



(51) International Patent Classification:

G01N 27/30 (2006.01)

G01N 33/74 (2006.01)

(21) International Application Number:

PCT/SG2021/050134

(22) International Filing Date:

16 March 2021 (16.03.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

10202002405U

16 March 2020 (16.03.2020)

SG

(71) Applicant: NATIONAL UNIVERSITY OF SINGAPORE [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors: XU, Gang; c/o National University of Singapore, Faculty of Engineering, Department of Chemical and Biomolecular Engineering, 21 Lower Kent Ridge Road,

Singapore 119077 (SG). TONG, Yen Wah; c/o National University of Singapore, Faculty of Engineering, Department of Chemical and Biomolecular Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG). LI, Cheng; c/o National University of Singapore, Faculty of Engineering, Department of Chemical and Biomolecular Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG). CHAI, Li Lin Christina; c/o National University of Singapore, Faculty of Science, Department of Pharmacy, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(74) Agent: VIERING, JENTSCHURA & PARTNER LLP; P.O. Box 1088, Rochor Post Office, Rochor Road, Singapore 911833 (SG).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN,

(54) Title: AN ELECTROCHEMICAL BIOSENSOR AND METHOD OF FABRICATING THE SAME

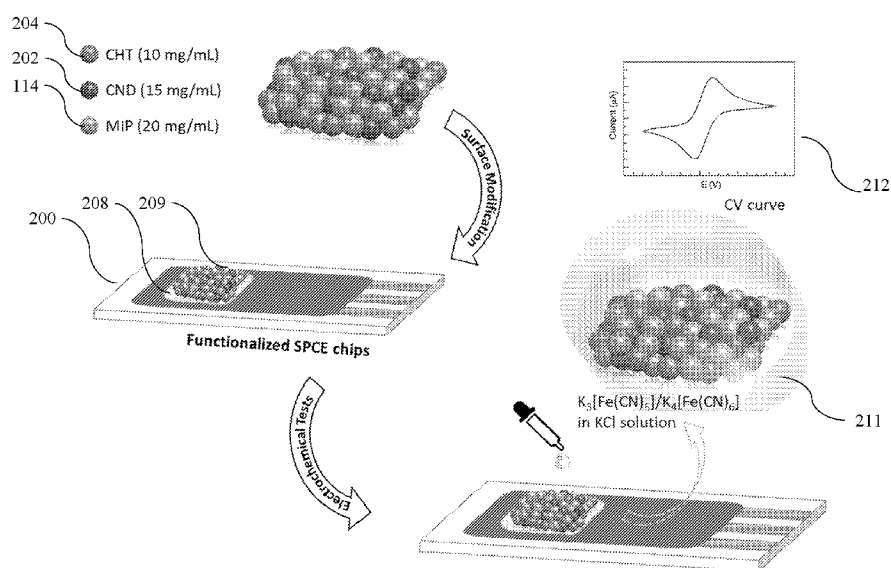


Figure 2

(57) Abstract: An electrochemical biosensor, a method of fabricating the electrochemical biosensor, and a method of electrochemical biosensing using the electrochemical biosensor. The electromechanical biosensor comprises an electrode substrate configured for electrical measurements of a functionalized area of a surface of the electrode substrate, wherein the functionalized area has formed therein a mixture of molecular imprinting polymer, MIP, particles and one or more immobilizer materials, the MIP particles having imprints of a molecule to be sensed by the biosensor. In a preferred embodiment, an electrochemical sensing system for cortisol detection based on a one-step modification of mixed chitosan, carbon nanodots, and MIP particles is provided.



KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

- (84) Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

AN ELECTROCHEMICAL BIOSENSOR AND METHOD OF FABRICATING THE SAME

FIELD OF INVENTION

5 The present invention relates broadly to an electrochemical biosensor and to a method of fabricating the same, more specifically to a sensitive and selective method for electrochemical measurement/detection, as well as to fabrication of a portable and electrochemical device. The present invention has particular, but not exclusive, application in cortisol detection and stress screening, hence to monitor customer wellness.

10

BACKGROUND

Any mention and/or discussion of prior art throughout the specification should not be considered, in any way, as an admission that this prior art is well known or forms part of common general knowledge in the field.

15 Biosensors have a myriad of application fields, for example in salivary measurements such as existing methodologies for salivary cortisol measurement. It is widely accepted that salivary cortisol is directly proportional to serum unbound cortisol in human beings, which is a steroid hormone released in response to stress or low blood glucose. Accordingly, cortisol is regarded as the ‘stress hormone’ and it plays a critical role in regulating people’s blood sugar level, adjusting metabolism, and influencing blood pressure etc.

Salivary measurements in the commercial market are typically complicated and cumbersome with multiple procedures. For example, customers are requested to collect their saliva samples by themselves in a specific time, and send back these samples to a supplier company for laboratory tests. In general, customers could receive the results in 5-7 working days upon departing of their samples. There are some disadvantages of the such methods, especially the uncertainties during sample collection and transportation. More importantly, the samples from patients/participants are open without necessary procedures to secure their privacies.

Alternatively, researchers have reported a stress monitoring (cortisol detection) technique, which combined lateral flow technology and a portable imaging device. With this technique, measurement of salivary cortisol could be completed at home within 20 min. The noninvasive and point-of-care measurement of cortisol levels is of great importance. However, fabrication of the lateral flow assay enabled sensing system is difficult and requires high skill. Also, professional expertise is necessary for analysis of the colorimetric signal.

Saliva is a complicated mixture, containing various types of electrolytes, proteins, enzymes, mucins and so on. Up to now, some sensing techniques have been developed for ultrasensitive and selective detection of pre-treated cortisol. However, the direct detection of salivary cortisol has been a challenge for decades. For example, the proteins in saliva are key interferences on

chromatography-based sensing strategies, due to their big size compared with the cortisol molecule. Also, salivary cortisol detection is currently a time-consuming process, which is operated by professionals in laboratory.

Embodiments of the present invention seek to address at least one of the above problems.

5

SUMMARY

In accordance with a first aspect of the present invention, there is provided an electrochemical biosensor comprising:

10 an electrode substrate configured for electrical measurements of a functionalized area of a surface of the electrode substrate,

wherein the functionalized area has formed therein a mixture of molecular imprinting polymer, MIP, particles and one or more immobilizer materials, the MIP particles having imprints of a molecule to be sensed by the biosensor.

15 In accordance with a second aspect of the present invention, there is provided a method of fabricating an electrochemical biosensor, comprising the steps of:

providing an electrode substrate configured for electrical measurements of a functionalized area of a surface of the electrode substrate, and

20 providing a mixture of molecular imprinting polymer, MIP, particles and one or more immobilizer materials in the functionalized area, the MIP particles having imprints of a molecule to be sensed by the biosensor.

