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Research Paper

Microchip electrophoresis-single wall carbon nanotube press-transferred electrodes for fast and reliable electrochemical sensing of melatonin and its precursors

In the current work, single-wall carbon nanotube press-transferred electrodes (SW-PTEs) were used for detection of melatonin (MT) and its precursors tryptophan (Trp) and serotonin (5-HT) on microchip electrophoresis (ME). SW-PTEs were simply fabricated by press transferring a filtered dispersion of single-wall carbon nanotubes on a nonconductive PMMA substrate, where single-wall carbon nanotubes act as exclusive transducers. The coupling of ME–SW-PTEs allowed the fast detection of MT, Trp, and 5-HT in less than 150 s with excellent analytical features. It exhibited an impressive antifouling performance with RSD values of ≤2 and ≤4% for migration times and peak heights, respectively ($n = 12$). In addition, sample analysis was also investigated by analysis of 5-HT, MT, and Trp in commercial samples obtaining excellent quantitative and reproducible recoveries with values of 96.2 ± 1.8%, 101.3 ± 0.2%, and 95.6 ± 1.2% for 5-HT, MT, and Trp, respectively. The current novel application reveals the analytical power of the press-transfer technology where the fast and reliable determination of MT and its precursors were performed directly on the nanoscale carbon nanotube detectors without the help of any other electrochemical transducer.

Keywords:
Carbon nanotubes / Melatonin / Press-transferred electrodes / Serotonin / Tryptophan
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1 Introduction

Microchip electrophoresis (ME) offer excellent opportunities to carry out novel and creative analytical works that feature short analysis times, low sample and reagent consumption, and extremely low waste generation [1,2]. Electrochemical detection in ME has been proven as an ideal and valuable analytical technique due to its inherent facility for miniaturization without loss of performance, high sensitivity, and extremely high compatibility with the micro and nanotechnologies [3,4]. One relevant example is the coupling of carbon nanotubes (CNTs) as electrochemical detectors to ME, which has enhanced the analytical performance in terms of selectivity, sensitivity, and reproducibility because these nanomaterials offer low detection potentials, high amperometric currents, resistance to fouling, and good stability versus usage time [5–7]. Commonly, the building of CNT-based detectors for microfluidic sensing has been carried out using thin film coating. The design of CNT thin film electrodes is very simple. The underlying bulk electrode is modified with CNT films, usually by deposition of a CNT suspension in the solvent and allowing its evaporation. The CNTs, consequently, lay randomly as CNT films on the electrode surface. The advantage of this design is the simplicity of the CNT film electrodes and the ability to choose from a wide range of underlying electrodes. However, such films are sometimes mechanically fragile.

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Abbreviations: **CNT**, carbon nanotube; **5-HT**, serotonin; **ME**, microchip electrophoresis; **MT**, melatonin; **PTE**, press-transferred electrode; **SWCNT**, single-wall carbon nanotube; **SW-PTE**, single wall carbon nanotube press-transferred electrode; **Trp**, tryptophan

Colour Online: See the article online to view Figs. 1 and 3 in colour.

Alternatively to this approach, recently, we have proposed novel single-walled press-transferred carbon nanotube electrodes (SW-PTEs) on PMMA substrates for electrochemical microfluidic sensing [8, 9]. In this approach, the electronic transfer is carried out directly on the target nanomaterial where (SWCNT) is the only one transducer in the electrochemical sensing and consequently the analytical advantages of these nanomaterials are directly exploited. In this work, we are exploring the electrochemical microfluidic sensing of melatonin (MT) and its precursors tryptophan (Trp), and its derivative serotonin 5-hydroxytryptamine (5-HT) using these unique nano-scaled detectors (SW-PTEs) coupled to ME.

