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PII: S0927-7765(18)30443-0
DOI: https://doi.org/10.1016/j.colsurfb.2018.07.006
Reference: COLSUB 9462

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 22-3-2018
Revised date: 13-6-2018
Accepted date: 3-7-2018

Please cite this article as: Brazaca LC, Bramorski CB, Cancino-Bernardi J, da Silveira Cruz-Machado S, Markus RP, Janegitz BC, Zucolotto V, An antibody-based platform for melatonin quantification, *Colloids and Surfaces B: Biointerfaces* (2018), https://doi.org/10.1016/j.colsurfb.2018.07.006

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Title: An antibody-based platform for melatonin quantification

Running Title: Immunosensor for melatonin detection

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Statistical Summary:

Number of words on the manuscript: 3.364

Number of figures: 6

Number of tables: 2

Supplementary Material: Yes

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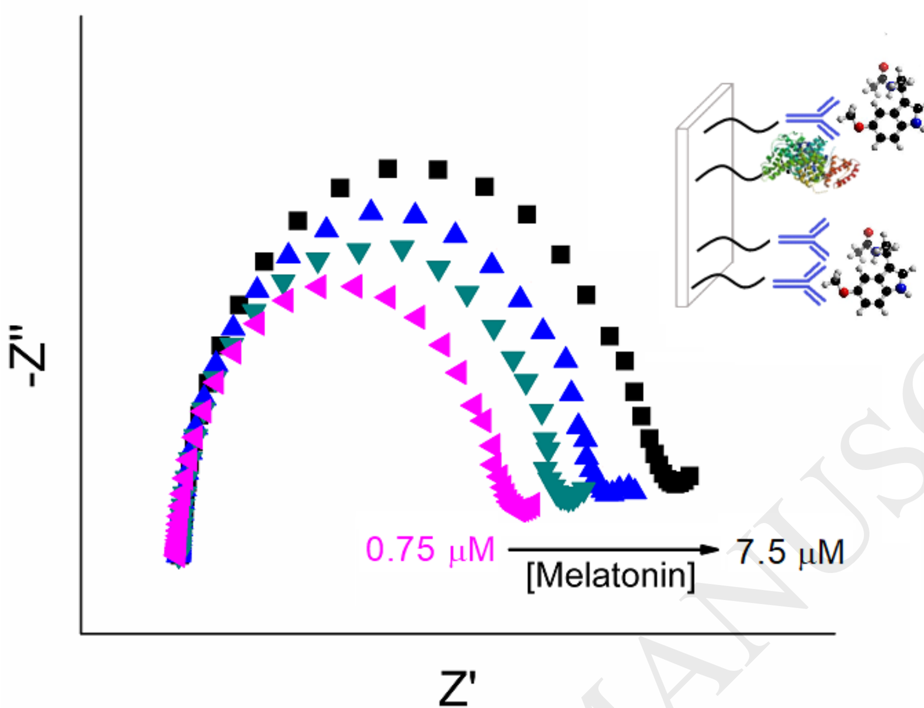
Graphical abstract



Highlights

- Novel, simple and low-cost electrochemical biosensor for melatonin quantification.
- From the best of our knowledge, this is the first immunosensor for the biomolecule detection;
- Melatonin is a biomarker for diverse diseases including some types of cancer.
- The device presented great stability and low interference with similar molecules.
- Results presented similar precision as ELISA when applied in real mouse samples.

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Abstract:

Melatonin, the 'chemical signal of darkness', is responsible to regulate biological rhythms and different physiological processes. It is mainly produced by the pineal gland as a hormone in a rhythmic daily basis, but it may also be synthesized by other tissues, such as immune cells, under inflammatory conditions. Its abnormal circulating levels have been related to several diseases such as type 2 diabetes, Alzheimer's disease and some types of cancer. Currently, melatonin is exclusively quantified by ELISA or radioimmunoassays, which although are very sensitive techniques and present low detection limits, usually require specialized personal and equipment, restricting the tests to a limited number of patients. To overcome such limitations, we developed a novel easy-to-use electrochemical immunosensor for rapid melatonin quantification. Anti-melatonin antibodies were immobilized into Indium tin oxide (ITO) platforms using (3-Aminopropyl)triethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) crosslinkers. The platforms were assayed with synthetic and biologically-present melatonin containing samples. The developed device displayed a linear response in the concentration range from 0.75 to 7.5 μmol/L and a limit of detection of 0.175 μmol/L using Electrochemical Impedance Spectroscopy (EIS) (R^2=0.989) and 0.513 μmol/L using Cyclic Voltammetry (CV) (R^2=0.953) for synthetic melatonin. Furthermore, the sensors exhibited a good stability and reproducibility (3.45% and 2.87% for EIS and CV, respectively, n=3), maintaining adequate response even after 30 days of assembly. On biologically-present melatonin-containing samples the device displayed a similar performance when compared to ELISA technique (deviation of 13.31%). We expect that the developed device contributes significantly to the medical

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area allowing precise and complete diagnosis of the diseases related to abnormal levels of melatonin.