In accordance with a third aspect of the present invention, there is provided a method of electrochemical biosensing using the electrochemical biosensor of the first aspect.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Embodiments of the invention will be better understood and readily apparent to one of ordinary skill in the art from the following written description, by way of example only, and in conjunction with the drawings, in which:

Figure 1 shows schematic drawings illustrating preparation of molecular imprinting polymers, MIPs, for use in an example embodiment via bulk polymerization.

30 **Figure 2** shows schematic drawings illustrating fabrication of a cortisol biosensor according to an example embodiment onto a screen-printed carbon electrode.

Figure 3 shows a chart illustrating selective binding capacity results for MIPs for use in an example embodiment, and for non-imprinting polymers, NIP, for comparison.

Figure 4A shows a graph illustrating cyclic voltammetry, CV, curves, for a cortisol biosensor according to an example embodiment, depending on cortisol concentration in the probing sample.

Figure 4B shows a graph illustrating the calibration curve and fit relative to the CV responses in Figure 4A at the 0.23 V peak at different cortisol concentrations.

Figure 5 shows a chart illustrating a comparison of the increase of CV current at the 0.23 V peak (Δ CV current), relative to CV current of SPCE-CND/CHT/MIP and SPCE-CND/CHT/NIP for different probing samples.

Figure 6 shows a schematic drawing illustrating an electrochemical biosensor according to an example embodiment.

Figure 7 shows a flowchart illustrating a method of fabricating an electrochemical biosensor, according to an example embodiment.

DETAILED DESCRIPTION

The biosensor according to example embodiments of the present invention has integrated the advantages of molecular imprinting technology and electrochemical sensing technique, which is highly selective, simple, fast and easy-to-operate. As such, the detection device according to example embodiments can advantageously facilitate the real-time monitoring of consumer wellness.

A portable and electrochemical sensing system for cortisol detection based on molecular imprinting technology is provided according to an example embodiment. The molecular imprinting polymers (MIP)-based electrochemical biosensor according to an example embodiment has particular, but not exclusive, applications in quantitative detection of cortisol, real-time measurement of cortisol concentration, and subsequent assessment of psychological stress of human beings. A portable and electrochemical sensing system for cortisol detection according to one example embodiment comprises: ferricyanide-containing buffer solutions as probing signal, MIP-modified surface-printed carbon electrode (SPCE) chips as sensing platform, and an electrochemical workstation assisted subsystem for data mining, analysis and screening.

Advantageously, the cortisol sensing system based on molecular imprinting polymers (MIPs) according to an example embodiment is easy to construct. Electrochemical signals are captured and processed by an inexpensive portable bipotentiostat, interacted with a personal computer according to an example embodiment. The sensing device according to an example embodiment is simple and easy to use, without requiring sophisticated instrument(s) and complicated processing. Therefore, the MIP-based biosensor according to an example embodiment can be an ideal candidate as point-of-care biosensor for cortisol detection.

The fabrication of a sensing system according to an example embodiment includes the preparation and evaluation of MIP, and the fabrication and optimization of MIP-based electrochemical biosensor. Molecular imprinting creates template-shaped cavities in a polymeric matrix with the specific binding affinity between the matrix and template molecules, according to an example embodiment. In comparison with other recognition strategies, MIP applied according to example embodiments is highly specific to target molecules. Additionally, MIP applied according to example embodiments has good chemical stability, low cost, and ease-to-prepare. Advantageously, rapid, sensitive and accurate CV measurements can be provided based on SPCE portable platform according to example embodiments of the present invention.

In a preferred embodiment, highly conductive carbon nanodots are also employed in an example embodiment, to promote the surface immobilization with MIP particles on sensing chips. The resulting modification method is easy-to-operating, and potentially applicable to other platforms. In addition, with the development of integrated circuit technology, more and more portable workstations have been emerging as an ideal platform to develop point-of-care biosensors with sensitivity, rapidness and practicality. It has been recognized by the inventors that advantages from both MIT and electrochemical sensing methods can be provided in an MIP-based electrochemical biosensor according to an example embodiment for sensitive and selective detection of cortisol, with potential in real-time measurement of salivary cortisol, hence to monitor consumer wellness in a non-invasive and simple manner.

Molecular imprinting polymers (MIPs) for use in an example embodiment and non-imprinting polymers (NIPs) for reference measurements were prepared via bulk polymerization, as shown in **Figure 1**. By way of a non-limiting example, in a reaction vial 100, cortisol 102 (template molecule, 72.5 mg), Azobisisobutyronitrile 101 (AIBN, initiator, 1.64 mg), 2,2-Dimethoxy-2-phenylacetophenone 103 (DMPA, initiator, 5.12 mg), methacrylic acid 104 (MAA, functional monomer, 101.7 μ L), and ethylene glycol methacrylate 106 (EGDMA, cross-linker, 1.13 mL) were thoroughly dissolved in 5 mL of chloroform 108 (porogenic solvent) under ultrasonic treatment (20 min) and subsequent mechanical stirring (20 min). Then, the reaction vial 100 was degassed under Argon phenomenon for 30 min, and sealed for reaction. Next, the polymerization (indicated at numeral 110) was initiated by UV irradiation 112 and the reaction was kept at 4 °C for 12 hours. NIPs were also prepared with the same method, except from the addition of template (cortisol) molecules. After polymerization reaction, the obtained polymers (MIP and NIP) were mechanically grinded into particles, e.g. MIP particles 114 (10 Hz, 10 min). Subsequently, the polymer particles (MIP/NIP) were thoroughly washed against acetic acid/methanol mixture (8:2, v/v) using Soxhlet extractor for 48 hours, during which the mixed solvent was refreshed every 12 hours. For the resulting MIP particles 114, the washing resulted in removal of the cortisol template molecule, indicated at numeral 116, leaving correspondingly shaped imprints e.g. 118 in the washed MIP particles 114. The final polymer particles (MIP/NIP) were dried under vacuum for 12 hours and stored in clean vials for future use.

Stock chitosan solution (pH 5.0, 5 mg/mL) was prepared by dissolving chitosan flakes in hot (85 °C) aqueous solution of 0.05 M HCl under mechanical stirring. After cooling to room

temperature, the pH of the aqueous solution was adjusted to 5.0 using concentrated NaOH solution. Next, the chitosan solutions were filtered using a 0.45 µm cellulose filter and stored at 4 °C for future use. To prepare carbon nanodots, 20 mg of candle soot was suspended in 20 mL of mixed solvent (water-ethanol 1:1 (v/v)), and sonicated for 2 hours. The black mixture was centrifuged at 3000 rpm for 4 min to remove large-size particles. The supernatant was collected and re-centrifuged at 8000 rpm for 15 min. The black precipitate was collected and dried by lyophilization for future applications.