MT is a neurohormone produced and released by pinealocytes of the pineal and photoreceptor cells of the retina under the control of light and darkness exposure (circadian rhythm) and the alleviation of sleep disorders, such as insomnia due to jet lag and shift work [10]. MT was also recently reported to be an effective free radical scavenger, an antioxidant and immunomodulator in cancer therapy [11]. Two key steps in melatonin biosynthesis involve the amino acid Trp and 5-HT; interestingly, both compounds possess notable antioxidant properties [12, 13]. Thereafter, MT was considered exclusively as an animal hormone, specifically a neurohormone for nearly four decades until the discovery in plants. Since then, it has been found in more than 140 different plant species and foods [14–24]. MT usually appears in the presence of its precursors; therefore, their simultaneous determination may be extremely important to understand its biosynthesis pathway and biological function in plants.

HPLC coupled with different detectors such as fluorescence, electrochemical, and MS [25–28] as well as CE [29–31] have been employed for the determination of MT and its precursors [29–31]. Although these techniques are suitable for the determination of these molecules; however, they are time-consuming and use large amounts of reagents.

In this sense, ME technology, which offer inherent advantages such as fast analysis times with low sample and reagents consumption, have not been used for the detection of these analytes. Therefore, the objective of this work has been to explore the analytical possibilities of the press-transfer technology coupled to ME for the fast and reliable electrochemical sensing of MT and their precursor 5-HT and Trp.

2 Materials and methods

2.1 Reagents, samples, and sample preparation

5-HT, MT, and Trp were purchased from Sigma-Aldrich (Steinheim, Germany), methanol from Panreac (Barcelona, Spain), di-sodium tetraborate decahydrate from Merck (Darmstadt, Germany). SWCNT from Sigma-Aldrich, 1,2-dichloroethane (Fluka), PMMA (Maniplastic), Teflon filters with pore sizes of 0.1 μm (JVWP01300, Millipore Omnipore), silver conductive paint (Electrolube) for the electrical contacts, and epoxy protective overcoat (242-SB de ESL Europe) as an insulator were used to produce the single-walled carbon nanotube press-transfer electrodes (SW-PTEs).

Melatonin herb extract capsules were purchased at a local market. Extraction was carried out under dim light to prevent analyte degradation. The content of capsules were diluted with 5 mL of MeOH, then vortexed during 30 s and sonicated in an ultrasound bath for 10 min. The resulting extract was filtered through a 0.20 μm syringe filter (Sartorius Ministart®) and stored in an amber vial. All solutions were daily prepared.

2.2 Electrode fabrication

The electrode fabrication is based on preparing films by filtering homogenized SWCNT dispersions through Teflon filters that are transferred to PMMA [8, 32]. Briefly, a stock dispersion of SWCNTs (0.50 mg per 100 mL) was prepared in 1,2-dichloroethane. Good dispersions were achieved using an ultrasound bath for 1 h and a tip sonicator applying a power of 250 W for 5 min. Then, 5 mL of the dispersion were filtered under vacuum using a Teflon filter that was dried for 5 min. SWCNT films collected on the filter were cut to obtain a SWCNT wire (13 × 1 mm). The SWCNT wire was transferred in the middle of the PMMA substrate by pressure to a piece of PMMA (33 × 9 mm) applying 5 ± 1 ton for 30 s. PMMA pieces were cleaned with ethanol and deionized water and then dried, prior to the transfer. The Teflon filter was removed slowly and carefully using tweezers. SWCNTs were transferred to PMMA providing a very homogeneous film. The electrical contacts were made using conductive silver. These contacts were later electrically isolated using insulating paint allowing electrode dimensions of 7 × 1 mm.

2.3 ME set-up

The analytical microsystem set-up was originally reported [33], and then adapted [34]. The glass chip was fabricated by Micronit Microfluidics (Model X8050, Enschede, The Netherlands) and consisted of a glass plate (82 x 15 mm) with a four-way injection cross, a 75 mm-long separation channel, and side arms measuring 5 mm long. The amperometric detector consisted of an Ag/AgCl wire as a reference electrode, a platinum wire as a counter electrode, and an SW-PTE as a working electrode, which was vertically placed and aligned at the end of the main channel (in an *end-channel* detection configuration). The working electrode is positioned on the cross-section of the main microchannel (50 × 20 μm) at a 60 μm distance to end of channel. Counter and reference electrodes are all arranged in one external electrochemical cell. Amperometric detection was performed using a Potentiostat AutolabPGSTAT101 from Eco Chemie. A LabSmith HVS448 high-voltage sequencer with eight independent high-voltage channels and programable sequencing for an entire level of voltage manipulation (Lab-Smith, Livermore, CA, USA) was