Keywords: *Melatonin; Electrochemical Biosensor; Electrochemical Impedance Spectroscopy; Antibody; Hormone;*

Introduction

Melatonin (N-acetyl-5-methoxy tryptamine) is an indoleamine present in most biological interfaces. In several species, melatonin plays important roles as a hormone and it is believed that its biological signaling is one of the oldest existing in nature.[1] In humans, melatonin biosynthesis is rhythmically active in the pineal gland. Some other tissues, which includes the retina and gastrointestinal tract, may also synthesize melatonin in a rhythmic manner.[2] Melatonin may also be synthesized on demand by macrophages, lymphocytes and microglia when activated by pathogens or abiotic injuries.[2-4]. Under normal conditions its maximum concentration in human plasma is about 50-200 pg/mL (0.22-0.86 nmol/L) during the night and lower than 2.0 pg/mL (8.6 pmol/L) at daylight,[5-7] which is in accordance with one of its major functions: translating environmental darkness to adjust endogenous physiology. Excretion of melatonin in the urine is also a pivotal marker of melatonin bioavailability. It is noteworthy to mention that melatonin can also be found in human saliva at concentrations ten times lower than in plasma, which highlights the possibility for the development of non-invasive and sensitive methods for detection of the indoleamine in different biological fluids.

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The idea that melatonin is only linked to the circadian rhythms or seasonal behavior was long challenged by the scientific community. In the last decades, intense efforts have been spent in understanding the influence of this hormone in other actions and physiological systems[3,8-10]. For example, studies have shown that melatonin presents a very important role on the organization of the immune response[11]. In this case, the hormone derived from the pineal gland is ceased but is locally produced by defense cells and acts locally, as regulator of the immune response. Also, under inflammatory frames, nocturnal pineal melatonin suppression favors the mounting of inflammation by allowing the rolling and adherence of leukocytes as well as its vascular permeability.[11]

Melatonin has also been pointed as an important biomarker for a wide variety of diseases. Daily melatonin profile was validated as an interesting noninvasive tool to diagnose tumors of the pineal region and its external administration as a possible approach to prevent the post-pinealectomy syndrome[12]. Moreover, diseases as sleep disorders[13], depression[14,15], hypertension[16], Alzheimer's disease[17,18], type 2 diabetes[19] and certain types of cancer, specially breast cancer[20], are related to abnormal levels of melatonin. Furthermore, the exogenous administration of the indoleamine has been studied as a tool for regulating the circadian rhythm in elderly individuals, as well as in patients suffering from different diseases[17,21-23]. It has been shown that melatonin production depends on age, gender and varies significantly between individuals[24,25], which highlights the need for a simple and low-cost methodology that allows the monitoring of the biomolecule, as subjective dose administration is not recommended. In this scenario, quantification of melatonin brings great potential to determine the optimal exogenous dosage and facilitate diagnosis and treatment of the above cited diseases.

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Currently, the most widely used methods for the quantification of melatonin in biological fluids are Enzyme-Linked Immunosorbent Assay (ELISA) and radioimmunoassays (RIA).[26] Although these methods are capable of detecting extremely low levels of the hormone in biological samples (detection limit in the order of fmol-to-pmol/mL)[27], they present disadvantages such as high cost, need for sample processing, in addition to the need of specialized personnel, materials and equipment. Furthermore, in the case of RIA, the personnel also must deal with radioactive material, posing threats to themselves as well as for the environment. Thus, the development of faster, simpler and low-cost tools that require low or no sample preparation are of great interest to the medical area[28,29].

We developed an electrochemical immunosensor for the simple and low-cost melatonin quantification based on electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements. Specific melatonin antibodies (Ab) were immobilized on indium tin oxide (ITO) platforms modified using (3-Aminopropyl)triethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) crosslinkers. To maximize the electrochemical signal, an optimal electrode modification protocol was determined using synthetic samples of melatonin. The developed sensor was then tested in liver extracts from rats added with synthetic melatonin to mimic real samples and our results were compared to ELISA technique. We show for the first time the use of anti-melatonin specific antibodies onto the electrode surface as an effective, simple and versatile alternative to melatonin quantification.

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Materials and Methods

Materials

Potassium hexaferrocyanide (II) ($K_4[Fe(CN)_6]$), (3-Aminopropyl)triethoxysilane (APTES), Bovine Serum Albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Brazil). Anti-melatonin specific antibodies were acquired from Thermo Pierce (Brazil) and melatonin was acquired from Sigma Aldrich (Brazil). Indium tin oxide (ITO) electrodes were purchased from Delta Technologies (USA) (surface resistivity = 12 Ω), and used as working electrodes. Phosphate buffer (PB) solution was prepared using monosodium and disodium phosphate (0.1 mol/L, pH 7.2) (Sigma Aldrich-Brazil). Phosphate buffered saline (PBS) solution was prepared using potassium chloride, sodium chloride, sodium phosphate dibasic and potassium phosphate monobasic (1 x, pH 7.4) (Sigma Aldrich-Brazil). All chemicals used were of analytical grade. Ultrapure water was obtained from Mega UP water purification system (Mega Purity) with resistivity of 18.2 MΩ/cm.