To fabricate a cortisol biosensor according to a non-limiting example embodiment onto a screen-printed carbon electrode 200 (SPCE, DRP-110-U75, Metrohm), a 5 µL of mixture solution (carbon nanodots 202 (CND, 15 mg/mL), chitosan 204 (CHT, 10 mg/mL) and MIP 114, or NIP for the reference measurements, (20 mg/mL)), was gently dropped onto an area 208 of the SPCE 200 surface, as shown in **Figure 2**. That is, a one-step modification of mixed chitosan/carbon nanodots/MIP is advantageously carried out for the MIP-based biosensors according to example embodiments, including, but not limited to, as described in the above example embodiment. In this example embodiment, a uniform film 209 was formed on the SPCE 200 surface after complete evaporation of residual solvent. For sensing evaluations, the cyclic voltammetry (CV) test of each modified electrode was performed using a testing apparatus capable of being coupled to the SPCE 200. By way of example, not limitation, a µStat 400 bipotentiostat/Galvanostat (Metrohm Dropsens) was used, in the presence of 5 mM of $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ and 0.1 M KCl as probing buffer (indicated as droplet 211. The potential range of CV test in a non-limiting example, indicated at numeral 212, is from -0.3 to 0.5 V with a step potential of 2 mV, and the scan rate is 50 mV/s.

It is noted that the present invention is not limited to the specific SPCE 200 configuration as shown in Figure 2. For example, the number of electrodes may differ depending on the type of electrochemical measurement to be performed for the testing and/or the testing apparatus used.

Binding performance and selectivity of MIP applied according to an example embodiment

The as-prepared MIP/NIP (compare Figure 1) applied according to example embodiments was incubated with 1000 ppb of cortisol and other competitors individually, to evaluate their respective binding parameters. Specifically, the as-prepared polymers (MIP/NIP), 1.0 mg of MIP/NIP was incubated with 1.0 mL of cortisol aqueous solution (1000 ppm) in vial for 10 min under agitation. After that, 900 µL of the mixture was sampled, filtered (0.22 µm nylon filter), and transferred into HPLC vials for measurements. Reverse-phase HPLC analysis was used for quantitation of cortisol concentration, in which the detection wavelength was fixed at 254 nm. The mobile phase consisted of acetonitrile-ultrapure water 3:7 (v/v), with a fixed flow rate of 0.8 mL/min. Reference-used cortisol standards, with concentrations from 10, 50, 100, 500 to 1000 ppb, were thrice analyzed. Characteristic peak areas (HPLC spectrum) were integrated and plotted against concentration for calibration. Binding capacity was denoted as the amount of cortisol bound to the MIP/NIP, which was calculated by subtracting the concentration of free cortisol (detected by HPLC) from the given initial concentration (1000 ppb). Imprinting factor was defined as the ratio of binding capacity of MIP to that of NIP.

Similarly, MIP was also challenged against other competitors, and the selectivity index was defined as the amount of cortisol bound to MIP to that of competitors bound to MIP.

As shown in **Figure 3**, MIP according to an example embodiment exhibited a significantly higher affinity to cortisol molecules than NIP, as well as a significantly higher affinity to cortisol than to other molecular competitors. For instance, the binding capacity of MIP against cortisol was determined at 771 $\mu\text{g}/\text{mg}$, while that of NIP was only 159 $\mu\text{g}/\text{mg}$, hence the imprinting factor was calculated at 4.85. In comparison to cortisol, the amount of bond competitive molecules was markedly reduced, as the binding capacity of MIP against prednisolone, cortisone and corticosterone was 126, 211, and 204 $\mu\text{g}/\text{mg}$, respectively. Meanwhile, binding phenomena of competitive molecules observed on NIP were similar to that on MIP, indicating that the as-prepared MIP has a good binding specificity on the templated cortisol molecule.

Alternatively, MIP/NIP were also incubated with 1000 ppb of mixed solution (prednisolone, cortisol, cortisone and corticosterone), to further evaluate their binding performance in complicated and harsh systems. The binding results were in good agreement with that from **Figure 3**, the largest number of cortisol were re-assembled on MIP according to an example embodiment, while that on NIP was quite small. The selectivity was also validated as the binding capacities of MIP against other competitors were over 3 times smaller, compared with that against cortisol. In summary, the binding capacity is as high as 771 $\mu\text{g}/\text{mg}$, with a selectivity index at 3.65 against cortisone. Therefore, the cortisol-templated MIP applied according to an example embodiment was prepared successfully, and its binding performance was also ascertained to fabricate subsequent electrochemical cortisol sensors.

Electrochemical sensing measurements according to an example embodiment

With reference again to **Figure 2**, carbon nanodots 202 (CND) and chitosan 204 were employed to promote the immobilization of MIP/NIP on SPCE 200 surfaces. As for the anchoring layer, homogeneous dispersion of CND and MIP particles occurred in chitosan (CHT) solution according to an example embodiment, resulting in forming the uniform thin film 209 on SPCE 200 surface (SPCE-CND/CHT/MIP). Additionally, CND is known for its high conductivity, which facilitated electron transfer and hence reduced the electronic resistance. After immobilization of MIP/NIP on the SPCE 200 surface, cyclic voltammetry (CV) response of the SPCE-CND/CHT/MIP system against different concentrations of cortisol was studied in the presence of probing buffer, indicated as droplet 211 deposited onto the thin film 209 on the SPCE 200. In a non-limiting example, 5 mM of $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ in 0.1 M KCl was used as the probing buffer. The specific concentration of cortisol was obtained by mixing into the probing buffer, and the CV measurements were subsequently carried out. In **Figure 4A**, oxidation and reduction peaks 400, 402 with a respective potential of 0.23 and 0.09 V are discernible in all CV curves, which are induced by oxidation and reduction processes of $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ species. With the increase of concentration of cortisol from 50 ppb to 700 ppb (specifically: 50 (a), 100 (b), 150 (c), 200 (d), 250 (e), 300 (f), 400 (g), 500 (h), 600 (i) to 700 ppb (j)), the current of CV curve, located at the 0.23 V peak 400, is increasing accordingly. The increase of CV current at the 0.9 V peak 402 is resulting from embedded

cortisol molecules, which serve as conductive bridge between MIP and SPCE. In this hypothesis, once most cavities of MIP have been occupied by template molecules, these bridge-like cortisol molecules could provide a shortcut for electronic transitions during reduction and oxidation processes. In consequence, the largest CV current of 131.6 μA is registered upon the addition of 700 ppb of cortisol with the probing buffer solution. Under optimized conditions, the detection limit of the MIP-based sensor according to example embodiments is achieved as low as 50 ppb ($\text{S/N}=3$), which is competitive with previously reported biosensors, including MoS_2 nanosheets integrated electrochemical sensor, and zinc oxide membrane-based electrochemical impedance spectroscopy biosensor. In addition, the calibration curve relative to the CV responses at the 0.23 V peak 400 at different cortisol concentrations is depicted in **Figure 4B** - a linear relationship between CV current and cortisol concentration, in the range of 50-700 ppb, has been fitted with a R^2 value of 0.9923.

