Molecularly imprinted electrochemical sensing of urinary melatonin in a microfluidic system

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Melatonin levels may be related to the risks of breast cancer and prostate cancer. The measurement of urinary melatonin is also useful in monitoring serum melatonin levels following oral administration. In this work, melatonin is the target molecule, which is imprinted onto poly(ethylene-co-vinyl alcohol) by evaporation of the solvent on the working electrode of an electrochemical sensing chip. This sensing chip is used directly as a tool for optimizing the imprinting polymer composition, flow rate, and injection volume of the samples. Microfluidic sensing of the target and interference molecules revealed that the lowest detection limit is as low as $\sim$ pM, and the electrochemical response is weak even at high interference concentrations. Poly(ethylene-co-vinyl alcohol), containing 44 mol. % ethylene, had an imprinting effectiveness of more than six-fold. In random urine analysis, the microfluidic amperometric measurements of melatonin levels with an additional and recovery of melatonin, the melatonin recovery achieved $94.78 \pm 1.9\%$ for melatonin at a concentration of 1.75–2.11 pg/mL. © 2014 AIP Publishing LLC.
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I. INTRODUCTION

Molecularly imprinted polymers (MIPs) show good potential for modifying electrochemical and other sensors¹ and offer the advantage of being relatively stable in storage, overcoming the obvious disadvantages associated with biological recognition elements such as enzymes or antibodies. Schirhagl *et al.* reviewed surface-imprinted polymers in microfluidic devices.² Surface plasmon resonance (SPR) transducer and a quartz crystal microbalance (QCM) are commonly integrated with MIPs. Piletsky and Turner,³ Blanco-López *et al.*,⁴ McCluskey *et al.*,⁵ Rao and Kala,⁶ and Suryanarayanan *et al.*⁷ reviewed electrochemical sensors using molecularly imprinted polymers as sensing elements.

A robotic microfluidic sensing chip may reduce not only the time consumed in flow injection analysis but also the amounts of buffer and sample used during sensing. High-throughput protocols have been developed for rapid biological examinations with many samples.^{8–10} They may also reduce the waiting time in multiplex target sensing (for example, immune cell analysis¹¹) using a home-care system;¹² for example, Miller *et al.* demonstrated the integration of carbon fiber electrodes within hollow polymer microneedles for transdermal electrochemical sensing to measure the hydrogen peroxide and ascorbic acid.¹³ Kimmel *et al.* recently reviewed electrochemical sensors and biosensors¹⁴ and discussed two basic microfluidic systems that

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involved continuous flow and droplet assays. The first microfluidic device using MIPs in conjunction with amperometric detection was reported by Weng *et al.*¹⁵ The detection of biomolecules that employs microfluidic electrochemical methods involving molecular imprinting polymers remains poorly understood.

Melatonin, which is secreted by the pineal gland, exhibits strong antioxidant activity and helps to regulate other hormones, maintaining the body's circadian rhythm. Numerous investigations have suggested that low melatonin levels may be associated with risks of breast cancer,¹⁶ prostate cancer,¹⁷ and type 2 diabetes.¹⁸ Melatonin supplements may help sufferers of insomnia¹⁹ and menopause.²⁰ The measurement of urinary melatonin²¹ is also useful for monitoring levels of serum melatonin following oral administration. The reference concentration range of melatonin in urine is reported to be 2.0–35.4 pg/mL.²² Melatonin in urine is typically measured using commercially available radioimmunoassay (RIA)²² or enzyme-linked immunosorbent assay (ELISA) kits.²¹

In this study, the concentration and ethylene mol. % of EVAL were systematically varied and applied to stationary electrochemical reactors. Sub-microliters of melatonin-containing samples were then injected into a microfluidic system with melatonin-imprinted EVAL sensors. Flow rates and injection sample volumes were then considered to the optimized parameters of the microfluidic system. Finally, measurements were continuously made using melatonin-imprinted EVAL sensors to plot the melatonin calibration curve.

II. EXPERIMENTAL

A. Reagents

Melatonin (minimum $\geq 98.0\%$), creatinine (minimum $\geq 99.0\%$), urea (minimum $\geq 98.0\%$), albumin from bovine serum (minimum $\geq 98.0\%$), poly(ethylene-co-vinyl alcohol), EVAL, with ethylene 27, 32, 38, and 44 mol. % (product nos. 414077, 414093, 414085, and 414107) were from Sigma-Aldrich Co. (St. Louis, MO) and used as received. Dimethyl sulfoxide (DMSO, product # 161954) was purchased from Panreac (Barcelona, Spain) and used as the solvent to dissolve EVAL polymer particles. Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich Co. (St. Louis, MO) and used for the removal of target molecules. Deionized water used in the preparation of buffers and for rinse solutions was 18.2 M Ω , as produced by a PURELAB Ultra (ELGA, Albania). All chemicals were used as received unless otherwise mentioned.