Biosensor preparation

ITO electrodes were cleaned with acetone under sonication for 10 minutes. The process was repeated using isopropyl alcohol, ethanol and ultrapure water. The substrate was immersed for 15 minutes in 5 H_2O:1 H_2O_2:1 NH_4OH (v/v/v) at 80ºC. Finally ITO electrodes were abundantly rinsed using ultrapure water and dried using N_2.

Anti-melatonin antibodies were covalently bounded to ITO substrates (Figure 1). Initially, the ITO electrodes were immersed in an APTES solution (2% v/v, ethanol) for 1h. Following, the electrodes were rinsed with PBS solution (pH 7.4) and immersed in an

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EDC-NHS/Ab solution (8 mmol/L, 5 mmol/L and 4 ng/µL respectively, PBS 1x pH 7.4) for 1h. It is critical for the procedure to prepare the EDC-NHS/Ab 2 hours before immersing the electrode on it in order for the crosslinkers to interact with the Ab functional groups. This will cross-link Ab molecules, and their immobilization will be performed in clusters. The electrodes were rinsed using PBS and immersed in a BSA solution (1%, PBS, pH 7.4) for 30 minutes for blocking functional groups and avoiding unspecific interactions. Following, the electrodes were rinsed using PB, dried with N_2 and frozen in PBS (-10°C). The devices can be frozen up to 30 days with no loss in activity. For hormone detection, the biosensor was immersed in melatonin solutions for 1h 30 (PBS, pH 7.4). All experiments were performed at room temperature (25°C).

Electrochemical measurements

Electrochemical measurements were performed in a potentiostat/galvanostat PGSTAT 12 (Autolab), in a conventional three-electrode cell. The Ab/EDC-NHS/APTES/ITO platforms were used as working electrodes (0.5 cm^2 of working area), while a platinum plate (1 cm^2) and Ag/AgCl electrode (saturated with 3 mol/L KCl) were used as counter and reference electrodes, respectively. The measurements were performed in 10 mL of 4 mmol/L $K_4[Fe(CN)_6]$ solution in PB at room temperature (25°C). CV measurements were performed in the range from -0.2 to 0.8 V (2 cycles, 100 mV/s). EIS measurements were performed in an open cell potential. All measurements were recorded three times for each electrode, at room temperature (25°C). The software FRA (Autolab) was used to fit the expression of the equivalent circuit over experimental points, returning the values of charge transfer resistance (R_{ct}). The calibration curve was obtained by measuring the values of R_{ct} in a melatonin concentration range of 0.75 to 7.5

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μmol/L. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the following relations:

$$LOD = \frac{3\ x\ SD_{buffer}}{s} \qquad LOQ = \frac{10\ x\ SD_{buffer}}{s}$$

being SD_{buffer} the standard deviation for 3 buffer measurements and s the slope of the calibration curve.

Reproducibility tests were performed with three parallel assemblies equally constructed and their response to CV and EIS were evaluated after the interaction with 2.5 μmol/L of melatonin. In repeatability tests, biosensor responses to CV and EIS were investigated upon successive measurements, also after the interaction with 2.5 μmol/L of melatonin.

Real Samples

Adult male Wistar rats (10-weeks-old, 300–320 g) were obtained from the animal facility of the Department of Physiology (IB-USP, São Paulo, Brazil), and kept under a 12/12 h light/dark cycle (LD, lights on at 07:00; Zeitgeber time 0 or ZT0). Animals received water and food *ad libitum*, and were killed by decapitation at ZT18. Procedures were approved by IB-USP Ethical Committee (CEUA license number 198/2014) and performed according to Brazilian law for scientific use of animals (Federal Law number 11.794/2008).

After the euthanasia, the liver was removed, frozen in dry ice and pulverized using liquid nitrogen and a mortar and pestle. The resulting material was diluted in methanol (100%, Sigma Aldrich - Brazil) in the concentration of 500 mg/mL. The samples were vortexed for 1 minute and then centrifuged at 4,500 × g for 5 minutes, 4°C. The methanol

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was then evaporated by using a SpeedVac for approximately 1 hour, at 45ºC. The dried material was suspended in ultrapure water with synthetic melatonin. All the prepared concentrations were diluted from a 100 μmol/L stock solution.

An ELISA kit acquired from IBL International (Germany) was used for the quantification of melatonin in the real samples. The test was performed according to the manufacturer`s instructions. All samples were diluted 10.000x to fit in the analytical range of the kit.