Selectivity and other evaluations according to an example embodiment

In addition to SPCE-CND/CHT/MIP, CV responses of the SPCE-CND/CHT/NIP were also investigated. In **Figure 5**, the increase of CV current at the 0.23 V peak (Δ CV current), relative to CV current of SPCE-CND/CHT/MIP in the pure probing buffer, was summarized for step-wise comparison. For example, in the presence of 700 ppb of cortisol, Δ CV current observed from MIP-modified SPCE is 51.6 μA . In comparison, in the presence of 200 and 500 ppb of cortisol, Δ CV current obtained from NIP-modified SPCE is 11.2 and 15.2 μA , respectively. Although the concentration of cortisol was increased, CV current from SPCE-CND/CHT/NIP was kept relatively constant. These results indicated that free cortisol molecules played a negligible role during electronic transition processes. Meanwhile, the selectivity of the MIP-based biosensor was also studied by accessing CV responses of the SPCE-CND/CHT/MIP in the presence of 700 ppb of competitive molecules, including cortisone, corticosterone and prednisolone. In **Figure 5**, the competitors have registered much lower Δ CV current, as compared to that of cortisol. Among the three competitors, Δ CV current with 700 ppb of prednisolone was highest, at 18.8 μA . Therefore, these results demonstrated that MIP-based electrochemical biosensor for cortisol detection according to an example embodiment was successfully developed, with a promising selectivity against the other three competitors tested.

In an example embodiment, a point-of-care sensing system for cortisol detection integrating molecular imprinting technology and electrochemical detection has been provided. The sensing system according to an example embodiment comprises: molecular imprinting polymers prepared via bulk polymerization; and carbon nanodots/chitosan-assisted surface functionalization of surface-printed carbon electrode chips for portable and real-time measurements.

An advantage of the example embodiment is that the sensing system is simple, fast, and there is no strict requirement on complicated instruments or professional operations. The whole detection procedure could be completed within 5 min with a real-time screening of cortisol concentration. More importantly, the sample collection is non-invasive and can be performed

without skill/location/professionalness requirements. Prior to detection settings, the collected samples can be mixed with probing buffer, then CV measurements are performed for concentration quantitation.

To enhance the sensing performance, molecular imprinting technology empowered the binding affinity between polymeric matrix and template/cortisol molecules. In addition, carbon nanodots contributed to an increased surface area and reduced electronic resistance. Moreover, the selectivity was also ascertained by co-incubation with mixed solutions. Thanks to the excellent binding performance, the MIP-based electrochemical biosensor according to an example embodiment is capable to detect cortisol in the range of 50-700 ppb, and the limit of detection is as low as 50 ppb. These results indicate that the MIP-based electrochemical biosensor according to an example embodiment is potentially useful for real-time measurements of salivary cortisol, as well as to monitor customer wellness in the future.

Figure 6 shows a schematic drawing illustrating an electrochemical biosensor 600 according to an example embodiment. The electrochemical biosensor 600 comprises an electrode substrate 602 configured for electrical measurements of a functionalized area 604 of a surface 606 of the electrode substrate 602, wherein the functionalized area 604 has formed therein a mixture 608 of molecular imprinting polymer, MIP, particles and one or more immobilizer materials, the MIP particles having imprints of a molecule to be sensed by the biosensor.

The electrode substrate 602 may comprise a screen-printed carbon electrode, SPCE.

The mixture 608 may be provided as a thin film.

The one or more immobilizer materials may comprise chitosan, CHT.

The one or more immobilizer materials may comprise carbon nanodots, CND.

The mixture 608 may comprise one or more conductive materials. The conductive material may comprise one or more of a group consisting of carbon nanodots, CND, graphene, Gr, graphene oxide, GO, and reduced graphene oxide, rGO.

The electrode substrate may be configured for one or more of a group consisting of cyclic voltammetry, CV, measurements of the functionalized area 604, differential pulse voltammetry, DPV, measurements of the functionalized area, 604 and electrochemical impedance spectroscopy, EIS, measurements of the functionalized area 604.

The molecule to be sensed may comprise one or more of a group consisting of cortisol, prednisone, prednisolone, steroids, and biomarkers. The molecule to be sensed may be present in saliva.

Figure 7 shows a flowchart 700 illustrating a method of fabricating an electrochemical biosensor, according to an example embodiment. At step 702, an electrode substrate configured for electrical measurements of a functionalized area of a surface of the electrode substrate is provided. At step 704, a mixture of molecular imprinting polymer, MIP, particles, and one or

more immobilizer materials is provided in the functionalized area, the MIP particles having imprints of a molecule to be sensed by the biosensor.

The electrode substrate may comprise a screen-printed carbon electrode, SPCE.

The method may comprise forming the MIP particles by bulk polymerization.

- 5 The method may comprise providing the mixture as a thin film.

The one or more immobilizer materials may comprise chitosan, CHT.

The one or more immobilizer materials may comprise carbon nanodots, CND.

- 10 The mixture may comprise one or more conductive materials. The conductive material may comprise one or more of a group consisting of carbon nanodots, CND, graphene, Gr, graphene oxide, GO, and reduced graphene oxide, rGO.

The electrode substrate may be configured for one or more ore of a group consisting of cyclic voltammetry, CV, measurements of the functionalized area, differential pulse voltammetry, DPV, measurements of the functionalized area, and electrochemical impedance spectroscopy, EIS, measurements of the functionalized area.

- 15 The molecule to be sensed may comprise one or more of a group consisting of cortisol, prednisone, prednisolone, steroids, and biomarkers. The molecule to be sensed may be present in saliva.

In one embodiment, a method of electrochemical biosensing using the electrochemical biosensor of Figure 6 is provided.

20

A portable and simple biosensor for cortisol detection according to an example embodiment can have one or more of the following features and associated benefits/advantages:

Feature	Benefit/Advantage
Real-time measurement of cortisol concentration	Rapid and point-of-care detection of cortisol in human saliva for wellness monitoring
Molecular imprinting technology (MIT)-based sensing system	Integrating specificity, stability, reproducibility and low-cost
Electrochemical methods for signaling and detection	Rapid, stable and easy-to-use hardware/software
Portable and integrated sensing system with small size	A space-saving sensing device for personal and commercial usages in terms of cortisol/wellness monitoring

The above description of illustrated embodiments of the systems and methods is not intended to be exhaustive or to limit the systems and methods to the precise forms disclosed. While specific embodiments of, and examples for, the systems components and methods are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the systems, components and methods, as those skilled in the relevant art will recognize. The teachings of the systems and methods provided herein can be applied to other processing systems and methods, not only for the systems and methods described above.