B. The preparation of molecularly imprinted polymers coated sensing chips

The synthesis of melatonin- and non-imprinted EVAL thin film on the working electrodes consisted of three steps: (1) dropwise addition the EVAL solution (EVAL/DMSO = 0.01–2 wt. %, c.a. 2 μ L totally) either with or without 0.1 wt. % of template molecules on a screen-printed gold substrate (4 mm diameter, DropSens, Spain); (2) solvent evaporation in an oven at 50 °C for 12 h to completely remove DMSO; and then (3) removal of the template molecule by injection 200 μ L of 0.1 wt. % aqueous SDS with the modified electrode installed in a purpose-built commercially available thin-layer flow-cell for screen-printed electrodes (DRP-FLWCL, DropSens, Spain) or rinsing in 5 mL of 0.1 wt. % SDS and then deionized water for 10 min and repeating. The surface element analysis of nitrogen atomic % of melatonin-imprinted poly(ethylene-co-vinyl alcohol) thin film, containing 44 mol. % ethylene, before, after washing and non-imprinted polymers (NIPs) are 5.45, 3.54, and 2.53.²³ The thickness of EVAL thin films is around 300 nm when prepared at the polymer concentration of 0.02 wt. %.²⁴

C. The evaluation of the melatonin-imprinted EVAL coated electrodes for the determination of melatonin in human urine samples

The electrochemical reactions were controlled and monitored with a potentiostat (410B, CH Instruments, Inc., Austin, TX). The current response of the imprinted polymeric sensing electrodes was assessed using differential pulse voltammetry (DPV) in phosphate buffered

saline (PBS) at pH 7.4, with potassium ferrocyanide and potassium ferricyanide as the electroactive tracer (both at 20 mM concentration)^{25,26} The effect of different concentrations of melatonin, covering the reported reference concentration range, was examined. The effect of interferences (urea, creatinine, and albumin) was also examined in PBS and in urine samples.

Flow injection analysis was accomplished using an analytical isocratic HPLC pump (PU-2080, JASCO International Co., Ltd., Tokyo, Japan) equipped with an manual sample injector valve (Rheodyne 7725i, IDEX Health & Science, Oak Harbor, WA), a flow-cell for screen-printed electrodes (DRP-FLWCL, dimension: length $\times$ width $\times$ height = 3.3 $\times$ 6.0 $\times$ 3.3 cm, DropSens, Spain) and a screen-printed gold substrate (4 mm diameter, DropSens, Spain) with integrated counter electrodes and Ag/AgCl reference electrode (to which all potentials are referred). The details of the flow-cell and screen-printed electrode could be found on the web page: <http://www.dropsens.com/>. The working electrode is coated with melatonin-imprinted EVAL thin film as described above and illustrated schematically in Scheme 1.

The preliminary tests of the effect of EVAL concentration and ethylene mol. % were examined in stationary solution in a 20 mL vial filled with the above mentioned solution by differential DPV. The pulse interval, height, and amplitude are 500 ms, 60 ms, and 30 mV, respectively, in the potential range of ± 400 mV.

The suitability of the devices for melatonin analysis was performed in the flow injection mode. Random urine samples from the author and colleagues were analyzed 4 h before the test. Urine samples were injected (200 μ L loop was used unless other mentioned) to continuous electrochemical flow cell. As an added check on the calibration and the sensitivity to melatonin, the current change for urine samples was also measured after adding 0.2 or 0.4 pg (0.2 or 0.4 μ L of 1 ng/mL) of additional (exogenous) melatonin. The recovery of melatonin is estimated on the basis of the calibration curve in Fig. 4(b), *vide infra*, and defined as the percentage of melatonin detected.

An ELISA kit (CEA908Ge, Cloud-Clone Corp., Houston, TX) was employed to examine the melatonin concentration in random urine samples from the author and colleagues. The details of the protocol could be found on the web page: <http://www.cloud-clone.us/manual/ELISA-Kit-for-Melatonin-MT-E90908Ge.pdf>. All measurements in this work were carried out with at least two replicates; data are expressed as means and standard deviations.