Results and Discussion

Electrode Modification

EIS and CV measurements were performed after each modification step during electrode assembly. EIS results are shown in Figure 2. ITO substrates displayed a R_{ct} of 535 ± 40 Ω. After modifying the surface with APTES R_{ct} dropped to 106 ± 12 Ω, which may be due to the protonation of the molecules on the electrode surface. In this case, APTES presents a positive charge, attracting the electrochemical mediator ($K_4[Fe(CN)_6]$, negatively charged) to the electrode surface. After EDC-NHS/Ab modification layer is added to the film, the R_{ct} increased to 488 ± 10 Ω. Most antibodies are charged positively[30] and, therefore, attract the electrochemical mediator to the electrode – but these also present great molecular volume, reducing electron transfer. Consequently, an increase in R_{ct} is observed after the immobilization of the EDC-NHS/Ab layer. After blocking the surface with BSA, the R_{ct} increased to 678 ± 47. Finally, after the incubation of the Ab/EDC-NHS/APTES/ITO immunosensor in 1 μmol/L melatonin solution - which presents neutral charge under biological conditions[31] - it was observed a huge increase in the R_{ct}

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to 4050 ± 333 Ω, revealing the great affinity between antigen and antibody molecules, and showing that the assembled sensor is adequate for melatonin detection.

The assessment of film growth using CV has led to analogous results (Figure 3). In this case, an increase in R_{ct} leads to the decrease in the current of $K_4[Fe(CN)_6]$ oxidation peaks and a shift to more positive potentials. For example, ITO electrode showed a peak in 0.34 ± 0.01 V with an anodic current of 0.204 ± 0.009 mA. After the APTES modification step, the peak shifted to 0.319 ± 0.008 V, with an increase of current to 0.312 ± 0.009 mA. The modification step with EDC/NHS/Ab revealed a peak in 0.373 ± 0.008 V, with a decrease in the anodic current to 0.218 ± 0.001 mA. After blocking the sensors surface with BSA, the peak shifted to 0.41 ± 0.02 with a current decrease to 0.185 ± 0.009 mA. Upon incubation of the Ab/EDC-NHS/APTES/Ab immunosensor on a 1 μmol/L melatonin solution, it was observed a shift to 0.538 ± 0.008 V to the anodic peak of the molecular probe and a decrease in the current to 0.112 ± 0.007 mA. Therefore, both techniques corroborate with their results and indicate an efficient electrode modification to the immunosensor construction and melatonin detection.

Biosensor Assembly Optimization

After observing that the Ab/EDC-NHS/APTES/ITO immunosensor can efficiently detect melatonin, the immunosensor assembly was optimized to obtain an increased sensibility to melatonin detection. For this end, the concentration and time of immersion of the electrodes into each component solution were carefully studied.

First, ITO electrodes were placed in contact with APTES (2% v/v) for 30, 60, 90, 120 and 150 minutes, having R_{ct} measured right after these periods in pursuit of the

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lowest value. A mean value of 12 kΩ was obtained for electrodes immersed for 30 minutes in this solution. After 60 minutes of immersion, a decrease of about 9 kΩ occurred (R_{ct} = 3 kΩ), if compared to the 30 minutes measurement. For periods longer than 60 minutes, charge transfer resistance remained constant (R_{ct} = 3 kΩ). Therefore, 60 minutes was the chosen time for subsequent assemblies. APTES concentration was also optimized. For this purpose, the ITO electrodes were immersed for 60 minutes in 0.25, 0.5, 1.0, 2.0 and 4.0% (v/v) APTES solutions. The electrodes response was assessed using the $K_4[Fe(CN)_6]$ redox probe after the modification with EDC/NHS/Ab and the contact with 6 x 10^{-8} mol/L melatonin. The study showed a maximum R_{ct} signal using a 2% solution, which was the concentration selected for further studies.

In sequence, the ideal time for the interaction of EDC/NHS and anti-melatonin Ab was assessed. Those molecules were mixed for 30, 60, 90, 120 and 150 minutes before the electrode functionalization and their R_{ct} were recorded after the interaction of the fully assembled immunosensors with 6 x 10^{-8} mol/L melatonin. Greater values of R_{ct} indicates higher sensitivity of the sensor for melatonin detection. A maximum R_{ct} signal was obtained after 120 minutes of contact between the solutions. Following, the anti-melatonin Ab concentration was varied from 1 to 5 μmol/L, maintaining the EDC/NHS concentration constant and in excess. R_{ct} was assessed using EIS after the immunosensor interaction with 6 x 10^{-8} mol/L melatonin (Figure SD-1). It was observed that a maximum R_{ct} value was obtained upon the use of 4 μmol/L of anti-melatonin antibodies. Possibly, higher concentrations of antibodies lead to the formation of a more packed film structure, which difficult the adequate interaction with the hormone.

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The immobilization time of EDC/NHS/Ab on the ITO modified platforms was then tested. They were put in contact for 30, 60, 120 and 150 minutes. A difference of approximately 2.5 kΩ was observed between 30 and 60 minutes measurements. After 60 minutes of immersion time, R_{ct} values were constant. Therefore, 60 minutes was the chosen period for the following studies.

Finally, the ideal time for the interaction of the biosensor with melatonin was studied. The biosensor was immersed in a melatonin solution (6×10^{-8} mol/L) for 30, 60, 90, 120 and 150 minutes (Figure SD-2). Initially, an increase in R_{ct} was observed, which reached a steady state after 90 minutes – which was the time chosen to give continuity to our studies.

