solution to room temperature.

17. The method of any one of claims 14-16, wherein the cortisol binding ligand is a cortisol aptamer, and step b) comprises: adding the cortisol aptamer in a buffer solution, optionally shaking the buffer solution comprising the cortisol aptamer; centrifuging the buffer solution to produce a supernatant, preferably the buffer solution is transferred to a 3kDa centrifugal filter; and adding water to the supernatant to provide the second solution.

18. The method of any one of claims 14-17, wherein step c) further comprises adding an acidic solution, preferably hydrochloric acid, and wherein the second solution is added to the first solution and subsequently the acidic solution is added thereafter, optionally the acidic solution is in an amount of about 0.5-50µl, preferably about 10 µl, optionally the concentration of the acidic solution is about 0.01M-1M, preferably 0.1M.

19. The method of any one of claims 14-18, wherein step e) further comprises adding an alkaline solution, preferably sodium hydroxide, optionally the alkaline solution is in an amount of about 0.5-50µl, preferably about 10 µl, optionally the concentration of the alkaline solution is about 0.01M-1M, preferably 0.1M.

20. The method of any one of claims 14-19, wherein the production of the cortisol sensor is verified by:

i) UV-vis spectroscopy, wherein the UV-vis spectroscopy comprises comparing a UV-vis spectrum of the cortisol sensor to a UV-vis spectrum of the metal nanoparticle in the first solution; and/or,

ii) a salt stability test, wherein the salt stability test comprises adding the cortisol sensor to a saline solution, and evaluating the dispersion of the cortisol sensor for aggregation.

21. Use of the cortisol sensor according to any one of claims 1-13 for detecting the presence or absence, and optionally the quantity, of cortisol in a sample.

22. A method to detect the presence or absence, and optionally the quantity, of cortisol in a sample, comprising or consisting the steps of:

- a) providing a cortisol sensor according to any one of claims 1-13;
- b) contacting the cortisol sensor to the sample in a saline environment that is suitable for binding the cortisol binding ligand to cortisol, to produce a sample mixture; and
- c) detecting the presence or absence, and optionally the quantity, of cortisol in the sample mixture by determining the degree of aggregation of the cortisol sensor in the sample mixture, wherein the degree of aggregation of the sample mixture is compared with the degree of aggregation of a control sample and a differential degree of aggregation is indicative of the presence or absence, and optionally the quantity, of cortisol in the sample.

23. The method of claim 22, wherein a bivalent salt ion is added to the sample mixture in step b), wherein the bivalent salt ion is selected from the group consisting of a calcium chloride ( $\text{CaCl}_2$ ), calcium carbonate ( $\text{CaCO}_3$ ), calcium sulfate ( $\text{CaSO}_4$ ), magnesium chloride ( $\text{MgCl}_2$ ), magnesium sulfate ( $\text{MgSO}_4$ ), magnesium carbonate ( $\text{MgCO}_3$ ), barium chloride ( $\text{BaCl}_2$ ), barium sulfate ( $\text{BaSO}_4$ ), lead acetate ( $\text{Pb}(\text{CH}_3\text{COO})_2$ ), lead nitrate ( $\text{Pb}(\text{NO}_3)_2$ ), ferrous sulfate ( $\text{FeSO}_4$ ), ferrous chloride ( $\text{FeCl}_2$ ) and combinations thereof, preferably the bivalent salt ion is  $\text{MgCl}_2$ .

24. The method of claim 22 or 23, wherein stability of the cortisol sensor increases in the presence of cortisol in the sample, such that the degree of aggregation is reduced in the presence of cortisol in the sample compared to the control sample.

25. The method of any one of claims 22-24, wherein the degree of aggregation is measured by UV-Visible Spectroscopy, microscopy techniques, dynamic light scattering techniques, visual observation of colour, or surface plasmon resonance techniques.

26. The method of claim 25, wherein the degree of aggregation is measured by UV-vis spectroscopy to obtain a UV-vis spectrum of the sample mixture, wherein the UV-vis spectrum of the sample mixture is compared with the UV-vis spectrum of a control sample and a differential UV-vis spectrum is indicative of the presence or absence, and optionally the quantity, of cortisol in the sample.