III. RESULTS AND DISCUSSION

The critical issue associated with the use of MIPs as sensing materials is not only generating sufficient template cavities but also optimizing the signal response. Figure 1(a) shows that the applied potential is approximately 0.05 V, and the current response falls dramatically from 18 μ A to under 4 μ A as the EVAL concentration is decreased from 2.0 to 0.01 wt. %. Various ethylene concentrations in EVAL were tested to find the one that yielded the strongest

SCHEME 1. The sensing of melatonin with molecularly imprinted polymer coated sensing chip.

FIG. 1. Electrochemical response of 50 pg/mL of melatonin to melatonin-imprinted EVAL-coated electrodes with (a) concentrations of EVAL (44 mol. % ethylene) in the imprinting solution; (b) EVALs contains different mol. % of ethylene.

electrochemical response. The inset of Figure 1(a) plots the DPV electrochemical responses using different EVAL concentrations in the MIP materials coated on the working electrode. The higher concentration of EVAL applied may give the thicker molecular imprinted thin film and then increase the resistance. The current changes with melatonin exist compared with buffer solution also increased dramatically with the ethylene concentration of the EVALs. In Figure 1(b), the largest change in current, $2.80 \pm 0.04 \mu\text{A}$, was obtained using EVAL that contained 44 mol. % of ethylene. NIPs exhibited a weak response of $0.5 \mu\text{A}$ for all EVALs used.

Interestingly, the current change in the presence of 50 pg/mL melatonin was significantly larger with higher ethylene concentration. The hydrogen bonding and hydrophobic interaction of the chemical structure of melatonin with optimum composition of EVALs gave the best performance for the detection of melatonin.²⁷ At low ethylene ratios (27 and 33 mol. %), the change in current associated with MIPs is 2- to 3-fold that associated with NIPs. Moreover, as the ethylene concentration increased in EVAL, 38 and 44 mol. %, 5.1- and 6.5-fold differences in the current of MIPs relative to that in NIPs were obtained, respectively. The imprinting effectiveness (IF) is defined as the ratio of signal on MIP to signal on NIP.

The flow rate of samples to the sensing area critically affects the electrode response in two ways: at low flow, exhaustive (effectively coulometric) performance prevails whereas at high flow rates we would expect the diffusion limited current to depend on the cube root of the volume flow rate.²⁸ Flow rates varying from 0.1 to 1.5 mL/min were tested (see Figs. 2(a) and 2(b)). The electrochemical response is substantially different at low and high flow rates: at a flow rate of lower than 0.5 mL/min or higher than 1.0 mL/min, the differences between current densities were around 6 and 18 mA/cm², respectively. Fewer target molecules produce weaker response signals, but the less precious sample volume required for a sensing system may have the advantage of more tests. Figures 2(c) and 2(d) indicate that the electrochemical response

FIG. 2. Microfluidic melatonin sensors under (a) different flow rate and (c) injection volumes of testing sample. Electrochemical response of MIPs sensors with (b) flow rates and (d) injection volume at 40 pg/mL of melatonin.

FIG. 3. The effect of the interferents (e.g., urea (circles), creatinine (triangles), and albumin (squares)) were tested and compared with melatonin (inset) up to 50 pg/mL. The test concentration for urea, creatinine, and albumin are from 0 to 8, 5, and 0.5 mg/mL, respectively. (b) The relative response of melatonin (2 pg/mL) and coexist with urea (20 mg/mL).

increases linearly with increasing the number of target molecules that are injected into the system.

In urine, the most common causes of interference with melatonin sensing were urea, creatinine, and albumin. Figure 3 presents the interferences of reference concentrations of these compounds. In Fig. 3(a), the highest concentrations of urea, creatinine, and albumin were 0.5, 5, and 8 mg/mL, respectively; the corresponding differences in current density ranged from 1 to 2.5 $\mu\text{A}/\text{cm}^2$. A melatonin concentration of as low as 20 pg/mL produces a difference in current

densities of at least $5 \mu\text{A}/\text{cm}^2$. The complementary structure between target molecules and MIPs may reduce the non-specific binding of interferences (e.g., urea) when coexisted, as shown in Fig. 3(b). Less than 3% of response difference is found at the reference concentration (ca. 2 pg/mL) of melatonin and mixture with 20 mg/mL of urea.

Figure 4(a) demonstrates the continuous electrochemical measurements on MIP- and NIP-coated electrodes. The stable current responses of MIP and NIP-coated electrodes were

FIG. 4. (a) The continuously current-time measurement with melatonin injections. (b) Current differences of melatonin- and non-imprinted EVAL coated electrodes under different melatonin concentrations and controls were re-plotted to give the calibration curve of urinary melatonin.

FIG. 5. Repeatedly measurement of 3 pg/mL of melatonin with melatonin- and non-imprinted EVAL-coated electrodes.