Melatonin Detection

The Ab/EDC-NHS/APTES/ITO immunosensor response was then tested in solutions containing from 0.75 to 7.5 μmol/L of melatonin using both EIS and CV. For EIS technique, the biosensor presented an increase in R_{ct} linearly proportional to melatonin concentration (R^2=0.989) (Figure 4), as increase of concentration of hormone leads to the deposition of more neutral material on the electrodes surface, making it more difficult for electrons to reach it. Linear regression showed that the electrodes response follows the equation $R_{ct} = (3542 \pm 197) + (245 \pm 45)$ [melatonin] (μmol/L), with a sensitivity of 4.91×10^2 ohm L/μmol cm². The limit of detection was 0.175 μmol/L and the limit of quantification 0.583 μmol/L, with a standard deviation of 136.47 Ω for 5 parallel blank measurements.

Using CV, a calibration curve with a corresponding behavior was obtained (Figure 5). The amplitude of $K_4[Fe(CN)_6]$ oxidation peak decreased proportionally (R^2=0.953) with

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the melatonin concentration, following the equation $i_{peak} = (1.15 \pm 0.03) \times 10^{-4} - (3.6 \pm 0.7) \times 10^{-6}$ [melatonin] (μmol/L). The calculated sensitivity was -7.23 × 10^{-6} A.L/μmol.cm² with a limit of detection of 0.154 μmol/L and limit of quantification 0.513 μmol/L, with a standard deviation of 0.626 μA for 5 parallel blank measurements.

The electrochemical characterization of the Ab/EDC-NHS/APTES/ITO modified electrodes showed that they are very stable under successive CV cycles (Figure SD-3), presenting a variation of only 6.71% in $K_4[Fe(CN)_6]$ oxidation peak in 100 cycles (melatonin 2.5 μmol/L, PB). To verify the applicability of the constructed devices in clinical analyses, the stability of the biosensor over the time was also assessed. The devices were frozen in -10°C for one month. Even after this period, the Ab/EDC-NHS/APTES/ITO biosensor still presented adequate responses to 2.5 μmol.L^{-1} of melatonin (Figure SD-4).

In reproducibility tests EIS technique presented a difference of 3.45% in R_{ct} in 3 different assemblies, while CV technique displayed a difference of 2.87%.

Regarding the repeatability tests, it was observed a variation of 1.81% in the oxidation peak of after 18 measurements using CV technique, and a variation of 23.6% after 12 measurements for EIS technique (Figure SD-5). Despite the interesting results obtained for the biosensor repeatability, it is important to notice that the developed devices are intended for medical diagnosis and should be disposed after use.

Considering that the anti-melatonin antibody may present cross-reactivity with some of its intermediary metabolites, we have also accessed the response of the developed sensor after the interaction with two of these biomolecules: N-Acetylserotonin (NAS) and 5-Hydroxytryptamine creatinine sulfate complex (C5-HT). EIS measurements

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showed that NAS and C5-HT presented low interaction levels with the anti-melatonin antibody, and its signals were 85.43 and 78.36% lower than the ones showed by melatonin in the same concentration (2.5 μmol/L), respectively (Figure SD-6). Furthermore, NAS is usually found in concentrations 10 times lower than melatonin in blood, and, therefore, is not considered a potential interferent.[32] No representative data was found for the concentration of C5-HT in plasma.

Real Samples

To test the applicability of our Ab/EDC-NHS/APTES/ITO sensor, liver extract samples were tested. As the concentration of the hormone obtained in these tissues stands below the LD of this sensor, spiked samples were prepared by adding synthetic melatonin to the real samples in the concentrations of 0, 0.8, 1.5, 3.0, 5.0 and 7.0 μmol/L. From EIS results, it was possible to obtain a new analytical curve that presented the same overall behavior as in ideal conditions (Figure 6). The curve presented a coefficient of linearity (R^2) of 0.995 in the 0.0 to 7.0 μmol/L melatonin range, with a sensitivity of 1.34×10^3 Ω.L/μmol.cm². Linear regression generated the equation $R_{ct} = (852 \pm 97) + (673 \pm 80)$ [melatonin] (μmol/L). The calculated LOD and LOQ were 0.222 and 0.741 μmol/L, respectively and the sensors presented good reproducibility (4.69% for 3 parallel assemblies, 1.5 μmol/L melatonin). The analytical curve was then used to determine the concentration of melatonin in 12 liver extracts samples (Table 1). The mean deviation for the measurements was 13.31%, presenting good agreement with the initially expected values.

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In order to compare the sensor developed here with the standard methods current existent, the concentration of melatonin on real samples was also assessed using ELISA technique. The obtained results are in agreement with the expected values and a summary of the measurements can be found on Table 2. The mean deviation for 6 samples was 16.27% which is slightly higher than what is presented by our sensor.