used as the voltage source. Injection was performed by the unpinched approach; +1500 V were applied to the sample reservoir for 5 s with the electrochemical reservoir held at ground (injection). After the injection was completed, the high voltage was switched back to the buffer reservoir and the separation was initiated.

2.4 ME protocols

The channels of the ME were treated before using and between groups of runs by rinsing them with 0.5 M NaOH for 40 min and de-ionized water for 10 min. This procedure was carefully monitored to obtain reproducible results. The optimum electrophoresis buffer consisted of 20 mM di-sodium tetraborate buffer (pH = 9.2) with 15% (v/v) of methanol. The running buffer and sample reservoirs were filled with their respective solution. The detection reservoir was filled with running buffer. A voltage of +1500 V was applied for 5 min to the buffer reservoir to fill the separation channel, while the detection reservoir was grounded and the others were floating. This process was performed for each sample reservoir for 30 s to facilitate filling the injection channel (between the separation channel and the sample reservoir), and then, the voltage was applied for 5 min to the running buffer reservoir to eliminate the remains of the previously introduced samples from the separation channel.

2.5 Amperometric detection

The amperometric detection was made with a detection voltage of +0.70 V that was applied to the working electrode for MT and its precursor detection. All experiments were performed at room temperature.

2.6 Calculations

The resolution (R) was calculated using the following equation:

$$R = \frac{1.18\ (t_2 - t_1)}{w_1 + w_2}$$

Where t_1 and t_2 are migration times of corresponding analytes and w_1 and w_2 are the corresponding peak width at the half height of two adjacent analyte bands.

2.7 Safety considerations

The high-voltage supply should be handled with extreme care in order to avoid electrical shock.

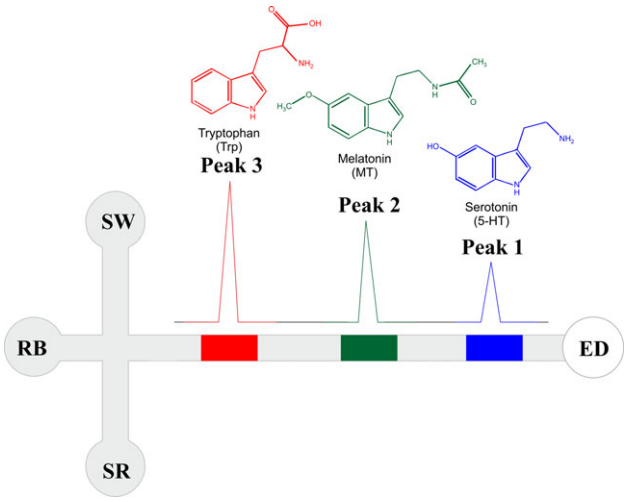

Figure 1. Chemical structures of MT and its precursors Trp and 5-HT on the ME layout. RB: running buffer reservoir, SR: sample reservoir, SW: sample waste reservoir, ED: electrochemical detection cell.

3 Results and discussion

3.1 Optimization of microchip separation and electrochemical detection of melatonin and its precursors

Figure 1 illustrates the chemical structures of MT and its precursors Trp and 5-HT on the ME layout used in this work. Since chosen analytes exhibit acid-base properties, CZE was explored for their microchip separation. Indeed, 5-HT is basic ($pK_a = 9.8$) and Trp is an amino acid, their ionic form significantly depends on the pH ($pK_{a1} = 2.4$, $pK_{a2} = 9.4$), while MT ($pK_a = 15.8$) is neutral in wide range of pH.