230–240 and 80–90 $\mu\text{A}/\text{cm}^2$, respectively. Figure 4(b) exhibits the corresponding current density differences. The differences between the current densities on MIP and NIP were 5.5 and 0.5 $\mu\text{A}/\text{cm}^2$, respectively. It is noteworthy that the electrochemical responses on the NIP-coated electrodes in the stationary (Figure 1(b)) and microfluidic systems were very close to each other (Fig. 4(b)). The imprinting effectiveness in the microfluidic system reached as high as 12-fold.

Before measurements were made on real samples, the MIP-coated electrode was packed into the microfluidic electrochemical reactor, which was alternately injected using 200 μL of 1 wt. % SDS or 3 pg/mL melatonin. Figure 5 plots the electrochemical responses of the MIP and NIP-coated electrodes when used repeatedly. The MIP-coated electrode exhibits a very small (less than 1.0%) variability in the current densities.

Table I presents the measurements of the real samples. Three samples were injected into the microfluidic MIP electrochemical system. Melatonin spikes with urine samples were also injected to calculate the recovery of melatonin. The electrochemical responses of those samples range from 0.89 to 1.09 $\mu\text{A}/\text{cm}^2$, corresponding to 1.75 to 2.11 pg/mL of melatonin. To further confirm the sensitivity to melatonin in real samples, 200 μL of each 100 times diluted urine sample was doped with additional melatonin²³ by adding either 0.1 or 0.2 μL in a 1 ng/mL melatonin stock solution. The electrochemical response of the doped samples increased more than the undoped samples; the doped melatonin concentrations subjected to the calibration curve obtain “recoveries” (of the doped amount) of 91.78%–97.56% in Table II. The average recovery of the doped melatonin, $94.78 \pm 1.90\%$, was achieved using this microfluidic MIP electrochemical system.

TABLE I. The spike melatonin measurement of melatonin in urine. The recovery of melatonin was defined as the percentage of exogenous melatonin spikes detected in the urine sample.

Urine sample	Urine (μL)	Melatonin added (pg)	$\Delta\text{Current}$ ($\mu\text{A}/\text{cm}^2$)	Convert conc. (pg/ML)	Melatonin recovered (pg)	Recovery (%)
I	200	...	0.89	1.75	0.35	
	200	0.2	1.37	2.67	0.53	91.78
	200	0.4	1.83	3.64	0.73	94.70
II	200	...	0.93	1.82	0.36	
	200	0.2	1.43	2.78	0.56	96.32
	200	0.4	1.86	3.72	0.74	95.13
III	200	...	1.09	2.11	0.42	
	200	0.2	1.55	3.04	0.61	93.19
	200	0.4	2.01	4.06	0.81	97.56

TABLE II. Comparison of melatonin in urine measured by the microfluidic MIP sensor and ELISA.

Samples	Microfluidic MIP sensor			ELISA			Accuracy (%)
	Δ Current (μ A/cm ²)	Convert conc. (pg/mL)	Avg. conc. (pg/mL)	Optical density (a.u.)	Convert conc. (pg/mL)	Avg. conc. (pg/mL)	
A	5.20	23.85	24.33 $\pm$ 0.48	1.34	24.65	25.48 $\pm$ 0.83	95.50
	5.28	24.80		1.29	26.30		
B	3.83	10.17	11.29 $\pm$ 1.12	2.25	10.26	11.31 $\pm$ 1.05	99.82
	4.11	12.40		2.01	12.35		

Table II lists the analyses of two additional real urine samples from the authors and his colleague using an ELISA and the microfluidic MIP sensor, which measured melatonin concentrations fell in the 11.31–25.48, and 11.29–24.33 pg/mL, respectively. Their average accuracies for urinal melatonin were 95.49% and 99.82% for each sample. Therefore, the high accuracy of the microfluidic electrochemical system was also demonstrated.

IV. CONCLUSIONS

The integration of a sensing chip and a microfluidic system is of interest because of the high throughput of samples by using a small amount of sample which leads to reduced analysis time. In this work, the developed microfluidic system enhances double sensitivity and uses of much less buffer solution (1 mL vs. 20 mL/sample) as compared the electrochemical sensing in stagnant solution. Furthermore, the imprinting effectiveness, based on the fluidic electrochemical response at 20 pg/mL of melatonin, was as high as 6.5. The microfluidic electrochemical system may be extended to sensing arrays at very low cost, offering high sensitivity in applications with multiple biomarkers compared with conventional radioimmunoassay (RIA)²² or ELISA.²¹

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