A price estimative was carried out to compare the value expended to perform a single test using our device as well as using the ELISA technique. For that, the price of each product used for building the sensor was assessed individually. The results can be seen in Table SD-1. The total test price (US$ 1.66) is about 5x cheaper than ELISA technique (US$ 8.28 per test, US$ 795.00 for 96 tests[34]). This can be achieved mainly due to the direct format of the test – which does not require additional biological reagents to perform the measurements.

The developed biosensors also present other advantages if compared to the currently used method. First, the total time for performing the tests is much shorter. While ELISA tests take around 6h to be performed [35,36], our sensor generates the result in 1h40. Furthermore, the hands-on time is also significantly shorter. Our sensor requires the user to act during 5 min only, while ELISA requires around 20-30 min due to the diverse steps involved in the assay. Another disadvantage of ELISA technique, if compared to our sensor, is the requirement of several reagents. While our sensor requires only the measuring solution to perform the assay – which is composed by a stable chemical compound - ELISA requires secondary antibodies labeled with enzymes and their substrate. These are biological reagents, which are prone to degradation and instabilities. Therefore, although our sensor acts in a higher concentration range (developed sensor:

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0.75 to 7.5 µM; ELISA: 0.6 fmol/L - 4.3 pmol/L[37]), it presents advantages, including the test price, time requirement and stability of reagents. Furthermore, the developed sensor can be used with biomolecules concentration techniques, allowing biological samples to be read directly on the device.

Therefore, the developed sensor presented a similar performance when compared to ELISA, having the advantages of requiring less sample preparation, as well as being simpler and cheaper than the existing methods.

Conclusions

We have developed a low cost, simple and efficient electrochemical immunosensor for melatonin quantification. The device presented a linear response to melatonin concentrations between 0.75 and 7.5 µmol/L using both EIS (R^2=0.989) and CV (R^2=0.953) techniques. Furthermore, it presented great stability, reproducibility and displayed similar results to the ones obtained using other traditional methods for detection of melatonin in biological fluids and tissues, such as ELISA, with the advantages of being simpler and more rapid than the current techniques. We expect that the developed biosensors may lead to more accessible melatonin quantification tests. Moreover, it is an interesting device that could be used in point-of-care diagnosis in the future.

Declarations of interest: none

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Acknowledgements

The authors are grateful to São Paulo Research Foundation - FAPESP (Grant number: 2015/02623-0, 2013/13691-1, 2015/04557-5 and 2015/19099-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Grant number: 140625/2015-1) for financial support. We also would like to thank the researchers from Nanomedicine and Nanotoxicology Group and from Laboratory of Chronopharmacology for their cooperation in our studies.

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Figures

Figure 1. Schematic configuration of Au-APTES-EDC/NHS-Ab biosensor. Upper part: Method of film growth; Lower part: Chemical bonds established on the thin film. BSA protein structure was acquired from the data deposited on Protein Data Base by Majorek et al.[38]

Figure 2. Nyquist plots displaying each stage of the electrode's functionalization. ITO (blue), APTES-ITO (red), Ab/EDC-NHS/APTES/ITO (black) responses are shown, as well as the impedance after the interaction of the Ab/EDC-NHS/APTES/ITO sensor with 1 μmol/L melatonin (green). Support electrolyte: PB; Redox probe: 4 mmol/L $K_4[Fe(CN)_6]$.

Figure 3. Cyclic voltammograms displaying each stage of the electrode's functionalization. ITO (blue), APTES-ITO (red), Ab/EDC-NHS/APTES/ITO (black) responses are shown as well as the voltammogram after the interaction of the Ab/EDC-NHS/APTES/ITO sensor with 1 μmol/L melatonin (green). Support electrolyte: PB; Redox probe: 4 mmol/L $K_4[Fe(CN)_6]$.

Figure 4. Analytical curve for melatonin concentrations in the range of 0.75 to 7.5 μmol/L using EIS technique. Left: Nyquist plots. Right: Linear regression showing the increase in R_{ct} with hormone concentration raise. Support electrolyte: PB; Redox probe: 4 mmol/L $K_4[Fe(CN)_6]$.

Figure 5. Analytical curve for melatonin concentrations in the range of 0.75 to 7.5 μmol/L using CV technique. Left: Cyclic voltammograms. Right: Linear regression. In this case,

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the magnitude of the oxidation peak of the electrochemical label decreases with increase of hormone concentration. Support electrolyte: PB; Redox probe: 4 mmol/L $K_4[Fe(CN)_6]$.

Figure 6. Analytical curve for melatonin in mouse organ extract samples constructed using EIS and hormone concentrations in the range of 0.0 to 7.0 μmol/L. Left: Nyquist plots. Right: Linear regression showing the increase in R_{ct} with hormone concentration raise. Support electrolyte: PB; Redox probe: 4 mmol/L $K_4[Fe(CN)_6]$.