For example, while the sensing device according to the example embodiment described herein is specifically applicable for cortisol detection, it is believed that in different embodiments, sensing devices for detection of other molecules can be provided using appropriate template molecules during the MIP fabrication process. For example, the molecule to be sensed may comprise cortisone, prednisone, prednisolone or other steroids or biomarkers present in saliva.

Also, in different embodiments the thin film may comprise different conductive materials such as Gr, GO, and/or rGO.

Also, it will be appreciated that the electrode substrate may be configured for different measurements of the functionalized area, such as DPV or EIS.

It will be appreciated by a person skilled in the art that numerous variations and/or modifications may be made to the present invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive. Also, the invention includes any combination of features described for different embodiments, including in the summary section, even if the feature or combination of features is not explicitly specified in the claims or the detailed description of the present embodiments.

In general, in the following claims, the terms used should not be construed to limit the systems and methods to the specific embodiments disclosed in the specification and the claims, but should be construed to include all processing systems that operate under the claims. Accordingly, the systems and methods are not limited by the disclosure, but instead the scope of the systems and methods is to be determined entirely by the claims.

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise," "comprising," and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in a sense of "including, but not limited to." Words using the singular or plural number also include the plural or singular number respectively. Additionally, the words "herein," "hereunder," "above," "below," and words of similar import refer to this application as a whole and not to any particular portions of this application. When the word "or" is used in reference to a list of two or more items, that word covers all of the following interpretations of the word: any of the items in the list, all of the items in the list and any combination of the items in the list.

CLAIMS

1. An electrochemical biosensor comprising:
 - an electrode substrate configured for electrical measurements of a functionalized area of a surface of the electrode substrate,
 - 5 wherein the functionalized area has formed therein a mixture of molecular imprinting polymer, MIP, particles and one or more immobilizer materials, the MIP particles having imprints of a molecule to be sensed by the biosensor.
2. The biosensor of claim 1, wherein the electrode substrate comprises a screen-printed carbon electrode, SPCE.
- 10 3. The biosensor of claims 1 or 2, wherein the mixture is provided as a thin film.
4. The biosensor of any one of claims to 3, wherein the one or more immobilizer materials comprise chitosan, CHT.
5. The biosensor of any one of claims to 4, wherein the one or more immobilizer materials comprise carbon nanodots, CND.
- 15 6. The biosensor of any one of claims 1 to 5, wherein the mixture comprises one or more conductive materials.
7. The biosensor of claim 6, wherein the conductive material comprises one or more of a group consisting of carbon nanodots, CND, graphene, Gr, graphene oxide, GO, and reduced graphene oxide, rGO.
- 20 8. The biosensor of any one of claims 1 to 7, wherein the electrode substrate is configured for one or more of a group consisting of cyclic voltammetry, CV, measurements of the functionalized area, differential pulse voltammetry, DPV, measurements of the functionalized area, and electrochemical impedance spectroscopy, EIS, measurements of the functionalized area.
- 25 9. The biosensor of any one of claims 1 to 8, wherein the molecule to be sensed comprises one or more of a group consisting of cortisol, prednisone, prednisolone, steroids, and biomarkers.
10. The biosensor of claim 9, wherein the molecule to be sensed is present in saliva.
11. A method of fabricating an electrochemical biosensor, comprising the steps of:
 - 30 providing an electrode substrate configured for electrical measurements of a functionalized area of a surface of the electrode substrate, and

providing a mixture of molecular imprinting polymer, MIP, particles and one or more immobilizer materials in the functionalized area, the MIP particles having imprints of a molecule to be sensed by the biosensor.

12. The method of claim 11, wherein the electrode substrate comprises a screen-printed carbon electrode, SPCE.

13. The method of claims 11 or 12, comprising forming the MIP particles by bulk polymerization.

14. The method of any one of claims 11 to 13, comprising providing the mixture as a thin film .

15. The method of any one of claims 11 to 14, wherein the one or more immobilizer materials comprise chitosan, CHT.

16. The method of any one of claims 11 to 15, wherein the one or more immobilizer materials comprise carbon nanodots, CND.

17. The method of any one of claims 11 to 16, comprising providing one or more conductive materials in the mixture.

18. The method of claim 17, wherein the conductive material comprises one or more of a group consisting of carbon nanodots, CND, graphene, Gr, graphene oxide, GO, and reduced graphene oxide, rGO.

19. The method of any one of claims 11 to 18, comprising configuring the electrode substrate for one or more ore of a group consisting of cyclic voltammetry, CV, measurements of the functionalized area, differential pulse voltammetry, DPV, measurements of the functionalized area, and electrochemical impedance spectroscopy, EIS, measurements of the functionalized area.

20. The method of any one of claims 11 to 19, wherein the molecule to be sensed comprises one or more of a group consisting of cortisol, prednisone, prednisolone, steroids, and biomarkers.