The separation media used consisted of 20 mM disodium tetraborate buffer (pH = 9.2) with different concentrations of methanol added as additive. At this pH the migration order obtained was 5-HT, MT, and Trp since, 5-HT was positively charged, MT remains neutral and Trp was negatively charged. Also, it is well known that the use of additives such as organic solvents could enhance the resolution of the analytes in electrophoresis. This is owing to the effect of decreasing both the conductivity of the buffer and EOF in presence of organic solvents. In such cases, the subsequent enhancement in resolution may result from a combination of the decreased EOF, decreased thermal diffusion, and improved analyte solubility [35]. Figure 2A shows the effect of methanol concentration over the separation of the analytes in connection with a separation voltage of +1500 V. A 15% (v/v) of MeOH was chosen as optimal concentration since it gave well-defined and resolved analyte peaks with migration times of 71.5 ± 0.3 s, 100.6 ± 0.2 s, and 120.9 ± 0.7 s for 5-HT, MT, and Trp, respectively; and with a good resolution ($R_{5\text{-HT-MT}} = 2.0$, $R_{\text{MT-Trp}} = 1.5$) in less than 150 s.

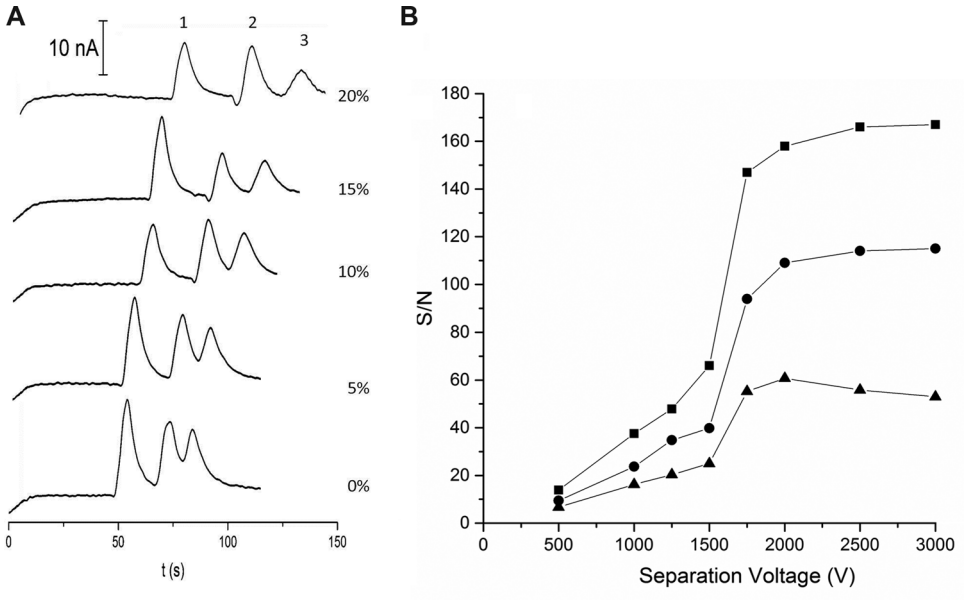

Figure 2. (A) Microchip electropherograms of 0.5 mM 5-HT (peak 1), 1 mM MT (peak 2) and 1 mM Trp (peak 3) for different concentrations of methanol added to the BGE. Conditions: 20 mM di-sodium tetraborate buffer (pH 9.2), separation voltage +1500 V, injection voltage +1500 V for 5 s, detection potential +0.70 V. (B) Effect of separation voltage on S/N ratio (0.5 mM 5-HT (■), 1 mM MT (●), and 1 mM Trp (▲)).