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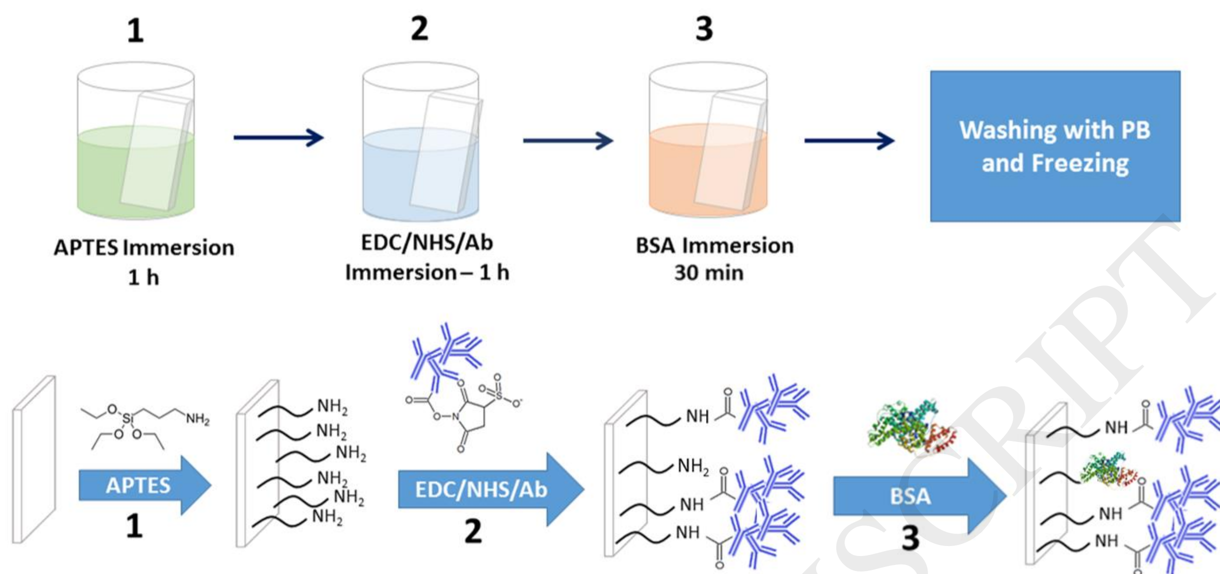

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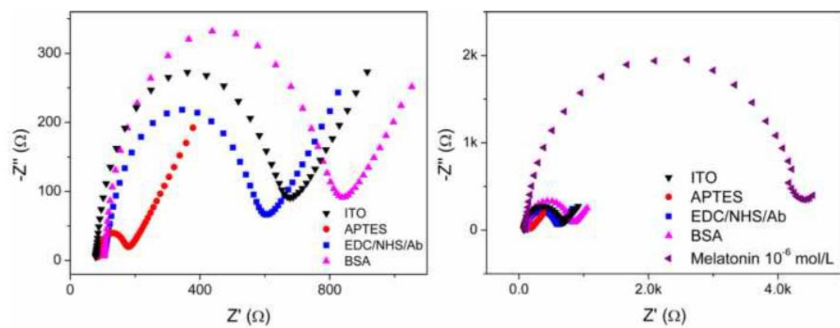

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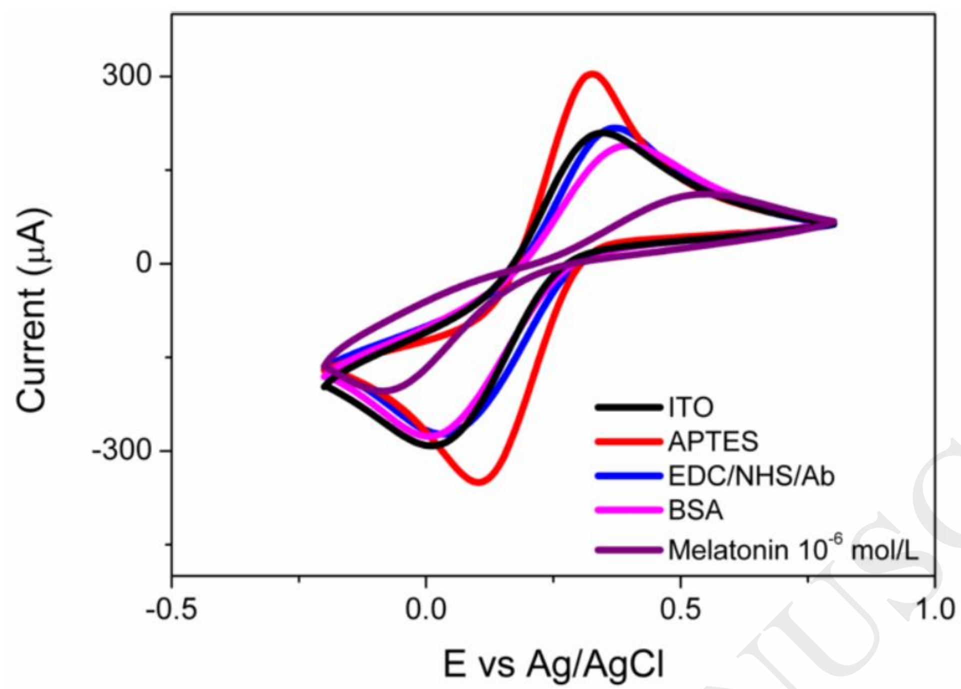