21. The method of claim 20, wherein the molecule to be sensed is present in saliva.

22. A method of electrochemical biosensing using the electrochemical biosensor of any one of claims 1 to 10.

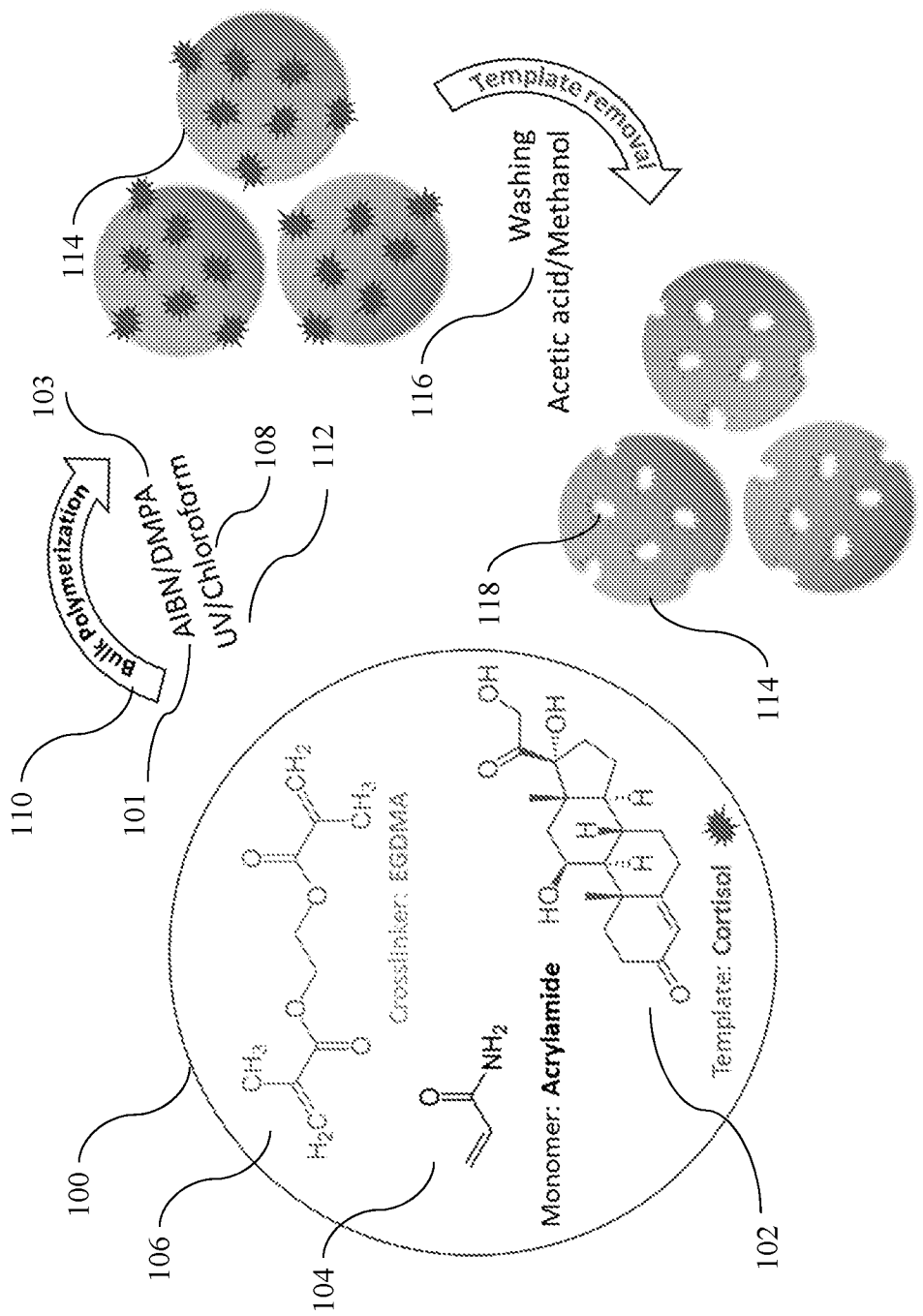


Figure 1

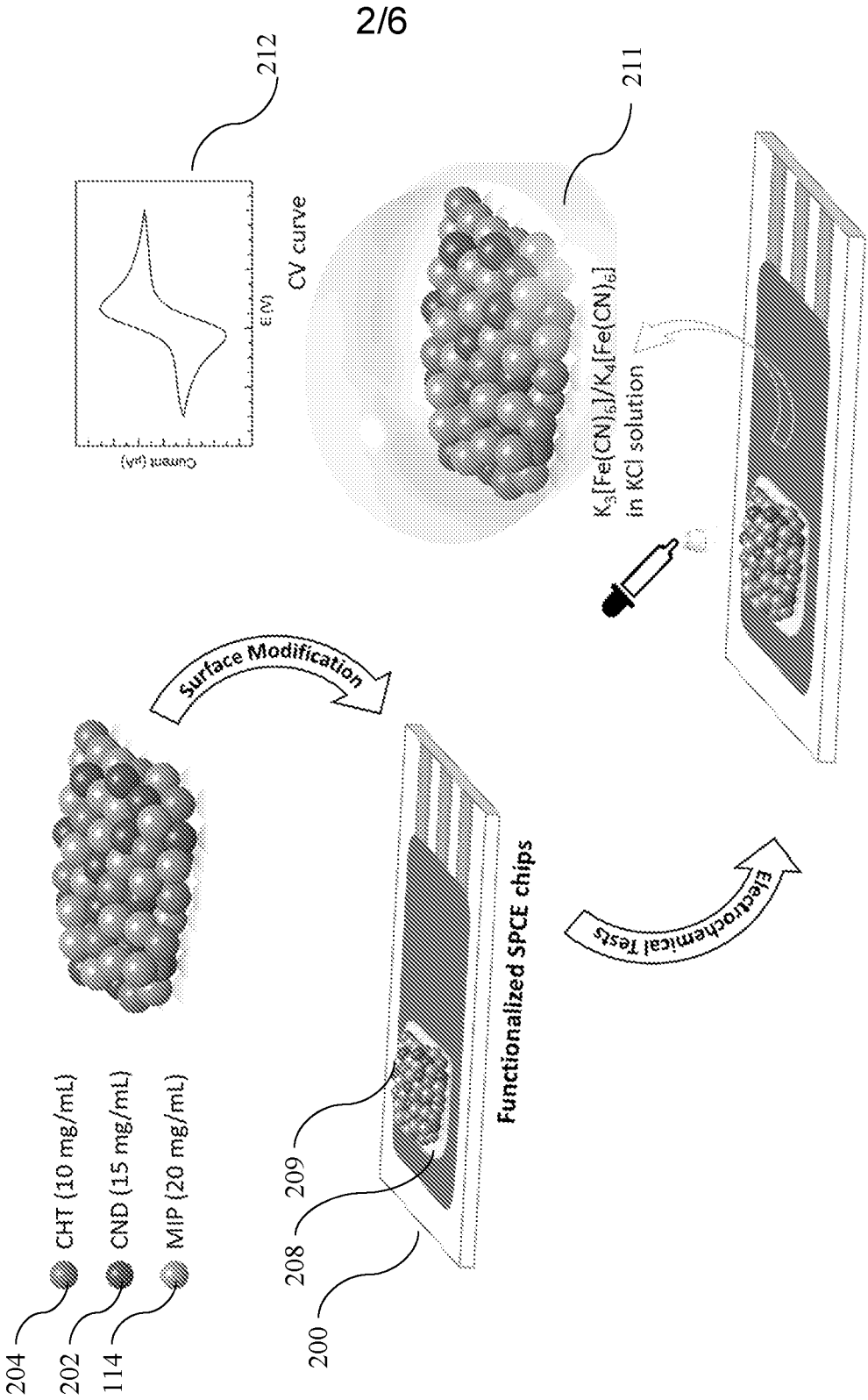


Figure 2

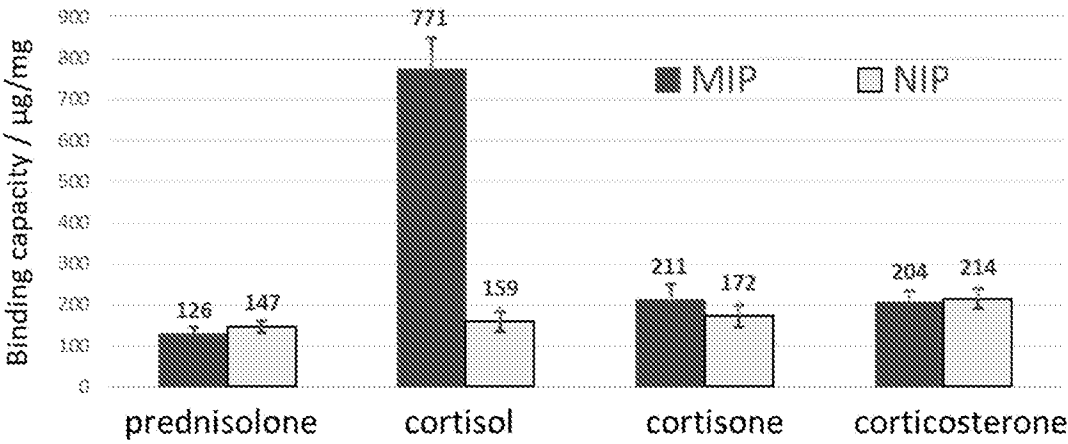


Figure 3

4/6

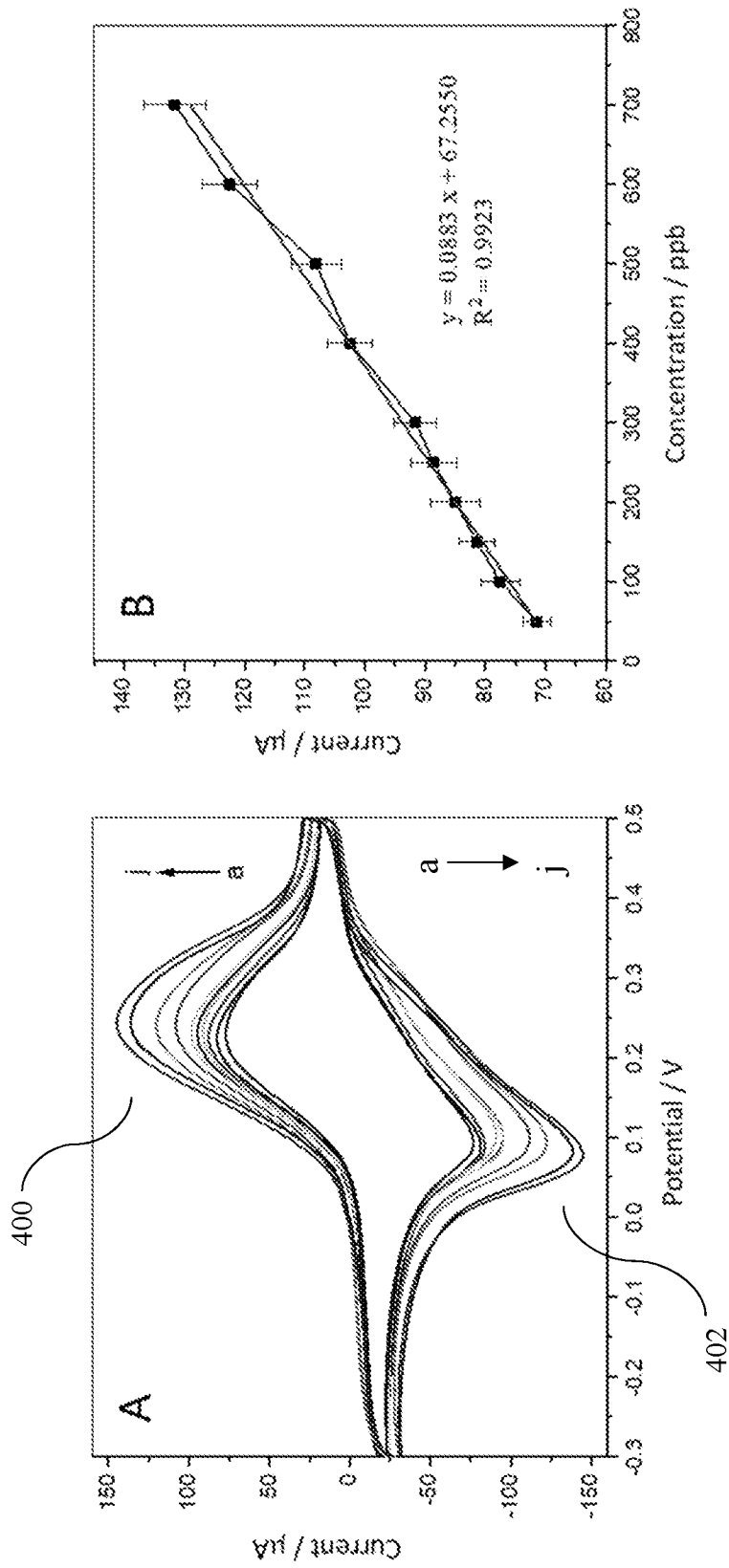


Figure 4

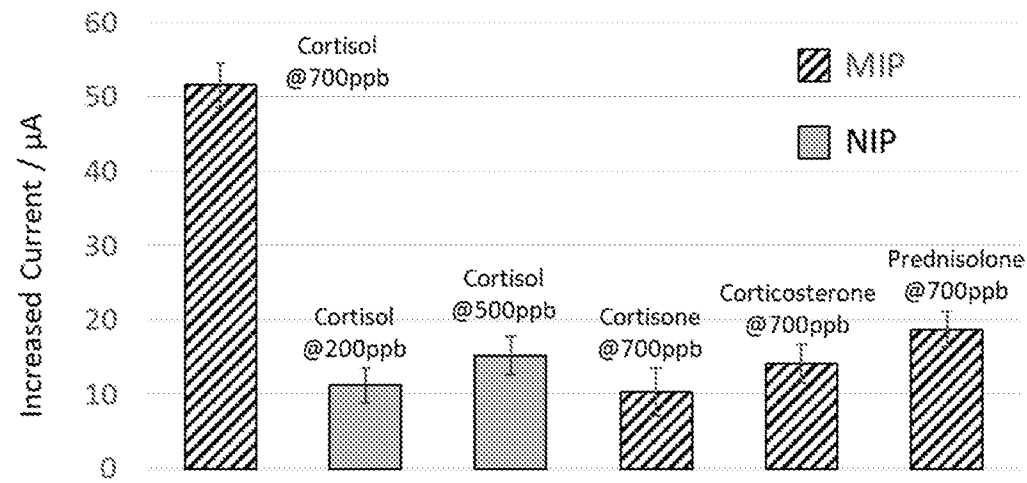


Figure 5

6/6

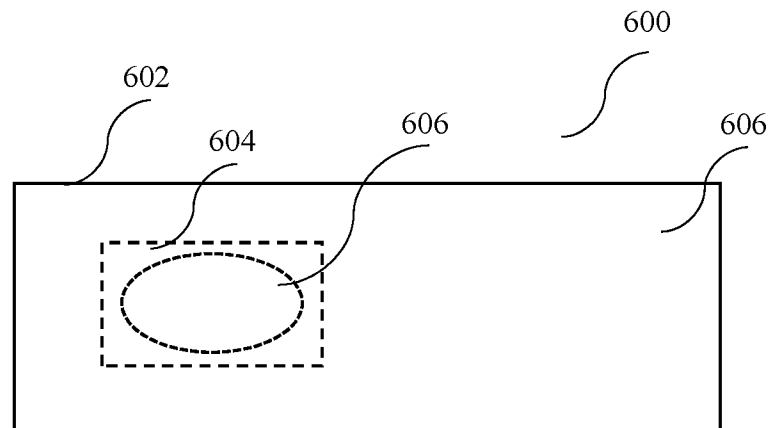


Figure 6

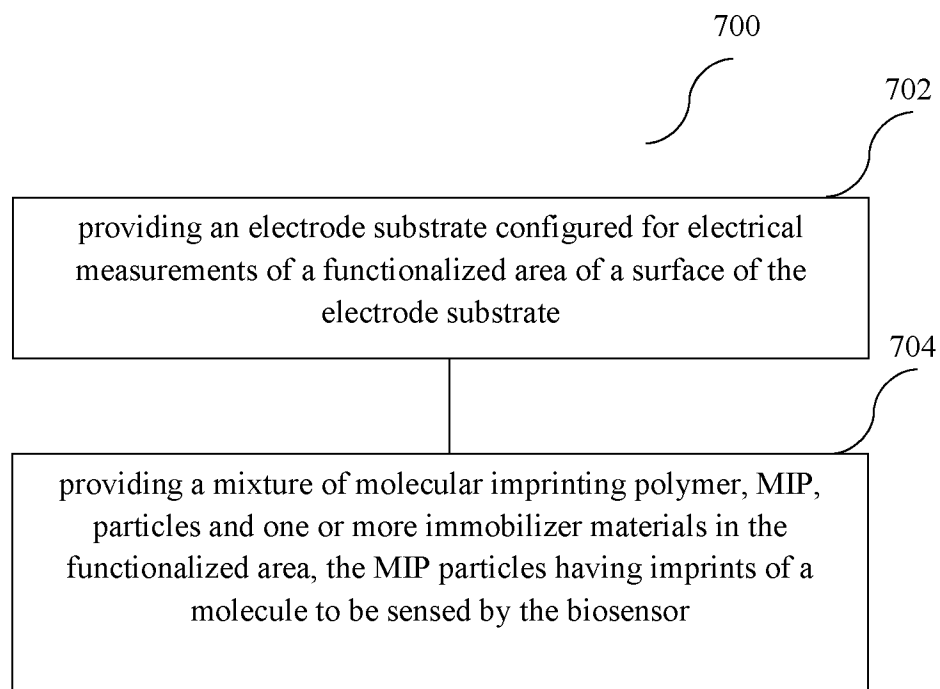


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2021/050134

A. CLASSIFICATION OF SUBJECT MATTER

G01N 27/30 (2006.01) G01N 33/74 (2006.01)

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: CAPLUS, and EPODOC

Search terms: biosensor, electrode, molecular imprinting polymer particles, carbon nanodots, chitosan, biomarker, steroid, cortisol, and other related terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 105675683 A (UNIV JIANGSU) 15 June 2016 paras [0006]-[0011] and examples 1-3 of the original non-English language document (a machine translation is enclosed only for your reference)	1-22