27. The method of any one of claims 22-24, wherein the degree of aggregation is determined by measuring the absorbance ratio of the sample mixture, wherein the absorbance ratio of the sample mixture is compared with the absorbance ratio of the control sample, and a differential absorbance ratio is indicative of the presence or absence, and optionally the quantity, of cortisol.

28. The method of claim 27, wherein an absorbance ratio of  $A_{520}/A_{650}$  is measured for the sample mixture and control sample, wherein a differential absorbance ratio of  $A_{520}/A_{650}$  between the sample and control sample is indicative of the presence or absence of cortisol in the sample.
29. The method of claim 25, further comprising quantifying the cortisol in the sample mixture based on the absorbance ratio of  $A_{520}/A_{650}$ , wherein the absorbance ratio of the sample mixture is compared with the absorbance ratio of a control sample, wherein a differential absorbance ratio between the sample and control sample is indicative of the presence, and quantity, of cortisol in the sample mixture, and the absorbance ratio of the control sample is subtracted from the absorbance ratio of the sample mixture.
30. The method of claim 29, wherein the differential absorbance ratio is in the range of 0.2-1.2 and corresponds to cortisol concentrations ranging from 0.1-1000 nM.
31. The method of any one of claims 22-30, wherein after step b), the sample mixture is not further processed, treated, incubated or washed before the detection step c) is carried out, such that the method does not comprise an additional or intermediary step between steps b) and c).
32. The method of any one of claims 22-31, wherein the detection step is completed in about 10-30 minutes, preferably about 15 minutes, after step b), preferably step c) immediately follows, or is immediately initiated after, step b).
33. The method of any one of claims 22-32, wherein the sample is a water sample obtained from an aquaculture tank, preferably a fish tank, or the sample is a biological sample, preferably a biological tissue or fluid sample that has been obtained from an animal, even more preferably a fish tissue sample, optionally the fish tissue sample is derived from the fin, scales, mucus, or plasma of the fish and may be in a fluid form or a solid form, optionally wherein the solid form is processed and extracted into a liquid form.
34. A method of measuring a stress level in a subject, comprising:  
a) contacting a biological sample that has been obtained from the subject with a cortisol sensor according to any one of claims 1-13 in a saline environment that is suitable for binding the cortisol binding ligand to cortisol, to produce a biological sample mixture; and  
b) detecting the quantity of cortisol in the biological sample mixture by determining the degree of aggregation, wherein the degree of aggregation of the biological sample mixture is compared with the degree of aggregation of a control sample and a differential degree of aggregation is indicative of the quantity of cortisol in the biological sample mixture and the stress level of the subject.
35. The method of claim 34, wherein the quantity of cortisol in the biological sample mixture is compared with known reference levels of cortisol associated with certain stress levels in the subject,

wherein each known reference level is specific for gender, age, mental condition, and/or predictive of a mental disorder of said subject.

36. A method for assisting in determining a subject's risk for developing an autoimmune disease, an inflammatory disease, and/or an allergic disease through measuring the subject's cortisol level with the method of claim 22, wherein the sample has been obtained from the subject.

37. A method for predicting a subject's response to a steroid treatment for treating a disorder through measuring the subject's cortisol level with the method of claim 22, wherein the sample has been obtained from the subject.

38. A method for assisting in determining the presence or absence of a mental disorder in a subject through measuring the subject's cortisol level with the method of claim 22, wherein the sample has been obtained from the subject, optionally wherein the mental disorder is one or more of an anxiety disorder, a major depressive disorder (MDD), a post-traumatic stress disorder (PTSD), a traumatic brain injury (TBI), a psychotic disorder, and/or a personality disorder.

39. A method for developing or identifying therapeutic agents capable of modifying the stress level of a subject, the method comprising:

- a) measuring the subject's stress level with the method of claim 34 or 35,
- b) administering a candidate therapeutic agent to the subject,
- c) measuring the subject's stress level with the method of claim 34 or 35 following the administration of the candidate therapeutic agent,
- d) characterizing the candidate therapeutic agent as capable of modifying the stress level of the subject, if the subject's measured cortisol level increases or decreases following administration of the candidate therapeutic agent.

40. A colloidal solution or composition comprising a plurality of cortisol sensors according to any one of claims 1-13.

41. A substrate comprising a plurality of cortisol sensors according to any one of claims 1-13, for use in detecting the presence or absence, and optionally the quantity, of cortisol in a sample.