In addition, the influence of separation voltage on S/N characteristics was also evaluated as it is illustrated in Fig. 2B. When separation voltages were increased from +500 to +3000 V, S/N ratio increased until separation voltages around +2000 V. This fact was due to the increase of the electrophoretic velocity with the consequent decrease of the diffusion effect and the narrowing of the peak widths (i.e. for 5-HT at +500 V peak width had an S/N of 42.6, while at +3000 V was 12.0). At higher voltages, S/N remained constant due to the noise caused by Joule effect. Although S/N characteristics for +1750 and +2000 V were more favorable than for +1500 V, resolutions obtained ($R_{5\text{-HT-MT}} = 1.8$, $R_{\text{MT-Trp}} = 1.2$, and $R_{5\text{-HT-MT}} = 1.6$, $R_{\text{MT-Trp}} = 1.1$ for +1750 and +2000 V, respectively) were not considered well-enough for an optimum separation in comparison with those obtained for +1500 V ($R_{5\text{-HT-MT}} = 2.0$, $R_{\text{MT-Trp}} = 1.5$). As expected, the increase of separation voltages from +500 to +3000 V also caused a dramatic decrease in the migration times (see Supporting Information Fig. 1) for the three analytes. From 225 to 40 s, from 325 to 50 s, and from 400 to 70 s for 5-HT, MT, and Trp, respectively, with a clear decrease in the analyte resolution as it was stated above. Also, initial charging-current baseline rise indicates incomplete isolation of the electrochemical detector from separation voltages higher than +2000 V. Therefore, +1500 V became the most favorable separation voltage.

On the other hand, Fig. 3 shows hydrodynamic voltammograms for the oxidation of 5-HT, MT, and Trp. The curves were taken stepwise in connection with +1500 V as separation voltages by making 0.20 V changes in the detection potential. The response rises gradually between +0.30 V and +0.60 V, after which it levels off. Detection potential of +0.70 V was chosen, because it offered the best S/N characteristics. Figure 3 (inset) also shows photography of the SW-PTE that consists of a SWCNTs press-transferred

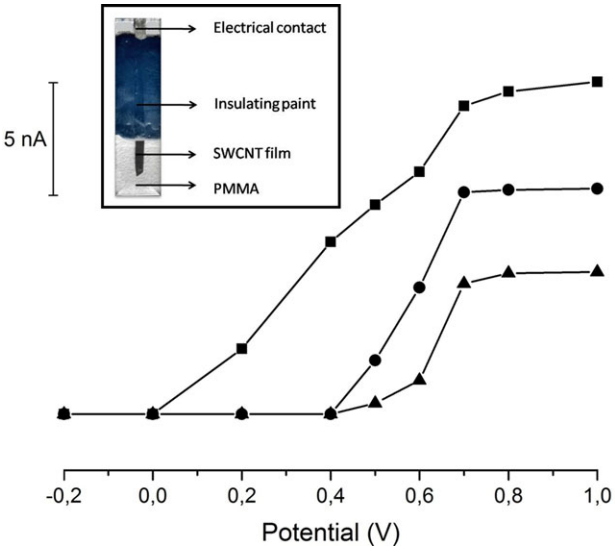

Figure 3. Hydrodynamic voltammograms for 0.5 mM 5-HT (■), 1 mM MT (●) and 1 mM Trp (▲). Inset: Photography of the SW-PTE.

line-film (7 × 1 mm) positioned and centered on the PMMA substrate (33 × 9 mm). The electrode geometry was suitable for ME coupled to an *end-channel* into a wall-jet configuration.

Summarizing, the oxidation potential of +0.70 V applied on SW-PTEs, in connection with a separation voltage of +1500 V, resulted in a well-defined and resolved peaks, a flat baseline, as well as a favorable signal-to-noise characteristics.

3.2 Analytical performance and sample analysis

Under these optimized conditions, precision was carefully evaluated. Supporting Information Table 1 lists quantitatively