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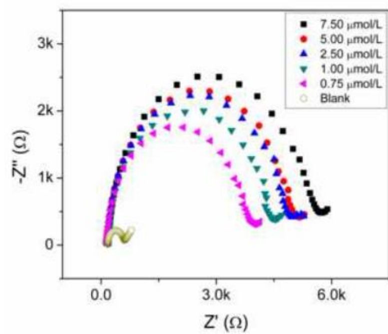

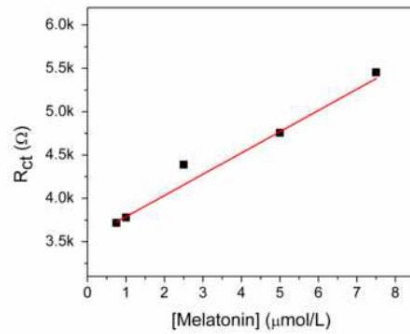

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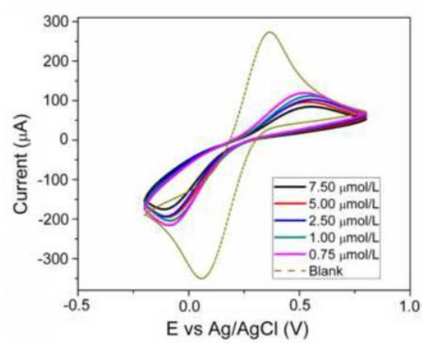

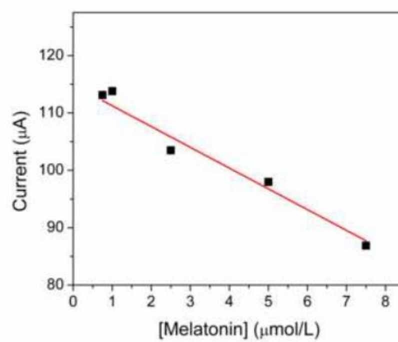

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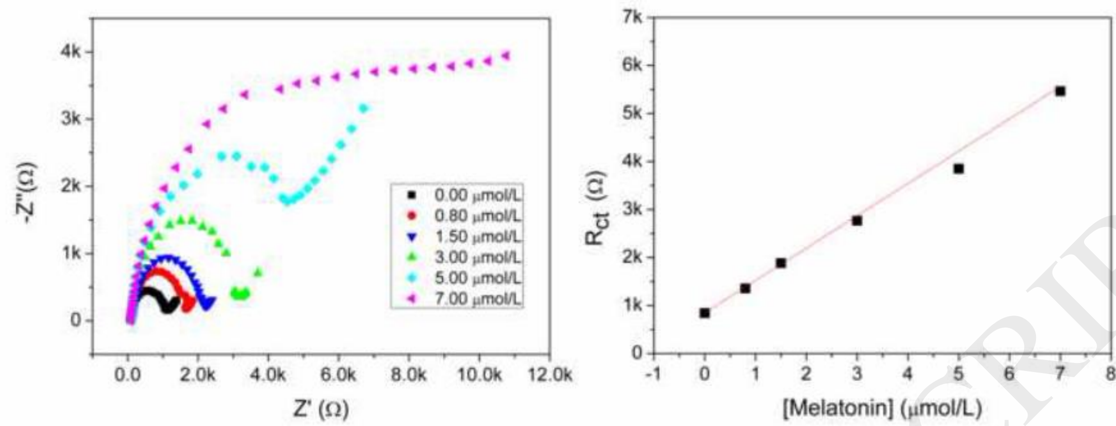

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Table 1

Expected Melatonin Concentration (μmol/L)	Melatonin concentration obtained using ITO-APTES/EDC/NHS/Ab sensor (μmol/L)	% relative to the expected concentration
0.0	-0.01	--
0.8	0.75	93.63
0.8	1.00	125.59
1.5	1.86	123.93
1.5	1.53	102.22
1.5	1.82	121.12
1.5	1.27	84.63
1.5	1.32	88.20
3.0	2.84	94.83
3.0	3.93	130.91
5.0	4.45	89.10
7.0	6.86	97.93

Table 1. Summary of Ab/EDC-NHS/APTES/ITO sensor quantifications in liver extracts.

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Table 2

Expected Melatonin Concentration (μmol/L)	Melatonin concentration obtained using ELISA kit (μmol/L)	% relative to the expected concentration
0.0	0	--
0.8	0	--
1.5	1.1 ± 0.3	73.33
3	2.8 ± 0.7	93.33
3	3.3 ± 0.8	110.00
5	4 ± 1	80.00
5	4 ± 1	80.00
7	8 ± 2	114.29

Table 2. Summary of ELISA quantifications in liver extracts. Errors were calculated using the maximum deviation on recuperation percentages presented on the Kit manual (25%)[33].

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