X	KLANGPHUKHIEW, S. ET AL., Cortisol Stress Biosensor Based on Molecular Imprinted Polymer. <i>Proceedings of Eurosensors</i> , 16 August 2017, Vol. 1, No. 4, pages 538 (1-4) [Retrieved on 2021-06-09] <DOI: 10.3390/PROCEEDINGS1040538> abstract and Figure 1	1-22
X	US 2016/0091505 A1 (TAKEUCHI, T. ET AL.) 31 March 2016 Paras [0058] and [0086]	1-22

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

09/06/2021

(day/month/year)

Date of mailing of the international search report

11/06/2021

(day/month/year)

Name and mailing address of the ISA/SG



Intellectual Property Office of Singapore
1 Paya Lebar Link, #11-03
PLQ 1, Paya Lebar Quarter
Singapore 408533

Email: pct@ippos.gov.sg

Authorized officer

Wang Lishan (Dr)

IPOS Customer Service Tel. No.: (+65) 6339 8616

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2021/050134

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 104181217 A (UNIV NANCHANG) 3 December 2014 para [0006]; examples and Figures 1-5 of the original non-English language document (a machine translation is enclosed only for your reference)	1-22
A	GUO, W. ET AL., A novel molecularly imprinted electrochemical sensor modified with carbon dots, chitosan, gold nanoparticles for the determination of patulin. <i>Biosensors and Bioelectronics</i> , 5 July 2017, Vol. 98, pages 299-304 [Retrieved on 2021-06-09] <DOI: 10.1016/J.BIOS.2017.06.036> abstract	-
A	DAI, H. ET AL., Electrochemical detection of triclosan at a glassy carbon electrode modifies with carbon nanodots and chitosan. <i>Electrochimica Acta</i> , 20 July 2012, Vol. 80, pages 362-367 [Retrieved on 2021-06-09] <DOI: 10.1016/J.ELECTACTA.2012.07.032> abstract	-
A	CN 110564807 A (UNIV NANJING TECH) 13 December 2019 see examples 1-3 of the original non-English language document (a machine translation is enclosed only for your reference)	-
A	UYGUN, H. D. E. ET AL., Non-invasive cortisol detection in saliva by using molecularly cortisol imprinted fullerene-acrylamide modified screen printed electrodes. <i>Talanta</i> , 8 August 2019, Vol. 206, pages 120225 (1-5) [Retrieved on 2021-06-09] <DOI: 10.1016/J.TALANTA.2019.120225> abstract and graphical abstract	-

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2021/050134

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 105675683 A	15/06/2016	NONE	
US 2016/0091505 A1	31/03/2016	DE 112014001792 T5 WO 2014/181662 A1 JP 2014219353 A	14/01/2016 13/11/2014 20/11/2014
CN 104181217 A	03/12/2014	NONE	
CN 110564807 A	13/12/2019	NONE	