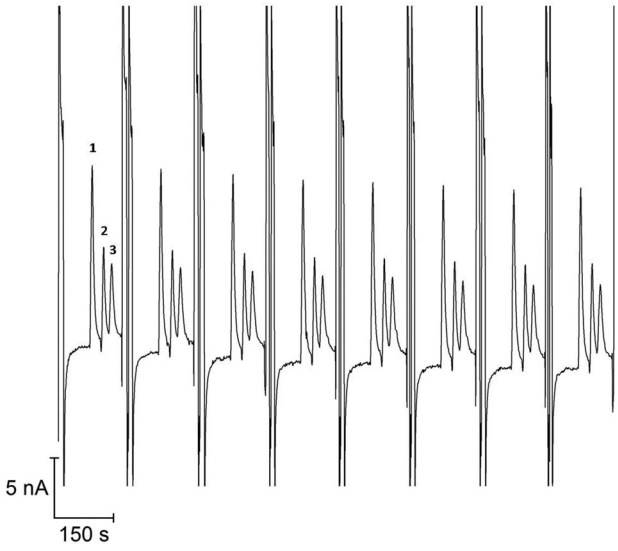

Figure 4. Microchip electropherograms corresponding to a mixture of 5-HT (peak 1), MT (peak 2), and Trp (peak 3) (500 μM each) ($n = 8$).

Table 1. Analytical features of the proposed method

Analyte	Lineal range (μM)	Equation[a)]	r	LOD (μM)	LOQ (μM)
5-HT	10–200	$y = 153\,x + 1.6$	0.999	1	4
MT	50–500	$y = 50\,x + 3.4$	0.990	4	12
Trp	50–500	$y = 40\,x + 0.7$	0.992	5	16

a) Regression equation is $y = bx + a$ where y is the amperometric current (nA) and X is the analyte concentration (μM).

the precision obtained for migration times and amperometric currents obtained for MT and its precursors. As it is observed, an excellent precision with RSD <2% for migration times and RSD <3% for amperometric currents were obtained. Electrode fabrication reproducibility was also evaluated using different electrodes and by evaluating their electro-analytical response. The results obtained showed good inter-electrode reproducibility in the migration times (RSD ≤2%) and peak heights (RSD ≤4%) ($n = 5$). This excellent interelectrode reproducibility indicates that these electrodes could be disposables, which constitute an extra advantage of this approach. Also, one of the most remarkable advantages derived from the use of CNTs is the resistance to the fouling that these materials offer. Figure 4 illustrates selected microchip electropherograms corresponding to MT and their precursors at SW-PTEs during more than 35 min of analysis. It exhibited an impressive anti-fouling performance with RSD values of ≤3 and ≤5% ($n = 12$) for migration times and peak heights, respectively. These excellent values gave information not only about the high resistance-to-fouling, but also about the stability of both EOF and the press-transferred SWCNTs films in PMMA substrates.

On the other hand, Table 1 lists the analytical features of the proposed method. The resulting calibration plots were highly linear ($r \geq 0.990$) in the concentration range assayed for each analyte. Combining the high sensitivity with its low noise level resulted in suitable LODs (S/N = 3) and LOQs (S/N = 10) in the μM range.

As early application, the analysis of commercial samples was also investigated (see Supporting Information Table 2). MT was found in a concentration of 1.78 ± 0.07 mg per capsule, in agreement with the declared by fabricant (1.8 mg per capsule). In addition, excellent quantitative and reproducible recoveries with values of 96.2 ± 1.8%, 101.3 ± 0.2%, and 95.6 ± 1.2% for 5-HT, MT, and Trp, respectively; were also obtained during the analysis of spiked samples. These results revealed a very good accuracy of the method as well as it did not show interferences during the analysis of these samples.

4 Concluding remarks

This work reveals the analytical merits of ME coupled to the SW-PTEs where the fast and reliable determination of MT and its precursors were performed directly on the nanoscale CNT detectors without any other electrochemical transducer under 150 s with excellent analytical performance. Press-transfer technology allowed the possibility to directly explore the advantages of the CNTs without the need of other sophisticated facilities (such as clean room) demonstrating a new way for detection and determination of these molecules.

Although the sample analysis explored here was chosen as an early example of the analytical potency of this new coupling, the excellent results obtained became a realistic promise for future coupling of SW-PTEs to ME. It suggests the incorporation of other separation modes such as MEKC to avoid potential interferences from neutral electroactive molecules in more complex samples.

In addition, in comparison with others methodologies that used HPLC and CE, it could be said that the presented approach is a faster and simpler alternative avoiding even the large use of toxic solvents.

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